INTERNATIONAL

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Standard Test Method for Determining Bacterial Retention of Membrane Filters Utilized for Liquid Filtration[1]

This standard is issued under the fixed designation F838; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

~~NOTE—Fig. 1 was editorially updated and the year date changed on Sept. 30, 2015.~~

~~ε1 NOTE—9.1 was editorially corrected in August 2018.~~

1. Scope

1.1 This test method determines the bacterial retention characteristics of membrane filters for liquid filtration using *Brevundimonas diminuta* as the challenge organism. This test method ~~may~~can be ~~employed~~used to evaluate any membrane filter system used for liquid sterilization.

1.2 This test method is not intended to be used in performance of product- and process-specific validation of the bacterial retention characteristics of membrane filters to be used in pharmaceutical or biopharmaceutical sterilizing filtration, or both. Process- and product-specific bacterial retention validation should be carried out using the intended product manufacturing process parameters and the product solution or surrogate as the carrier fluid.

1.3 The values stated in SI units are to be regarded as standard.

1.3.1 *Exception*—The inch-pound values given for units of pressure are to be regarded as standard; SI unit conversions are shown in parentheses.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.5 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D1193 Specification for Reagent Water

3. Terminology

3.1 *Definitions:*

[1] This test method is under the jurisdiction of ASTM Committee E55 on Manufacture of Pharmaceutical and Biopharmaceutical Products and is the direct responsibility of Subcommittee E55.03 on General Pharmaceutical Standards.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the ~~standard's~~standard's Document Summary page on the ASTM website.

3.1.1 *log reduction ~~value—~~value, n—*the logarithm to the base 10 of the ratio of the number of microorganisms in the challenge to the number of organisms in the filtrate.

4. Summary of Test Method

4.1 After sterilization, the test filter is challenged with a suspension of *B. diminuta* (ATCC 19146[3]) ~~at a concentration of~~ to provide 10^7 organisms per cm^2 of effective filtration area (EFA) at a maximum differential pressure across the test filter of 30 psig (206 kPa) and a ~~flow rate~~ flux of 2 to 4 × 10^{-3} ~~LPM per~~ L per min (3.3 to 6.7 × 10^{-8} m^3/s) per cm^2 of effective filtration area. The entire filtrate is ~~then filtered~~ passed through an analytical membrane ~~filer~~filter disc, which is subsequently incubated on a solidified growth medium. Microorganisms that are not retained by the filter being tested will develop into visible colonies on the analysis membrane and can then be enumerated.

5. Significance and Use

5.1 This test method is designed to assess the retentivity of a sterilizing filter under standard challenge conditions.

5.1.1 A challenge of 10^7 bacteria per cm^2 of effective filtration area is selected to provide a high degree of assurance that the ~~filter will be challenged uniformly across the membrane surface to assure it~~method has sufficient sensitivity to detect oversized pores and that the filter will quantitatively retain large numbers of organisms. The model challenge organism, *B. diminuta*, is widely considered to be a small bacterium and is recognized as an industry standard for qualifying sterilizing filters. Other species may represent a worst-case test in terms of ability to penetrate a filter. This test does not provide assurance that filters can completely retain such bacteria.

5.1.2 The analytical procedure utilized in this test method provides a method to assign a numerical value to the filtration efficiency of the filter being evaluated under standard filtration conditions. For the purpose of product sterility assurance, additional process-specific studies should be performed.

6. Apparatus

6.1 Assemble the apparatus described below as in Fig. 1:

6.1.1 *Stainless Steel Pressure Vessel,* 12-L capacity (or larger), fitted with a 0 to 50-psi (0 to 350-kPa) pressure gauge.

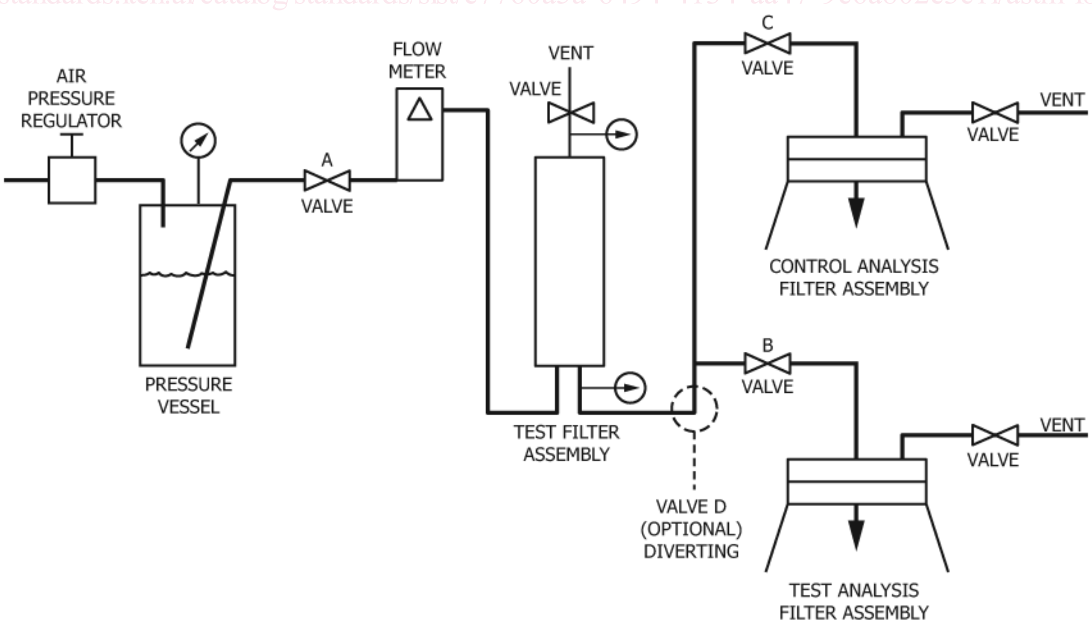

FIG. 1 Test Set-Up for ~~Bacteria~~Bacterial Retention Testing

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

6.1.2 *Air Regulator.*

6.1.3 ~~*47-mm–142-mm*~~ *47-mm or 142-mm Analysis Disc Filter Assemblies,* two or more, with hose or sanitary connections as applicable.

6.1.4 *Diaphragm-Protected 0 to 50-psi (0 to 350-kPa) Pressure Gauge,* for upstream pressure reading.

6.1.5 *Manifold,* with valves (autoclavable) and hose connections.

6.1.6 *Autoclavable Tubing,* (must be able to withstand a pressure of 50 psi (350 kPa)).

6.1.7 *Filter Housing,* with hose connections.

6.1.8 *Hose Clamps.*

~~6.1.9 *Incubator,* 30 ± 2°C.~~

~~6.1.10 *Laminar Flow Bench.*~~

~~6.1.11 *Smooth-Tip Forceps.*~~

6.1.9 *Test Filter.*

7. Purity of Reagents and Materials

7.1 *Purity of Reagents*—Reagent grade chemicals shall be used. Unless otherwise indicated, all reagents shall conform to the specifications of the American Chemical Society, where such specifications are available.[4]

7.2 *Purity of Water*—Unless otherwise indicated, references to water shall mean reagent water, Type IV as defined in Specification D1193.

7.2.1 Additionally, any water used in this test method must conform to the requirements for non-bacteriostatic water specified in the current edition of *Standard Methods for the Examination of Water and Wastewater*.[5]

8. Reagents and Materials

8.1 *Saline Lactose Broth Medium:*

8.1.1 *Lactose Broth*—Dissolve 1.3 g of dehydrated lactose broth medium in 100 mL of water.

8.1.2 *Sodium Chloride Solution*—Dissolve 7.6 g of sodium chloride (NaCl) in 970 mL of water in a 2-L flask with an appropriate closure.

8.1.3 Add 30 mL of lactose broth (8.1.1) to 970 mL of sodium chloride solution. Autoclave at ~~121°C~~ 121 °C for 15 min.

8.2 *Frozen Cell Paste Method:*

8.2.1 *Growth Medium A*—Dissolve in water and dilute to 1 L. Autoclave at ~~121°C~~ 121 °C for 15 min (pH 6.8 to 7.0).

[4] ~~*Reagent Chemicals, American Chemical Society Specifications,*~~ *ACS Reagent Chemicals, Specifications and Procedures for Reagents and Standard-Grade Reference Materials,* American Chemical Society, Washington, ~~DC, www.chemistry.org.~~ DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see *Analar Standards for Laboratory Chemicals*, BDH Ltd., Poole, Dorset, U.K., and the *United States Pharmacopeia and National Formulary*, U.S. Pharmacopeial Convention, Inc. (USPC), Rockville, ~~MD, http://www.usp.org.~~ MD.

[5] Available from the American Public Health Association (APHA), 800 I Street, NW, Washington, DC 20001-3710, http://www.apha.org.

Tryptic Peptone (or Casitone)	7.5 g
Yeast Extract	2.5 g
Sodium Chloride (NaCl)	0.5 g
Magnesium Sulfate ($MgSO_4 \cdot 3H_2O$)	0.35 g

8.2.2 *Harvesting Buffer*—Dissolve 0.790 g of monobasic potassium phosphate (KH_2PO_4) and 1.0 g of Kdibasic potassium phosphate (K_2HPO_4) in 100 mL of glycerol ($C_3H_8O_3$). Adjust to pH 7.2 with 0.1 N potassium hydroxide solution. Dilute to 1 L with water and sterilize at 121°C 121 °C for 15 min.

8.2.3 *Potassium Hydroxide Solution (0.1 N)*—Dissolve 5.61 g of potassium hydroxide (KOH) in water and dilute to 1 L in a volumetric flask.

8.2.4 *Tryptic Soy Agar*—Prepare according to manufacturer's instructions.

8.2.5 *Tryptic Soy Broth*—Prepare according to manufacturer's instructions.

8.3 *Analytical Reagents and Materials:*

8.3.1 *M-Plate Count Agar*—Prepare according to manufacturer'smanufacturer's instructions.

8.3.2 *Peptone Water (1 g/L)*—Dissolve the peptone in water. Dispense suitable volumes, for preparing decimal dilutions, into screw-capsuitable containers. Autoclave at 121°C 121 °C for 15 min.

8.4 *B. diminuta* (ATCC 19146).

8.5 *Analytical Membrane Filters,* 47-mm or 142-mm diameter, 0.45 µm nominal pore size, 130 to 160 µm thick.

8.6 *Petri Dishes,* 150-mm diameter.

8.7 *Incubator,* 30 ± 2 °C.

8.8 *Unidirectional Airflow Bench.*

9. Methods for Preparation of Bacterial Challenge Stock Suspension

9.1 *General*—The following two methods have been used extensively for the preparation of *B. diminuta* challenge suspensions. The presentation of these methods is not meant to exclude other equally valid methods for the preparation of *B. diminuta*. It is important, however, that any *B. diminuta* challenge suspension used is monodispersemonodispersed and meets the criteria set forth in Section 10.

9.2 Reconstitute the culture according to directions provided by the American Type Culture Collection (ATCC). Check the purity of the reconstituted culture by means of streak plates. Examine for uniform colony morphology,morphology and identify single-cell isolates as *B. diminuta* in accordance with Section 10.

9.2.1 *Stock Cultures*—Prepare stock cultures from single cell isolates of 9.2. Inoculate tryptic soy agar slants and incubate at 30 ± 2°C 2 °C for 24 h. Overlay slants with sterile mineral oil and store at 4°C. 4 °C. Check weekly for viability and purity. Alternatively, tryptic soy semisolid agar stab cultures may be substituted for the slant cultures.

9.2.2 *Long Term Storage of Cultures*—Lyophilize or store in liquid nitrogen.

9.3 *Preparation of Challenge Stock Suspension in Saline Lactose Broth:*

9.3.1 Inoculate 10-mL sterile tryptic soy broth with stock culture (9.2.1) and incubate at 30 ± 2°C 2 °C for 24 h.

9.3.2 Transfer 2 mL of agitated broth culture to 1 L of sterile saline lactose broth, swirl to mix inoculum and incubate at 30 ± 2°C 2 °C for 24 h. h with agitation. Check purity of seed broth.

NOTE 1—Saline lactose broth suspension may be stored at ~~4°C~~ 4 °C for up to 8 h prior to use.

9.3.3 Determine the concentration of viable cells in the challenge suspension according to Section 11 (expected concentration is 10^7 to 10^8 cells/mL).

9.3.4 Identify the organisms as *B. diminuta* in accordance with Section 10.

9.4 *Preparation of Frozen Cell Paste of B. diminuta:*

9.4.1 Inoculate 10 mL of Sterile Growth Medium A (8.2.1) with the stock culture (9.2.1) and incubate at 30 ± ~~2°C~~ 2 °C for 24 h.

9.4.2 Transfer 10 mL of the bacterial suspension from ~~9.3.1~~9.4.1 into 500 mL of Sterile Growth Medium A and incubate at 30 ± ~~2°C~~ 2 °C for 24 h.

9.4.3 Prepare 10 L of a seed culture by transferring 200 mL of the bacterial suspension from 9.4.2 into 10 L of Sterile Growth Medium A. Incubate at 30 ± ~~2°C~~ 2 °C for 24 h.

9.4.4 Inoculate the 10 L of the seed culture into 500 L of Growth Medium A. Grow aerobically at 30 ± ~~2°C.~~ 2 °C. Monitor growth spectrophotometrically at 500 nm, and plot growth curve.

9.4.5 When the culture reaches the stationary phase, harvest the cells by continuous flow centrifugation.

9.4.6 Re-suspend cells in two to three volumes of cold sterile harvesting buffer.

9.4.7 Centrifuge suspension and re-suspend cells in an equal volume of harvesting buffer. Determine the cell concentration (expected concentration of viable cells is 1×10^{12} cells/mL).

9.4.8 Transfer aliquots (for example, 50 mL) of cell paste into sterile plastic centrifuge tubes, and freeze using dry ice-acetone batch or liquid nitrogen. Store frozen cell paste at ~~–70°C.~~ –70 °C.

9.5 *Preparation of Challenge Stock Suspension from Frozen Cell Paste:*

9.5.1 Disinfect the tube containing the cell paste by dipping tube in 80 % ethyl alcohol and flaming just long enough to burn off most of the alcohol. Use sterile tongs to hold tube.

9.5.2 Aseptically remove the cap from the tube and drop the tube into a sterile Erlenmeyer flask containing a sterile magnetic stirring bar and 20 cell volumes of a sterile solution of 0.9 % NaCl which contains 0.001 to 0.002 M $MgCl_2$ at room temperature (for example, transfer a 50-mL aliquot of frozen cell paste into 1 L of sterile solution).

NOTE 2—$MgCl_2$ must be in the solution prior to adding the frozen cell paste to prevent ~~dumping~~clumping during thaw.

9.5.3 Place the flask on a magnetic stirring unit, and mix until the entire contents of the tube is suspended evenly (about 40 min).

9.5.4 Determine the concentration of viable cells according to Section 11 (expected concentration of the cell suspension is 1 to 2×10^{10} cells/mL).

9.5.5 Identify the organism as *B. diminuta* in accordance with Section 10.

10. Identification of *B. diminuta*

10.1 *Colonial Morphology:*

10.1.1 Colonies of *B. diminuta* are yellow-beige, slightly convex, complete and shiny.

10.1.2 At ~~30°C~~ 30 °C (optimum growth temperature) colonies are microscopic to pinpoint after 24 h and 1 to 2-mm diameter after 36 to 48 h.

10.2 *Microscopic Examination:*

10.2.1 Prepare a Gram stain.

10.2.1.1 Examine the preparation with a compound light microscope fitted with a calibrated ocular micrometer and an oil immersion objective lens with good resolving power (for example, a ~~planachromatic~~ plan achromatic objective with a numerical aperture of 1.2 or greater). Observe several microscopic fields for organisms' size and arrangement of cells.

10.2.1.2 Stained preparations should reveal a Gram-negative, small, rod-shaped organism about 0.3 to 0.4 µm by 0.6 to 1.0 µm in size, occurring primarily as single cells.

10.2.2 Prepare a flagella stain (optional). *B. diminuta* is characterized by a single, polar flagellum.

10.3 *Biochemical Characterization:*

10.3.1 Perform a number of the following biochemical characterization tests. *B. diminuta* gives the results indicated:[6]

Test	*B. diminuta* (ATCC 19146)
Spore formation	–
OF glucose medium, open	–
OF glucose medium, sealed	–
OF ethanol (3 %) medium, open	+
OF ethanol (3 %) medium, sealed	–
Indole	–
Methyl red	–
Acetylmethylcarbionol	–
Gelatinase	–
Aerobe	+
Catalase	+
Cytochrome (Indophenol) oxidase	+
Growth on MacConkey agar	+
Denitrification	+
DNAase (BBL DNase Test agar or equivalent)	–
Centrimide tolerance	–

11. Preparation of Bacterial Challenge Suspension

11.1 Determine by direct microscopic count the bacterial ~~titre~~titer of the suspension. This will determine the total number, viable and nonviable, cells present.

11.2 Using the appropriate volume of a challenge stock suspension, prepare an appropriate volume of a challenge suspension of *B. diminuta* in a saline lactose broth or sterile saline to contain a minimum total of 10^7 organisms per ~~square~~cm^2 ~~centimetre~~(10^{10} ~~of test filter~~organisms per ft^2 ~~area 10~~) of test^{10} ~~m/ft~~filter^2~~.~~ area. Mix well.

11.3 Aseptically remove a sample from the prepared challenge suspension of *B. diminuta*.

11.4 Within a ~~laminar flow~~unidirectional airflow hood, aseptically prepare dilutions of the suspension through 10^{-6} using 0.1 % Peptone water.

11.5 Perform viable cell assay, in duplicate, using the membrane filter assay or direct spread plate assay under conditions that are similar to those specified for ~~sterility~~ microbial enumeration testing in the current edition of the *United States Pharmacopeia*.[7]

[6] Confirmation of the identity of *B. diminuta* may also be achieved using molecular-based or other qualified methods (for example, 16S rRNA Sequencing, FAME).

[7] Available from the U.S. Pharmacopeial Convention (USP) 12601 Twinbrook Pkwy, Rockville, MD 20852, http://www.usp.org.

11.5.1 For the membrane filter assay, use 1 mL from the 10^{-4} through the 10^{-6} dilutions. Place 50 mL of sterile 0.9 % NaCl solution into the funnel of the filter holder prior to adding the 1.0 mL aliquots of the decimal dilutions. Filter and wash the walls of the funnel with 50 mL of sterile 0.9 % NaCl solution. Remove assay membrane from funnel, and place on agar medium.

11.5.2 For the direct spread plate assay, use 0.1 mL from 10^{-3}, 10^{-4}, 10^{-5} dilutions.

11.6 Incubate the membrane or spread assay plates at 30 ± ~~2°C~~ 2 °C for 48 h.

11.7 Count the colonies on the plates showing between 30 and 300 colonies (20 to 200 colonies on membrane filters) and calculate the concentration cell/mL of the original suspensions.

11.8 Compare the viable ~~titre~~titer with the direct microscopic count determined in 11.1. The viable count should be no less than 25 % of the total cell count.

12. Equipment Preparation

12.1 Install the filter to be tested in the housing. Wrap the inlet and outlet connections with autoclave paper, and autoclave according to ~~manufacturer's~~manufacturer's instructions. Alternatively, the test filter may be steam sterilized in-situ or gamma irradiated according to ~~manufacturer's~~manufacturer's instructions. The sterilization procedure should be validated using biological indicators, thermocouples, or other appropriate devices (dosimeters).

12.1.1 Aseptically perform an integrity test on the filter using an appropriate procedure recommended by the filter manufacturer.

12.2 Assemble analysis filter membranes in filter assemblies. Attach autoclavable tubing 1 to 2 ft to the inlets and outlets. Wrap the hose ends with a single layer of autoclavable paper. Autoclave in accordance with manufacturer's instructions usually 30 to 45 min at 15 psi (103 kPa) and ~~121°C.~~121 °C.

12.3 Wrap the manifold and connecting hose (valves must be in open position) in autoclave paper and autoclave. Alternatively, the manifold may be connected to the test filter assembly outlet and autoclaved or in-situ steam sterilized simultaneously. This will eliminate one aseptic connection downstream prior to testing. This sterilization procedure should be validated using biological indicators or thermocouples.

12.4 Place all sterilized units in ~~laminar flow~~ the unidirectional airflow bench for assembly.

12.5 The pressure vessel and the upstream connecting tubing do not need to be autoclaved, but should be thoroughly cleaned, disinfected, and flushed with sterile water prior to the test. The vessel may be disinfected with a 1 + 999 dilution of 5 % sodium hypochlorite solution in water or 70 % ethanol, drained, and rinsed thoroughly with sterile water.

12.6 Make all connections aseptically in the ~~laminar flow~~unidirectional airflow bench (see Fig. 1 for test system).

13. Test Procedure[8]

13.1 *Control*—The control is run immediately prior to the bacterial challenge test, and the control and ~~challenge~~test analysis filters are incubated simultaneously.

13.1.1 Add a sufficient volume of sterile buffered water or sterile saline to the pressure vessel.

13.1.2 Close Valves A, B, C.

13.1.3 Increase vessel pressure to 30 psi (207 kPa).

13.1.4 Open Valve A slowly and fill the test filter assembly with liquid. Vent air from the test filter assembly into a suitable disinfectant. When the test filter assembly is full of liquid, close the vent valve.

[8] Hydrophobic filters must be pre-wetted prior to challenge. A method to accomplish this is described in Annex A1.