



Designation: E2196 – 12

Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with Medium Shear and Continuous Flow Using Rotating Disk Reactor¹

This standard is issued under the fixed designation E2196; 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.

1. Scope

1.1 This test method is used for growing a reproducible (1)² *Pseudomonas aeruginosa* biofilm in a continuously stirred tank reactor (CSTR) under medium shear conditions. In addition, the test method describes how to sample and analyze biofilm for viable cells.

1.2 Although this test method was created to mimic conditions within a toilet bowl, it can be adapted for the growth and characterization of varying species of biofilm (rotating disk reactor—repeatability and relevance (2)).

1.3 This test method describes how to sample and analyze biofilm for viable cells. Biofilm population density is recorded as \log_{10} colony forming units per surface area (rotating disk reactor—efficacy test method (3)).

1.4 Basic microbiology training is required to perform this test method.

1.5 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.6 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:³
D5465 Practice for Determining Microbial Colony Counts

¹ This test method is under the jurisdiction of ASTM Committee E35 on Pesticides, Antimicrobials, and Alternative Control Agents and is the direct responsibility of Subcommittee E35.15 on Antimicrobial Agents.

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² The boldface numbers in parentheses refer to a list of references at the end of this standard.

³ 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.

from Waters Analyzed by Plating Methods

2.2 Other Standards:

Method 9050 C.1.a Buffered Dilution Water Preparation (4)

3. Terminology

3.1 *biofilm, n*—microorganisms living in a self-organized community attached to surfaces, interfaces, or each other, embedded in a matrix of extracellular polymeric substances of microbial origin, while exhibiting altered phenotypes with respect to growth rate and gene transcription.

3.1.1 *Discussion*—Biofilms may be comprised of bacteria, fungi, algae, protozoa, viruses, or infinite combinations of these microorganisms. The qualitative characteristics of a biofilm, including, but not limited to, population density, taxonomic diversity, thickness, chemical gradients, chemical composition, consistency, and other materials in the matrix that are not produced by the biofilm microorganisms, are controlled by the physiochemical environment in which it exists.

3.2 *coupon, n*—biofilm sample surface.

4. Summary of Test Method

4.1 This test method is used for growing a reproducible *Pseudomonas aeruginosa* biofilm in a rotating disk reactor. The biofilm is established by operating the reactor in batch mode (no flow) for 24 h. Steady state growth (attachment is equal to detachment) is reached while the reactor operates for an additional 24 h with continuous flow of the nutrients. The residence time of the nutrients in the reactor is set to select for biofilm growth, and is species and reactor parameter specific. During the entire 48 h, the biofilm is exposed to continuous fluid shear from the rotation of the disk. At the end of the 48 h, biofilm accumulation is quantified by removing coupons from the disk, harvesting the biofilm from the coupon surface, disaggregating the clumps, then diluting and plating for viable cell enumeration.

5. Significance and Use

5.1 Bacteria that exist in a biofilm are phenotypically different from suspended cells of the same genotype. The study of biofilm in the laboratory requires protocols that account for this difference. Laboratory biofilms are engineered in growth

reactors designed to produce a specific biofilm type. Altering system parameters will correspondingly result in a change in the biofilm. The purpose of this method is to direct a user in the laboratory study of biofilms by clearly defining each system parameter. This method will enable a person to grow, sample, and analyze a laboratory biofilm.

6. Apparatus

6.1 *Wooden Applicator Sticks*, sterile.

6.2 *Inoculating Loop*.

6.3 *Petri Dish*, 100- by 15-mm, plastic, sterile and empty to hold rotor while sampling.

6.4 *Culture Tubes and Culture Tube Closures*, any with a volume capacity of 10 mL and minimum diameter of 16 mm. Recommended size is 16- by 125-mm borosilicate glass with threaded opening.

6.5 *Pipetter*, continuously adjustable pipetter with volume capacity of 1 mL.

6.6 *Vortex*, any vortex that will ensure proper agitation and mixing of culture tubes.

6.7 *Homogenizer*, any capable of mixing at $20\,500 \pm 5000$ r/min in a 5- to 10-mL volume.

6.8 *Homogenizer Probe*, any capable of mixing at $20\,500 \pm 5000$ r/min in a 5 to 10 mL volume that can withstand autoclaving or other means of sterilization.

6.9 *Sonicator*, any cavitating sonicating bath that operates at 50 to 60 Hz.

6.10 *Bunsen Burner*, used to flame inoculating loop and other instruments.

6.11 *Stainless Steel Dissecting Tools*.

6.12 *Stainless Steel Hemostat Clamp*, with curved tip.

NOTE 1—Alternatively, a coupon holder⁴ may be used.

6.13 *Environmental Shaker*, capable of maintaining temperature of $36 \pm 2^\circ\text{C}$.

6.14 *Analytical Balance*, sensitive to 0.01 g.

6.15 *Sterilizer*, any steam sterilizer capable of producing the conditions of sterilization is acceptable.

6.16 *Colony Counter*, any one of several types may be used, such as the Quebec, Buck, and Wolfhugel. A hand tally for the recording of the bacterial count is recommended if manual counting is done.

6.17 *Peristaltic Pump*, pump head capable of holding tubing with inner diameter of 3.1 mm and outer diameter of 3.2 mm.

6.18 *Digital Magnetic Stir Plate*, top plate 10.16 by 10.16 cm, capable of rotating at 200 ± 5 r/min.

6.19 *Silicone Tubing*, two sizes of tubing: one with inner diameter of 3.1 mm and outer diameter of 3.2 mm, and the other with inner diameter of 7.9 mm and outer diameter of 9.5 mm. Both sizes must withstand sterilization.

6.20 *Norprene Tubing*, inner diameter of 3.1 mm and outer diameter of 3.2 mm.

6.21 *Glass Flow Break*, any that will connect with tubing of inner diameter 3.1 mm and withstands sterilization.

6.22 *Clamp*, used to hold flow break, extension clamp with 0.5-cm minimum grip size.

6.23 *Clamp Stand*, height no less than 76.2 cm, used with clamp to suspend glass flow break vertically and stabilize tubing above reactor.

6.24 *Reactor Components*⁵:

6.24.1 *Berzelius Borosilicate Glass Beaker*, 1000-mL with-out pour spout, 9.5 ± 0.5 -cm diameter. Borosilicate barbed outlet spout added at 250 ± 15 -mL mark at 30 to 45° angle, spout should accommodate silicone tubing with an inner diameter of 8 to 11 mm.

NOTE 2—The rotor, described in 6.24.3, will displace approximately 50 mL of liquid. Therefore, an outlet spout at the 250 mL mark will result in an operating volume of approximately 200 mL. Before use, the user should confirm the actual liquid volume in the reactor, after the rotor is in place and the stir plate is turned on. The measured volume is used to calculate an exact pump flow rate.

6.24.2 *Reactor Top*, size 15 rubber or machined stopper, with three holes bored through top to accommodate 6-cm pieces of stainless steel tubing or other suitable rigid autoclavable tubing with an outside diameter of 4 to 6 mm. One port accommodates tubing for media, the second port is fitted with a short piece of silicone tubing that holds a bacterial air vent, and the third is an inoculum port as shown in Fig. 1.

6.24.3 *Rotor or Disk*, nominal 1.6-mm thick PTFE sheet cut into a disk with a diameter of 7.0 ± 0.2 cm containing six evenly spaced holes with a diameter of 1.27 ± 0.1 cm. The center of each hole is located 2.55 ± 0.03 cm from the center of the disk. 4.5 to 7.0 mm thick rubber sheet, or other suitable autoclavable material, cut into a disk with a diameter of 7.0 ± 0.2 cm containing six evenly spaced holes with a diameter of 1.27 ± 0.15 cm (the holes in the rubber are aligned with the holes in the PTFE) and a small hole in the center to house the disk retrieving port. PTFE washer with disk retrieving port. Four nylon screws. PTFE-coated 4.0- by 1.4-cm star-head magnetic stir bar, set flush against PTFE disk, with holes drilled for assembly using nylon screws. The PTFE ridges on one side of the magnet may be shaved to provide a flush mounting surface. Assemble the pieces conforming to the general details shown in Fig. 2.

NOTE 3—Nominal implies that the manufacturer's tolerance is acceptable.

⁴ The sole source of supply of the apparatus (coupon holder) known to the committee at this time is BioSurface Technologies, Corp., www.biofilms.biz. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,¹ which you may attend. The user may also build the holder.

⁵ The sole source of supply of the apparatus (rotating disk reactor) known to the committee at this time is BioSurface Technologies, Corp., www.biofilms.biz. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,¹ which you may attend. The user may also build the reactor.

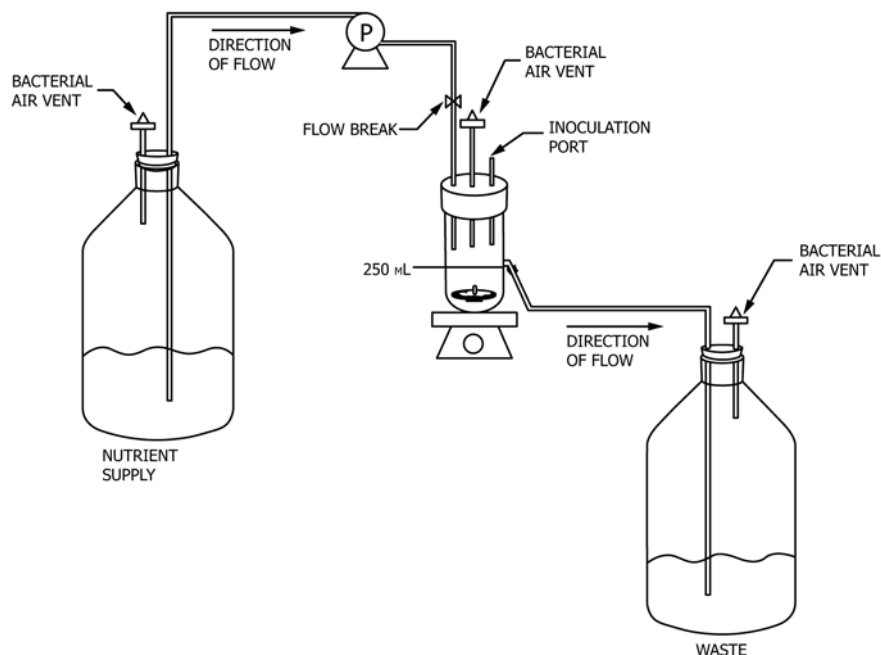


FIG. 1 Rotating Disk Reactor System

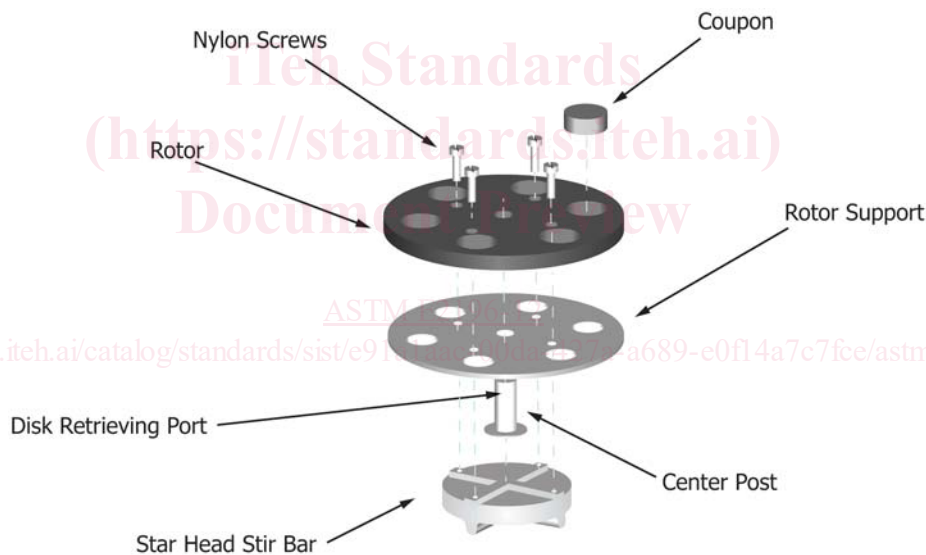


FIG. 2 Rotor Assembly

6.24.4 Six Cylindrical Polycarbonate Coupons, with a diameter of 1.27 ± 0.013 cm and a height of 1.5 to 4.0 mm.

6.25 Carboys, two 20-L autoclavable carboys, to be used for waste and nutrients.

6.25.1 Carboy Lids, two: one carboy lid with at least 2 barbed fittings to accommodate tubing ID 3.1 mm (one for nutrient line and one for bacterial air vent), one carboy lid with at least two 1-cm holes bored in the same fashion (one for effluent waste and one for bacterial air vent).

NOTE 4—Carboy tops can be purchased with fittings.

6.25.2 Bacterial Air Vent, autoclavable 0.2- μ m pore size, to be spliced into tubing on waste carboy, nutrient carboy, and reactor top (37-mm diameter recommended).

7. Reagents and Materials

7.1 Purity of Water—All reference to water as diluent or reagent shall mean distilled water or water of equal purity.

7.2 Culture Media:

7.2.1 Bacterial Liquid Growth Broth—Tryptic Soy Broth (TSB) is recommended.