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.

[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.

Microbiological Tests

⟨51⟩ ANTIMICROBIAL EFFECTIVENESS TESTING

Antimicrobial preservatives are substances added to nonsterile 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 microorganisms[1]: *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 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

[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
Escherichia coli (ATCC No. 8739)	Soybean–Casein Digest Broth; Soybean–Casein Digest Agar	32.5 ± 2.5°	18 to 24 hours	3 to 5 days
Pseudomonas aeruginosa (ATCC No. 9027)	Soybean–Casein Digest Broth; Soybean–Casein Digest Agar	32.5 ± 2.5°	18 to 24 hours	3 to 5 days
Staphylococcus aureus (ATCC No. 6538)	Soybean–Casein Digest Broth; Soybean–Casein Digest Agar	32.5 ± 2.5°	18 to 24 hours	3 to 5 days
Candida albicans (ATCC No. 10231)	Sabouraud Dextrose Agar; Sabouraud Dextrose Broth	22.5 ± 2.5°	44 to 52 hours	3 to 5 days
Aspergillus niger (ATCC No. 16404)	Sabouraud Dextrose Agar; Sabouraud Dextrose Broth	22.5 ± 2.5°	6 to 10 days	3 to 7 days

(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 ± 2.5°. 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.
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.