



Designation: E 2196 – 02

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

This standard is issued under the fixed designation E 2196; 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 repeatable² *Pseudomonas aeruginosa* biofilm in a continuously stirred flow reactor. In addition, the test method describes how to sample and analyze biofilm for viable cells.

1.2 In this test method, biofilm population density is recorded as log colony forming units per surface area.

1.3 Basic microbiology training is required to perform this test method. *This standard does not claim to address all of the safety problems associated with its use. It is the responsibility of the user of this standard to establish appropriate safety practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 Other Standards:

- Buffered Dilution Water Preparation—Method 9050 C.1a³
- Rotating Disk Reactor—Repeatability and Relevance⁴
- Rotating Disk Reactor—Efficacy Test Method⁵

3. Terminology

3.1 *biofilm*—an accumulation of bacterial cells immobilized on a substratum and embedded in an organic polymer matrix of microbial origin.

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

Current edition approved April 10, 2002. Published August 2002.

² Ellison, S.L.R., M. Rosslein, A. Williams. (Eds.) 2000. *Quantifying Uncertainty in Analytical Measurement*, 2nd Edition. Eurachem.

³ Eaton, A.D., L.S. Clesceri, A.E. Greenberg. (Eds.) 1995. *Standard Methods for the Examination of Water and Waste Water*, 19th Edition. American Public Health Association, American Water Works Association, Water Environment Federation. Washington D.C.

⁴ Zelter, N., M. Hamilton, B. Pitts, D. Goeres, D. Walker, P. Sturman, J. Heersink. 1999. Methods for measuring antimicrobial effects on biofilm bacteria: from laboratory to field. In: Doyle, R.J. (Ed.), *Methods in Enzymology-Biofilms* Vol 310, Academic Press, San Diego, CA, pp. 608-628.

⁵ Zelter, N., M. Hamilton, D. Goeres, J. Heersink. 2001. Development of a Standardized Antibiofilm Test. In: Doyle, R.J. (Ed.), *Methods in Enzymology-Biofilms* Vol 337, Academic Press, San Diego, CA, pp. 363-376.

NOTE 1—Biofilm is a dynamic, self-organized accumulation of microorganisms and microbial and environmental by-products that is determined by the environment in which it lives. Definition 3.1 does not capture every biofilm type that is known to exist.

3.2 *coupon*—biofilm sample surface.

4. Summary of Test Method

4.1 This test method is used for growing a repeatable *Pseudomonas aeruginosa* biofilm in a rotating disk reactor. The biofilm is established by operating the reactor in batch mode (no flow) for 24 h. A 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 experiences 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, scraping 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 capability of 10 mL and diameter no less than 6 cm. Recommended size is 16 by 125 mm borosilicate glass with threaded opening.

6.5 *Pipetter*, continuously adjustable pipetter with volume capability 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 $20\,500 \pm 5000$ rpm in a 5 to 10 mL volume.

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

6.9 *Sonicator*, any noncavitating sonicating bath that operates at 50 to 60 hertz.

6.10 *Syringe*, sterile, 1 mL syringe used during reactor inoculation.

6.10.1 *Needle*, sterile, 22 gauge needle used with syringe to inoculate reactor.

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

6.12 *Stainless Steel Dissecting Tools*.

6.13 *Stainless Steel Hemostat Clamp with Curved Tip*.

6.14 *Environmental Shaker*, capable of maintaining temperature of $35 \pm 2^\circ\text{C}$.

6.15 *Analytical Balance*, sensitive to 0.01 g.

6.16 *Sterilizers*, any steam sterilizer capable of producing the conditions of sterilization is acceptable.

6.17 *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.

6.18 *Peristaltic Pump*, pump head capable of holding tubing with ID 3.1 mm and OD 3.2 mm.

6.19 *Magnetic Stir Plate*, top plate 10.16 by 10.16 cm, capable of rotating at 200 ± 100 rpm.⁶

6.20 *Silicon Tubing*, two sizes of tubing: one with ID 3.1 mm and OD 3.2 mm and the other with ID 7.9 mm and OD 9.5 mm. Both sizes must withstand sterilization.

6.21 *Glass Flow Break*, any that will connect with tubing of ID 3.1 mm and withstand sterilization.

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

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

6.22 *Reactor Components*⁷.

6.22.1 *Berzelius Pyrex Beaker*, 1000 mL without pour spout, 9.5 ± 0.5 cm diameter. Pyrex barbed outlet spout added at $250\text{ mL} \pm 15\text{ mL}$ mark at 30 to 45° angle, spout should accommodate silicon tubing with an ID of 8 to 11 mm.

NOTE 2—The rotor, described in 6.22.3, will displace approximately 50 mL of liquid. Therefore, an outlet spout at the 250 mL mark will result in approximately a 200 mL operating volume. The user is encouraged to

confirm the actual liquid volume in the reactor, when the rotor is in place, before use. The measured volume is used to calculate an exact pump flow rate.

6.22.2 *Reactor Top*, size 15 rubber or machined stopper, 3 to 4 holes bored through stopper to accommodate 6 cm pieces of fire-polished glass tubing or other suitable rigid autoclavable tubing with OD 4 to 6 mm. Another hole can be added to the stopper to contain an inoculum port. The inoculum port consists of a 6 cm piece of fire-polished glass tubing or other suitable rigid autoclavable tubing fitted with a septum, as shown in Fig. 1.

6.22.3 *Rotor or Disk*, nominal⁸ 1.6 mm thick Teflon sheet cut into a disk with a diameter of 7.0 ± 0.2 cm containing 6 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 Viton sheet, or other suitable autoclavable material, cut into a disk with a diameter of 7.0 ± 0.2 cm containing 6 evenly spaced holes with a diameter of 1.27 ± 0.15 cm (the holes in the Viton are aligned with the holes in the Teflon) and a small hole in the center to house the disk retrieving port. Teflon washer with disk retrieving port. Four nylon screws. Teflon coated 4.0 by 1.4 cm starhead magnetic stir bar, set flush against Teflon disk and with holes drilled for assembly with nylon screws. The Teflon 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.

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

6.23 *Carboys*, two 15 to 20 L autoclavable carboys, to be used for waste and nutrients.

6.23.1 *Carboy Lids*, two carboy lids: 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 2-1 cm holes bored in the same fashion (one for effluent waste and one for bacterial air vent).⁹

6.23.2 *Bacterial Air Vent*, autoclavable 0.2 μm pore size, to be spliced into tubing on waste carboy, nutrient carboy and reactor top, recommended diameter 37 mm.

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*, soybean-casein digest medium, or an equivalent general bacterial growth medium. Tryptic soy broth is recommended.

7.2.2 *Bacterial Plating Medium*, R2A agar is recommended.

NOTE 3—Media concentration in this protocol differs from the manufacturer's recommendation. Two different concentrations are used in the protocol, 300 mg/L for the inoculum and batch reactor operation and 30 mg/L for the continuous flow reactor operation.

⁶ rpm may be measured using a strobe light.

⁷ Rotating disk reactor is available commercially from BioSurface Technologies, Corp. www.imt.net/~mitbst, or the user may build the reactor.

⁸ Nominal implies that the manufacturer's tolerance is acceptable.

⁹ Carboy tops can be purchased with fittings.