Designation: E2562 – 22

Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow using CDC Biofilm Reactor[1]

This standard is issued under the fixed designation E2562; 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 specifies the operational parameters required to grow a reproducible (1)[2] *Pseudomonas aeruginosa* ATCC 700888 biofilm under high shear. The resulting biofilm is representative of generalized situations where biofilm exists under high shear rather than being representative of one particular environment.

1.2 This test method uses the Centers for Disease Control and Prevention (CDC) Biofilm Reactor. The CDC Biofilm Reactor is a continuously stirred tank reactor (CSTR) with high wall shear. Although it was originally designed to model a potable water system for the evaluation of *Legionella pneumophila* (2), the reactor is versatile and may also be used for growing and/or characterizing biofilm of varying species (3-5).

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.

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.*

1.7 *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:*[3]

D5465 Practices for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

E2756 Terminology Relating to Antimicrobial and Antiviral Agents

2.2 *Other Standards:*

Method 9050 C.1.a Buffered Dilution Water Preparation according to Baird et al (6)

3. Terminology

3.1 For definitions of terms used in this standard refer to Terminology E2756.

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *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.

3.2.1.1 *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 physicochemical environment in which it exists.

3.2.2 *coupon, n*—biofilm sample surface.

4. Summary of Test Method

4.1 This test method is used for growing a reproducible *Pseudomonas aeruginosa* ATCC 700888 biofilm in a CDC Biofilm Reactor. The biofilm is established by operating the

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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.

*A Summary of Changes section appears at the end of this standard

reactor in batch mode (no flow of the nutrients) for 24 h. A steady state population is reached while the reactor operates for an additional 24 h with a 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 a baffled stir bar. Controlling the rate at which the baffle turns determines the intensity of the shear stress to which the coupons are exposed. At the end of the 48 h, biofilm accumulation is quantified by removing coupons from suspended rods, harvesting the biofilm from the coupon surface by scraping the biofilm from the coupon, homogenizing the removed biofilm to disaggregate the clumps, and diluting and plating for viable cell enumeration.

5. Significance and Use

5.1 Bacteria that exist in biofilms are phenotypically different from suspended cells of the same genotype. Research has shown that biofilm bacteria are more difficult to kill than suspended bacteria (**5**, **7**). 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. For example, research has shown that biofilm grown under high shear is more difficult to kill than biofilm grown under low shear (**5**, **8**). The purpose of this test method is to direct a user in the laboratory study of a *Pseudomonas aeruginosa* biofilm by clearly defining each system parameter. This test method will enable an investigator to grow, sample, and analyze a *Pseudomonas aeruginosa* biofilm grown under high shear. The biofilm generated in the CDC Biofilm Reactor is also suitable for efficacy testing. After the 48 h growth phase is complete, the user may add the treatment in situ or remove the coupons and treat them individually.

6. Apparatus

6.1 *Wooden Applicator Sticks*—sterile.

6.2 *Inoculating Loop.*

6.3 *Petri Dish*—100 mm by 15 mm, plastic, sterile, and empty to put beneath rod while sampling.

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

6.5 *Pipette*—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 that can mix at 20 500 r/min ± 5000 r/min in a 5 mL to 10 mL volume.

6.8 *Homogenizer Probe*—any that can mix at 20 500 r/min ± 5000 r/min in a 5 to 10 mL volume and can withstand autoclaving or other means of sterilization.

6.9 *Sonicating Water Bath*—any cavitating sonicating bath that operates at 45 kHz to 50 kHz for cleaning coupons.

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

6.11 *Stainless Steel Hemostat Clamp*—with curved tip.

NOTE 1—Alternatively, a coupon manipulating tool[4] may be used.

6.12 *Environmental Shaker*—that can maintain a temperature of 36 °C ± 2 °C.

6.13 *Analytical Balance*—sensitive to 0.01 g.

6.14 *Sterilizer*—any steam sterilizer that can produce the conditions of sterilization is acceptable.

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

6.16 *Peristaltic Pump*—pump head that can hold tubing with inner diameter 3.1 mm and outer diameter 3.2 mm.

6.17 *Digital Magnetic Stir Plate*—top plate 10.16 cm × 10.16 cm, that can rotate at 125 r/min ± 5 r/min.

6.18 *Silicone Tubing*—three sizes of tubing: one with inner diameter 3.1 mm and outer diameter 3.2 mm, one with inner diameter 7.9 mm and outer diameter 9.5 mm, and one with inner diameter 8 mm and outer diameter 11 mm. All sizes must withstand sterilization.

6.19 *Norprene*[5] Tubing—inner diameter 3.1 mm and outer diameter 3.2 mm.

6.20 *Glass Flow Break*—any that will connect with tubing of inner diameter 3.1 mm and withstand sterilization.

6.20.1 *Clamp*—Used to hold flow break, extension clamp with 0.5 cm minimum grip size.

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

6.20.3 *Laboratory Screw Clamp*—used to clamp effluent tubing during batch growth.

6.21 *Reactor Components.*[6]

6.21.1 *Berzelius Borosilicate Glass Tall Beaker*—1000 mL without pour spout, 9.5 cm ± 0.5 cm diameter. Barbed outlet spout added at 400 mL ± 20 mL mark. Angle the spout 30° to 45° to ensure drainage. Spout should accommodate flexible tubing with an inner diameter of 8 to 11 mm.

NOTE 2—The rods (see 6.21.3) and baffle (see 6.21.6) will displace approximately 50 mL of liquid when system is completely assembled. Therefore, an outlet spout at the 400 mL mark will result in approximately a 350 mL operating volume. The user should confirm the actual liquid

[4] The sole source of supply of the apparatus (coupon manipulating tool) 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] Trademarked by the Saint-Gobain Performance Plastics Corporation.

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