

specified, when substances are to be “accurately weighed” for Assay, the weighing is to be performed with a weighing device whose measurement uncertainty (random plus systematic error) does not exceed 0.1% of the reading. Measurement uncertainty is satisfactory if three times the standard deviation of not less than ten replicate weighings divided by the amount weighed, does not exceed 0.001. Unless otherwise specified, for titrimetric limits tests, the weighing shall be performed to provide the number of significant figures in the weight of the analyte that corresponds to the number of significant figures in the concentration of the titrant.

The class designations below are in order of increasing tolerances.

Class 1.1 weights are used for calibration of low-capacity, high-sensitivity balances. They are available in various denominations from 1 to 500 mg. The tolerance for any denomination in this class is 5 µg. They are recommended for calibration of balances using optical or electrical methods for accurately weighing quantities below 20 mg.

Class 1 weights are designated as high-precision standards for calibration. They may be used for weighing accurately quantities below 20 mg. (For weights of 10 g or less, the requirements of class 1 are met by USP XXI class M.)

Class 2 weights are used as working standards for calibration, built-in weights for analytical balances, and laboratory weights for routine analytical work. (The requirements of class 2 are met by USP XXI class S.)²

Class 3 and class 4 weights are used with moderate-precision laboratory balances. (Class 3 requirements are met by USP XXI class S-1; class 4 requirements are met by USP XXI class P.)²

A weight class is chosen so that the tolerance of the weights used does not exceed 0.1% of the amount weighed. Generally, class 2 may be used for quantities greater than 20 mg, class 3 for quantities of greater than 50 mg, and class 4 for quantities of greater than 100 mg. Weights should be calibrated periodically, preferably against an absolute standard weight.

Microbiological Tests

(51) ANTIMICROBIAL EFFECTIVENESS TESTING

Antimicrobial preservatives are substances added to non-sterile dosage forms to protect them from microbiological growth or from microorganisms that are introduced inadvertently during or subsequent to the manufacturing process. In the case of sterile articles packaged in multiple-dose containers, antimicrobial preservatives are added to inhibit the growth of microorganisms that may be introduced from repeatedly withdrawing individual doses.

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

All useful antimicrobial agents are toxic substances. For maximum protection of patients, the concentration of the

preservative shown to be effective in the final packaged product should be below a level that may be toxic to human beings.

The concentration of an added antimicrobial preservative can be kept at a minimum if the active ingredients of the formulation possess an intrinsic antimicrobial activity. Antimicrobial effectiveness, whether inherent in the product or whether produced because of the addition of an antimicrobial preservative, must be demonstrated for all injections packaged in multiple-dose containers or for other products containing antimicrobial preservatives. Antimicrobial effectiveness must be demonstrated for multiple-dose topical and oral dosage forms and for other dosage forms such as ophthalmic, otic, nasal, irrigation, and dialysis fluids (see *Pharmaceutical Dosage Forms* (1151)).

This chapter provides tests to demonstrate the effectiveness of antimicrobial protection. Added antimicrobial preservatives must be declared on the label. The tests and criteria for effectiveness apply to a product in the original, unopened container in which it was distributed by the manufacturer.

PRODUCT CATEGORIES

For the purpose of testing, compendial articles have been divided into four categories (see *Table 1*). The criteria of antimicrobial effectiveness for these products are a function of the route of administration.

Table 1. Compendial Product Categories

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

TEST ORGANISMS

Use cultures of the following microorganisms¹: *Candida albicans* (ATCC No. 10231), *Aspergillus niger* (ATCC No. 16404), *Escherichia coli* (ATCC No. 8739), *Pseudomonas aeruginosa* (ATCC No. 9027), and *Staphylococcus aureus* (ATCC No. 6538). The viable microorganisms used in the test must not be more than five passages removed from the original ATCC culture. For purposes of the test, one passage is defined as the transfer of organisms from an established culture to fresh medium. All transfers are counted. In the case of organisms maintained by seed-lot techniques, each cycle of freezing, thawing, and revival in fresh medium is taken as one transfer. A seed-stock technique should be used for long-term storage of cultures. Cultures received from the ATCC should be resuscitated according to directions. If grown in broth, the cells are pelleted by centrifugation. Resuspend in 1/20th the volume of fresh maintenance broth, and add an equal volume of 20% (v/v in water) sterile glycerol. Cells grown on agar may be scraped from the surface into the 10% glycerol broth. Dispense small aliquots of the suspension into sterile vials. Store the vials in liquid nitrogen or in a mechanical freezer at no more than -50°. When a fresh seed-stock vial is required, it may be removed and used to inoculate a series of working cultures. These

² Note that the designations S and P no longer designate weight classes but rather weight grades, that is, design limitations such as range of density of materials, surface area, surface finish, corrosion resistance, and hardness.

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

Table 2. Culture Conditions for Inoculum Preparation

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

working cultures may then be used periodically (each day in the case of bacteria and yeast) to start the inoculum culture.

MEDIA

All media used in the test must be tested for growth promotion. Use the microorganisms indicated above under *Test Organisms*.

PREPARATION OF INOCULUM

Preparatory to the test, inoculate the surface of a suitable volume of solid agar medium from a recently revived stock culture of each of the specified microorganisms. The culture conditions for the inoculum culture are described in *Table 2* in which the suitable media are Soybean–Casein Digest or Sabouraud Dextrose Agar Medium (see *Microbial Enumeration Tests* <61> and *Tests for Specified Microorganisms* <62>).

To harvest the bacterial and *C. albicans* cultures, use sterile saline TS, washing the surface growth, collecting it in a suitable vessel, and adding sufficient sterile saline TS to obtain a microbial count of about 1×10^8 colony-forming units (cfu) per mL. To harvest the cells of *A. niger*, use sterile saline TS containing 0.05% of polysorbate 80, and add sufficient sterile saline TS to obtain a count of about 1×10^8 cfu per mL.

Alternatively, the stock culture organisms may be grown in a suitable liquid medium (i.e., Soybean–Casein Digest Broth or Sabouraud Dextrose Broth) and the cells harvested by centrifugation, then washed and resuspended in sterile saline TS to obtain a microbial count of about 1×10^8 cfu per mL. [NOTE—The estimate of inoculum concentration may be performed by turbidimetric measurements for the challenge microorganisms. Refrigerate the suspension if it is not used within 2 hours.]

Determine the number of cfu per mL in each suspension, using the conditions of media and microbial recovery incubation times listed in *Table 2* to confirm the initial cfu per mL estimate. This value serves to calibrate the size of inoculum used in the test. The bacterial and yeast suspensions are to be used within 24 hours of harvest, but the fungal preparation may be stored under refrigeration for up to 7 days.

PROCEDURE

The test can be conducted either in five original containers if sufficient volume of product is available in each container and the product container can be entered aseptically (i.e., needle and syringe through an elastomeric rubber stopper), or in five sterile, capped bacteriological containers of suitable size into which a sufficient volume of product has been transferred. Inoculate each container with one of the prepared and standardized inoculum, and mix. The volume of the suspension inoculum used is between 0.5% and 1.0% of the volume of the product. The concentration of

test microorganisms that is added to the product (*Categories 1, 2, and 3*) are such that the final concentration of the test preparation after inoculation is between 1×10^5 and 1×10^6 cfu per mL of the product. For *Category 4* products (antacids) the final concentration of the test preparation after inoculation is between 1×10^3 and 1×10^4 cfu per mL of the product.

The initial concentration of viable microorganisms in each test preparation is estimated based on the concentration of microorganisms in each of the standardized inoculum as determined by the plate-count method.

Incubate the inoculated containers at $22.5 \pm 2.5^\circ$. Sample each container at the appropriate intervals specified in *Table 3*. Record any changes observed in appearance at these intervals. Determine by the plate-count procedure the number of cfu present in each test preparation for the applicable intervals (see *Procedure under Microbial Enumeration Tests* <61> and *Tests for Specified Microorganisms* <62>). Incorporate an inactivator (neutralizer) of the specific antimicrobial in the plate count or in the appropriate dilution prepared for plating. These conditions are determined in the validation study for that sample based upon the conditions of media and microbial recovery incubation times listed in *Table 2*. Using the calculated concentrations of cfu per mL present at the start of the test, calculate the change in \log_{10} values of the concentration of cfu per mL for each microorganism at the applicable test intervals, and express the changes in terms of log reductions.

CRITERIA FOR ANTIMICROBIAL EFFECTIVENESS

The requirements for antimicrobial effectiveness are met if the criteria specified under *Table 3* are met (see *Significant Figures and Tolerances under General Notices*). No increase is defined as not more than 0.5 \log_{10} unit higher than the previous value measured.

Table 3. Criteria for Tested Microorganisms

For Category 1 Products	
Bacteria:	Not less than 1.0 log reduction from the initial calculated count at 7 days, not less than 3.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 7, 14, and 28 days.
For Category 2 Products	
Bacteria:	Not less than 2.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 14 and 28 days.

Table 3. Criteria for Tested Microorganisms (Continued)

For Category 3 Products	
Bacteria:	Not less than 1.0 log reduction from the initial count at 14 days, and no increase from the 14 days' count at 28 days.
Yeast and Molds:	No increase from the initial calculated count at 14 and 28 days.
For Category 4 Products	
Bacteria, Yeast, and Molds:	No increase from the initial calculated count at 14 and 28 days.

(55) BIOLOGICAL INDICATORS— RESISTANCE PERFORMANCE TESTS

TOTAL VIABLE SPORE COUNT

For paper carrier biological indicators, remove three specimens of the relevant biological indicators from their original individual containers. Disperse the paper into component fibers by placing the test specimens in a sterile 250-mL cup of a suitable blender containing 100 mL of chilled, sterilized *Purified Water* and blending for a time known to be adequate to achieve a homogeneous suspension. It is not unusual for blending times of 15 minutes or more to be required for optimal recovery. Transfer a 10-mL aliquot of the suspension to a sterile, screw-capped 16- × 125-mm tube. For *Biological Indicator for Steam Sterilization, Paper Carrier*, heat the tube containing the suspension in a water bath at 95° to 100° for 15 minutes (heat shock), starting the timing when the temperature reaches 95°. For *Biological Indicator for Dry-Heat Sterilization, Paper Carrier*, and for *Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier*, heat the tube containing the suspension in a water bath at 80° to 85° for 10 minutes, starting the timing when the temperature of the spore suspension reaches 80°. Cool rapidly in an ice-water bath at 0° to 4°. Transfer two 1-mL aliquots to suitable tubes, and make appropriate serial dilutions in sterilized *Purified Water*, the dilutions being selected as calculated to yield preferably 30 to 300 colonies, but not less than 6, on each of a pair of plates when treated as described below. Where the biological indicator has a low spore concentration, it may be necessary to modify the dilution series and to use more plates at each dilution. Prepare a separate series of plates for each aliquot. Place 1.0 mL of each selected dilution in each of two 15- × 100-mm Petri dishes. Within 20 minutes, add to each plate 20 mL of *Soybean–Casein Digest Agar Medium* that has been melted and cooled to 45° to 50°. Swirl to attain a homogeneous suspension, and allow it to solidify. Incubate the plates in an inverted position at 55° to 60° for *Biological Indicator for Steam Sterilization, Paper Carrier*, and at 30° to 35° for *Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier* and for *Biological Indicator for Dry-Heat Sterilization, Paper Carrier* or at the optimal recovery temperature specified by the manufacturer. Examine the plates after 24 and 48 hours, recording for each plate the number of colonies; and use the number of colonies observed after 48 hours to calculate the results. Calculate the average number of spores per specimen from the results, using the appropriate dilution factor. The test is valid

if the log number of spores per carrier at 48 hours is equal to or greater than the log number after 24 hours in each case. For *Biological Indicator for Steam Sterilization, Self-Contained*, aseptically remove the three carriers from the container, and proceed as directed for *Biological Indicator for Steam Sterilization, Paper Carrier*.

For *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers*, aseptically remove the three carriers from their original packaging or container. Place each carrier in a suitable sterile container containing 100 mL of chilled *Purified Water*, and sonicate or shake on a reciprocal shaker for an appropriate time. Fifteen minutes or more may be required for optimal recovery. A previous study should be conducted that ensures that the recovery method results in at least 50% to 300% recovery of the labeled spore viable count. Transfer a 10-mL aliquot of the suspension to a sterile, screw-capped 16- × 125-mm tube. Heat the tubes containing suspensions of *Bacillus atrophaeus*, *Bacillus subtilis*, and *Bacillus coagulans* at 80° to 85° for 10 minutes. Heat the tubes containing a suspension of *Geobacillus stearothermophilus* at 95° to 100° for 15 minutes. Start the timing when the lowest temperature of the stated temperature ranges is reached. Cool rapidly in an ice-water bath at 0° to 4°. Transfer two 1-mL aliquots to suitable tubes, and make appropriate serial dilutions in *Purified Water*. The selected dilutions should be those that will preferably yield 30 to 300 colonies but not fewer than 6 on each pair of plates when treated as described below. When the biological indicator has a low spore concentration, it may be necessary to modify the dilution series and to use more plates at each dilution. Prepare a separate series of plates for each aliquot. Place 1.0 mL of each selected dilution in each of two 15- × 100-mm Petri dishes. Within 20 minutes add the aliquot to each plate containing 20 mL of agar that has been melted and cooled to between 45° and 50°. Swirl to attain a homogeneous suspension.

For *G. stearothermophilus*, *B. atrophaeus*, *B. subtilis*, and *B. coagulans*, use *Soybean–Casein Digest Agar Medium* and incubate the plates in an inverted position aerobically at the following respective temperatures for each microorganism: 55° to 60°, 30° to 35°, and 48° to 52°, or at the optimum temperature specified by the biological indicator manufacturer. Examine the plates after 24 and 48 hours. Record the number of colonies observed on each plate. Calculate the average number of spores per carrier from the results, using the appropriate dilution factor. The test is valid if the log number of spores per carrier at 48 hours is equal to or greater than the log number after 24 hours in each case.

For *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Liquid Spore Suspensions*, using *G. stearothermophilus*, *B. atrophaeus*, *B. subtilis*, and *B. coagulans* as biological indicators, prepare an appropriate serial dilution of the original spore suspension in chilled *Purified Water* contained in a sterile, screw-capped 16- × 125-mm tube, and proceed with the viable spore count procedures specified under *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers*.

D-VALUE DETERMINATION

Conduct all the tests described in this section under aseptic conditions, using sterilized equipment for nonthermophilic microorganisms. D-value determination for *G. stearothermophilus* and *B. coagulans* can be performed in a controlled but unclassified environment.

Apparatus

The test equipment for the determination of microbial resistance is described in substantial detail in ISO 18472, *Sterilization of Health Care Products—Biological and Chemical In-*