⟨55⟩ BIOLOGICAL INDICATORS—RESISTANCE PERFORMANCE TESTS

INTRODUCTION

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

TOTAL VIABLE SPORE COUNT

Sample Collection/Recovery

PAPER/FIBER INDICATORS

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

INDICATORS ON OTHER SUBSTRATES

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

SPORE SUSPENSIONS

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

Viable Spore Count

Transfer a 10-mL aliquot of the suspension to a sterile tube. For BIs using spores of *Geobacillus stearothermophilus*, *Bacillus coagulans*, and other thermophilic spore formers, heat the tube containing the suspension in a water bath at 95°–100° for 15 min (heat shock), starting the timing when the temperature reaches 95°. For BIs containing nonthermophilic spore formers, heat the tube containing the suspension in a water bath at 80°–85° for 10 min, starting the timing when the temperature of the spore suspension reaches 80°. Cool suspensions rapidly in an ice-water bath at 0°–4°. Transfer two 1-mL aliquots to suitable tubes, and make appropriate serial dilutions in sterilized *Purified Water*. Calculate the dilutions to yield 30–300 colonies on each plate in a pair, when treated as described below. Where the BI 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 min, add to each plate 20 mL of *Soybean–Casein Digest Agar Medium* that has been melted and cooled to approximately 45°. Swirl to attain a homogeneous suspension, and allow it to solidify. Incubate the plates in an inverted position at 55°–60° for thermophilic spore formers and at 30°–35° for nonthermophilic spore formers or at the optimal recovery temperature specified by the manufacturer. Examine the plates after at least 48 h, recording for each plate the number of colonies. Calculate the average number of spores per test sample from the results, using the appropriate dilution factor. When evaluating vendor-supplied BIs, the viable spore count shall be between 50% and 300% of the manufacturer's stated value.

Change to read:

D-VALUE DETERMINATION

Apparatus

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

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

Procedure

Carry out the tests for D-value at sterilization conditions consistent with those intended for use. Use 20 replicate test sample BIs in their original individual containers, subjected to at least five exposure conditions for a total of 100 tests. The number of exposure conditions is chosen to provide a range of observations from NLT one labeled D-value below the expected survival time through NLT one labeled D-value above the expected kill time. Place each group on a separate suitable sample holder that permits each sample 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 sample holders without test samples. Select a series of sterilizing times in increments from the shortest time for the samples to be tested. The differences in sterilizing times over the series are as constant as feasible, and the difference between adjacent times is NMT 75% of the expected 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*–*5*). The appropriate standard should be followed for the BI. The test methods and carriers used with the BIER may be adapted to the specifics of the BI. 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 BIs.

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 a manufacturer's label permits usage of the BI 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.

For BIs that are 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▲ (ERR 1-Nov-2019) 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 BIs and appropriate measurements made before use in sterilization validation activities.

Recovery

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

Incubate each tube at the optimal recovery temperature appropriate for the BI. 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 test sample(s) concerned may be omitted. Note the number of samples showing no evidence of growth at any time.

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

Calculation

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

Survival Time and Kill Time

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

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

REFERENCES

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