

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-*

*dicators—Test Equipment.*¹ The details of individual Biological Indicator Evaluation Resistometers (BIERs) vary with the specifics of their design and the particular sterilization process in conjunction with which they are used. Provided that the performance of the BIER vessel meets the requirements of the ISO standard for exposure of the biological indicator, design differences are acceptable.

Procedure

Carry out the tests for D value at each of the applicable sets of sterilization conditions for which the packaged biological indicator under test is labeled for use. Take a sufficient number of groups of specimens of biological indicators in their original individual containers, each group consisting of not less than 5 specimens. The number of groups provides a range of observations from not less than one labeled D value below the labeled survival time through not less than one labeled D value above the labeled kill time. Place each group on a separate suitable specimen holder that permits each specimen to be exposed to the prescribed sterilizing condition at a specific location in the sterilizing chamber of the BIER. Check the BIER apparatus for operating parameters using specimen holders without specimens. Select a series of sterilizing times in increments from the shortest time for the specimens to be tested. The differences in sterilizing times over the series are as constant as feasible, and the difference between adjacent times is no greater than 75% of the labeled D value.

Test procedures for the use of BIER vessels for the evaluation of microbial resistance are defined in a series of ISO standards under the 11138 series.^{2, 3, 4, 5} The appropriate standard should be followed for the biological indicator. The test methods and carriers used with the BIER may be adapted to the specifics of the biological indicator. The method and apparatus used for paper carriers may differ from those for other carriers and will be substantially different from those used for suspensions of biological indicators.

The D-value exposure conditions for alternative material carriers are the same as the conditions used to determine the D value for paper carriers. If the manufacturer's label permits usage of the biological indicator carrier with multiple sterilization methods, then data on D value, survival time, and kill time will need to be provided by the manufacturer for each sterilization method. It is possible that biological indicators inoculated onto carriers other than paper will be used for gaseous or vapor sterilization/decontamination methods such as vapor phase hydrogen peroxide and chlorine dioxide.

Standard physical conditions for the evaluation of biological indicators for use with vapor phase hydrogen peroxide or chlorine dioxide have not been defined. In the case of chlorine dioxide, concentration of the gas, relative humidity, and temperature are critical process control conditions that can be accurately measured. The manufacturer of biological indicators marketed for use with chlorine dioxide should state the conditions under which the D-value determination was conducted so that the user can at least discern the

resistance of a lot of biological indicators as compared to their own anticipated use conditions. The situation with vapor phase hydrogen peroxide is a more complex one. Various equipment manufacturers have proposed different decontamination or sterilization conditions. Thus, there is no standard process for the conduct of vapor phase hydrogen decontamination or surface sterilization. It follows, then, that there are no industry standard biological indicator evaluation methods for vapor hydrogen peroxide, and it has been reported that there may not be a direct correlation between vapor concentration and rate or even effectiveness of biological indicator inactivation. Additionally, it is difficult to accurately assess relative humidity, which is often defined as a critical process parameter, in the presence of vapor hydrogen peroxide. For these reasons it is more reasonable to consider resistance of biological indicators to be a relative or comparative measure from the manufacturer rather than a true D value. It follows that, depending upon equipment and processes employed, it may be impossible for an end user to duplicate the biological indicator resistance tests performed by the manufacturer.

For *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Liquid Spore Suspensions*, conduct D-value determinations for each of the microorganisms that are provided as a liquid spore crop suspension. The test is conducted using appropriate serial dilutions predicated upon the stated spore titer of the suspension in *Purified Water* in a sterile tube.

Where the suspension is placed on or in a substrate such as an elastomeric closure or formulated product, its resistance may differ from that determined in *Purified Water*. That difference may be significant to the usage of the biological indicators and appropriate measurements made prior to use in sterilization validation activities.

Recovery

After completion of the sterilizing procedure for *Biological Indicator for Dry-Heat Sterilization, Paper Carrier*; *Biological Indicator for Ethylene Oxide Sterilization, Paper Carrier*; or *Biological Indicator for Steam Sterilization, Paper Carrier*, which ever is applicable, and within a noted time not more than 4 hours, aseptically remove and add each strip to a suitable medium (see *Media* under *Sterility Tests* (71)) to submerge the biological indicator completely in a suitable tube. For each *Biological Indicator for Steam Sterilization, Self-Contained* specimen, the paper strip is immersed in the self-contained medium according to manufacturers' instructions, within a noted time not more than 4 hours. Incubate each tube at the optimal recovery temperature specified by the manufacturer. Observe each inoculated medium-containing tube at appropriate intervals for a total of 7 days after inoculation. (Where growth is observed at any particular observation time, further incubation of the specimen(s) concerned may be omitted.) Note the number of specimens showing no evidence of growth at any time.

For *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Nonpaper Carriers*, recovery of spores from the biological indicator carriers will follow recovery procedures described in the procedures under *Total Viable Spore Count*. D-value determination methods for paper carrier biological indicators may be used to calculate the D value for nonpaper carriers. Incubation conditions for the microorganisms that may be used for nonpaper biological indicators are described in the *Total Viable Spore Count* section.

For *Biological Indicators for Moist Heat, Dry Heat, and Gaseous Modes of Sterilization, Liquid Spore Suspensions*, the method of recovery following sterilization exposure conditions are those methods described in the *Total Viable Spore Count* section for liquid suspensions, and when a dry heat D-value determination is made from *B. atrophaeus* suspensions, the same recovery procedures as described under *Bio-*

¹ANSI/ AAMI/ ISO 18472 : 2006, Sterilization of Health Care Products—Biological and Chemical Indicators—Test Equipment. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA 22201-4795

²ANSI/AAMI/ISO 11138-1 : 2006, Sterilization of health care products—Biological indicators—Part 1: General requirements, 2nd ed. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.

³ANSI/AAMI/ISO 11138-2 : 2006, Sterilization of health care products—Biological indicators—Part 2: Biological indicators for ethylene oxide sterilization processes, 3rd ed. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.

⁴ANSI/AAMI/ISO 11138-3 : 2006, Sterilization of health care products—Biological indicators—Part 3: Biological indicators for moist heat sterilization processes. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.

⁵ANSI/AAMI/ISO 11138-4 : 2006, Sterilization of health care products—Biological indicators—Part 4: Biological indicators for dry heat sterilization processes. Association for the Advancement of Medical Instrumentation (AAMI), 1110 N. Glebe Road, Suite 220, Arlington, VA.

logical Indicator for Steam Sterilization, Paper Carrier are followed.

Where *C. sporogenes* is used as a biological indicator, methods for preparation, inoculation, and recovery methods and media must be adapted to accommodate the use of this anaerobic sporeformer.

Calculation

The determination of D values of biological indicators can be performed using the Limited Spearman-Kärber, Survival Curve Method or Stumbo-Murphy-Cochran procedures.^{6, 7, 8} It is preferable to use the same method as that defined by the biological indicator manufacturer to determine D values. The use of a different method can result in differences that are more an artifact of the method than a variation in the performance of the biological indicator.

Survival Time and Kill Time

Take two groups, each consisting of 10 specimens of the relevant biological indicator, in their original, individual containers. Place the specimens of a group in suitable specimen holders that permit each specimen to be exposed to the sterilizing conditions at a specific location in the BIER chamber.

Expose the specimens for the required survival time, enter the chamber, and remove the holder(s) containing the 10 specimens. Repeat the above procedure immediately, or preheat if a substantial interval has elapsed, so as to subject the second holder(s) containing 10 specimens similarly to the first conditions, but for the required kill time.

The *Survival time and kill time* for all monographed biological indicators is described in the official monograph under the heading for each.

(61) MICROBIOLOGICAL EXAMINATION OF NONSTERILE PRODUCTS: MICROBIAL ENUMERATION TESTS

INTRODUCTION

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

The tests are designed primarily to determine whether a substance or preparation complies with an established speci-

⁶ Pflug, I.J. *Syllabus for an Introductory Course in the Microbiology and Engineering of Sterilization Processes*, 4th ed. St. Paul, MN: Environmental Sterilization Services, 1980.

⁷ Pflug, I.J., and G.M. Smith. The Use of Biological Indicators for Monitoring Wet-Heat Sterilization Processes, in *Sterilization of Medical Products*, ed. E.R.L. Gaughran and K. Kereluk. New Brunswick, NJ: Johnson and Johnson, 1977, 193–230.

⁸ Holcomb, R.G., and I.J. Pflug. The Spearman-Kärber Method of Analyzing Quantal Assay Microbial Destruction Data, in *Microbiology and Engineering Sterilization Processes*, ed. I.J. Pflug. St. Paul, MN: Environmental Sterilization Services, 1979.

fication for microbiological quality. When used for such purposes, follow the instructions given below, including the number of samples to be taken, and interpret the results as stated below.

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

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

GENERAL PROCEDURES

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

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

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

ENUMERATION METHODS

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

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

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

General Considerations

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

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

Preparation of Test Strains

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