



Designation: F838 – 05

Standard Test Method for Determining Bacterial Retention of Membrane Filters Utilized for Liquid Filtration¹

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1. Scope

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

1.2 *This standard may involve hazardous materials, operations, and equipment. 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 and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards*:²
D1193 Specification for Reagent Water

3. Terminology

3.1 *Definitions*:

3.1.1 *log reduction value*—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 *Pseudomonas diminuta* (ATCC 19146)³ at a concentration of 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 of 0.5 to 1.0 GPM per ft^2 of effective filtration area (2 to 4×10^{-3} LPM per cm^2). The entire filtrate is then filtered through an analytical membrane filter disc which is subsequently incubated on a solidified

growth medium. Organisms 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 Since all sterilizing filtration processes are performed under positive pressure, this test method is designed to assess the retentivity of a sterilizing filter under process conditions.

5.1.1 A challenge of 10^7 bacteria per cm^2 of effective filtration area is orders of magnitude higher than one would expect to encounter in a sterilizing filtration process. This level was selected in order to provide a high degree of assurance that the filter would quantitatively retain large numbers of organisms. This concept is important, in view of the requirement to provide a quantitative assessment in validating a sterilization process.

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. This value, coupled with a knowledge of the number and types of organisms (bioburden) indigenous to the process, may then be utilized to determine the probability of obtaining a sterile filtrate. Conversely, the numerical value of the filtration efficiency may be used when one must meet a specified probability of sterility assurance to calculate the volume of fluid that may be filtered in order to maintain that level of assurance.

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 gage.

6.1.2 *Air Regulator*.

6.1.3 *142-mm Disc Filter Assemblies*, two or more, with hose connections.

6.1.4 *Diaphragm-Protected 0 to 50-psi Pressure Gage* (0 to 350-kPa), for upstream pressure reading. A second equivalent gauge for downstream pressure reading is optional.

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*.

¹ This test method is under the jurisdiction of ASTM Committee D19 on Water and is the direct responsibility of Subcommittee D19.24 on Water Microbiology.

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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 Document Summary page on the ASTM website.

³ Available from American Type Culture Collection, 21301 Parklawn Dr., Brookeville, MD 20833.

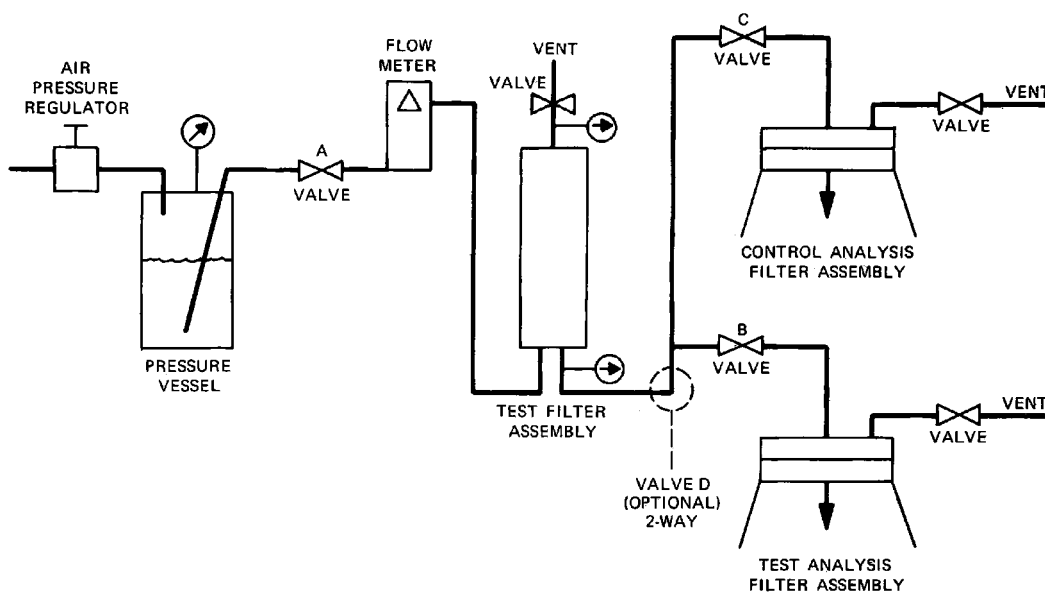


FIG. 1 Test Set-Up for Bacteria Retention Testing

- 6.1.9 Incubator, 30 ± 2°C.
- 6.1.10 Laminar Flow Bench.
- 6.1.11 Smooth-Tip Forceps.

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 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 for 15 min (pH 6.8 to 7.0).

Trypticase Peptone (or Casitone)	7.5 g
Yeast Extract	2.5 g
Sodium Chloride (NaCl)	0.5 g
Magnesium Sulfate (MgSO ₄ ·3H ₂ O)	0.35 g

8.2.2 *Harvesting Buffer*—Dissolve 0.790 g of monobasic potassium phosphate (KH₂PO₄) and 1.0 g of K₂HPO₄ in 100 mL of glycerol (C₃H₈O₃). Adjust to pH 7.2 with 0.1 N potassium hydroxide solution. Dilute to 1 L with water and sterilize at 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 *Trypticase Soy Agar*—Prepare according to manufacturer's instructions.

8.2.5 *Trypticase 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's instructions.

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

8.4 *Pseudomonas diminuta* (ATCC 19146).

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

8.6 *Petri Dishes*, 150-mm diameter.

9. Methods for Preparation of Bacterial Challenge Stock Suspension

9.1 *General*—The following two methods have been used extensively for the preparation of *P. diminuta* challenge suspensions. The presentation of these methods is not meant to exclude other equally valid methods for the preparation of *P. diminuta*. It is important, however, that any *P. diminuta*

⁴ "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC.

⁵ Available from the American Public Health Assoc., 1015 18th St., N.W. Washington, DC 20036.

challenge suspension used is monodisperse 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, and identify single-cell isolates as *P. diminuta* per Section 10.

9.2.1 *Stock Cultures*—Prepare stock cultures from single cell isolates of 9.2. Inoculate trypticase soy agar slants and incubate at $30 \pm 2^\circ\text{C}$ for 24 h. Overlay slants with sterile mineral oil and store at 4°C . Check weekly for viability and purity. Alternatively, trypticase 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 trypticase soy broth with stock culture (9.2.1) and incubate at $30 \pm 2^\circ\text{C}$ 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 $30 \pm 2^\circ\text{C}$ for 24 h. Check purity of seed broth.

NOTE 1—Saline lactose broth suspension may be stored at 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 *Pseudomonas diminuta* per Section 10.

9.4 *Preparation of Frozen Cell Paste of P. 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 $30 \pm 2^\circ\text{C}$ for 24 h.

9.4.2 Transfer 10 mL of the bacteria suspension from 9.3.1 into 500 mL of sterile growth medium A and incubate at $30 \pm 2^\circ\text{C}$ 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 $30 \pm 2^\circ\text{C}$ for 24 h.

9.4.4 Inoculate the 10 L of the seed culture into 500 L of growth medium A. Grow aerobically at $30 \pm 2^\circ\text{C}$. 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 Resuspend cells in two to three volumes of cold sterile harvesting buffer.

9.4.7 Centrifuge suspension and resuspend 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 -60°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 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 (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 *Pseudomonas diminuta* per Section 10.

10. Identification of *Pseudomonas diminuta*

10.1 *Colonial Morphology:*

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

10.1.2 At 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 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). *Pseudomonas diminuta* is characterized by a single, polar flagellum.

10.3 *Biochemical Characterization :*

10.3.1 Perform a number of the following biochemical characterization tests. *Pseudomonas diminuta* gives the results indicated:

Test	<i>P. diminuta</i> (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	+