Designation: E2647 – 13

Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown Using Drip Flow Biofilm Reactor with Low Shear and Continuous Flow[1]

This standard is issued under the fixed designation E2647; 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 repeatable[2] *Pseudomonas aeruginosa* biofilm close to the air/liquid interface in a reactor with a continuous flow of nutrients under low fluid shear conditions. The resulting biofilm is representative of generalized situations where biofilm exists at the air/liquid interface under low fluid shear rather than representative of one particular environment.

1.2 This test method uses the drip flow reactor. The drip flow reactor (DFR) is a plug flow reactor with laminar flow resulting in low fluid shear. The reactor is versatile and may also be used for growing and/or characterizing biofilms of different species, although this will require changing the operational parameters to optimize the method based upon the growth requirements of the new organism.

1.3 This test method describes how to sample and analyze biofilm for viable cells. Biofilm population density is recorded as log 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.*

2. Referenced Documents

2.1 *ASTM Standards:*[3]

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

2.2 *Other Standard:*

Method 9050 C.1.a Buffered Dilution Water Preparation, according to Eaton et al[4]

3. Terminology

3.1 *Definitions:*

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

3.1.1.1 *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.

3.1.2 *coupon, n*—biofilm sample surface.

3.1.3 *chamber, n*—reactor base containing four rectangular wells or channels.

3.1.4 *channel, n*—one of four rectangular wells in reactor chamber (base) where coupon is placed.

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

[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.

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

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4. Summary of Test Method

4.1 This test method is used for growing a repeatable *P. aeruginosa* biofilm in a drip flow reactor. The biofilm is established by operating the reactor in batch mode (no flow of nutrients) for 6 h. A mature biofilm forms while the reactor operates for an additional 48 h with a continuous flow of nutrients. During continuous flow, the biofilm experiences very low shear caused by the gravity flow of media dripping onto a surface set at a 10° angle. At the end of the 54 h, biofilm accumulation is quantified by removing coupons from the reactor channels, rinsing the coupons to remove the planktonic cells, scraping the biofilm from the coupon surface, disaggregating the clumps, then diluting and plating for viable cell enumeration.

5. Significance and Use

5.1 Vegetative biofilm bacteria are phenotypically different from suspended cells of the same genotype. Biofilm growth reactors are engineered to produce biofilms with specific characteristics. Altering either the engineered system or operating conditions will modify those characteristics.

5.2 The purpose of this test method is to direct a user in how to grow, sample, and analyze a *P. aeruginosa* biofilm under low fluid shear and close to the air/liquid interface using the DFR. The *P. aeruginosa* biofilm that grows has a smooth appearance and is loosely attached. Microscopically, the biofilm is sheet-like with few architectural details. This laboratory biofilm could represent those found on produce sprayers, on food processing conveyor belts, on catheters, in lungs with cystic fibrosis, and oral biofilms, for example. The biofilm generated in the DFR is also suitable for efficacy testing. After the 54 h growth phase is complete, the user may add the treatment *in situ* or harvest the coupons and treat them individually. Research has shown that *P. aeruginosa* biofilms grown in the DFR were less tolerant to disinfection than biofilms grown under high shear conditions.[5]

6. Apparatus

6.1 *Tetrafluoroethylene (TFE), Metal, or Rubber Spatulas*—Sterile, for scraping biofilm from coupon surface.

6.2 *Inoculating Loop.*

6.3 *Petri Dish*—100 by 15 mm, plastic, sterile, and empty for transporting coupons from reactor to work station.

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

6.5 *Glass Beakers*—Sterile, any with a volume capacity of 100 mL containing 45 mL sterile buffered water.

6.6 *Conical Centrifuge Tubes*—Sterile, any with 50-mL volume capacity containing 45 mL sterile buffered water.

6.7 *Vortex*—Any vortex that will ensure proper agitation and mixing of culture tubes.

6.8 *Homogenizer*—Any capable of mixing at 20 500 ± 5000 r/min in a 50 mL volume.

6.9 *Homogenizer Probe*—Any capable of mixing at 20 500 ± 5000 r/min in a 50 mL volume and with a gap between the rotor and stator of 0.25 mm. Both disposable probes and probes that can withstand autoclaving or other means of sterilization are acceptable.

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

6.11 *95 % Ethanol*—Used to flame sterilize hemostats or forceps.

6.12 *Stainless Steel Hemostat Clamp or Forceps*—For aseptic handling of coupons.

6.13 *Pipetter*—Continuously adjustable pipette with volume capability of 1 mL.

6.14 *Analytical Balance*—Sensitive to 0.01 g.

6.15 *Sterilizers*—Any steam sterilizer capable of producing the conditions of sterilization.

6.16 *Colony Counter*—Any one of several types may be used. A hand tally for the recording of the bacterial count is recommended if manual counting is done.

6.17 *Peristaltic Pump*—Four pump heads capable of holding tubing with inner diameter (ID) 3.1 mm and outer diameter (OD) 3.2 mm and operating at a flow rate of 200 mL per hour.

6.18 *Environmental Shaker*—Capable of maintaining a temperature of 35 ± 2°C.

6.19 *Tubing*—Two sizes of silicone 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.

6.20 *Glass Flow Break*—Any that will connect with tubing of ID 3.1 mm and withstands 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.

6.21 *Reactor Components*[6]—A schematic of the drip flow reactor is shown in Fig. 1. Fig. 2 is a picture of the assembled system.

6.21.1 *Chamber (Base)*—15.24 by 15.88 cm polysulfone chamber with four 3.05- by 10.16-cm channels and four 1.27-cm barbed effluent ports (one at the end of each channel). The underside holds four adjustable inserts (legs) providing a 10° angle for continuous flow conditions. Each channel contains two pegs to guide coupon placement.

[5] Buckingham-Meyer, K., Goeres, D. M., and Hamilton, M. A., "Comparative Evaluation of Biofilm Disinfectant Efficacy Tests," *J. Microbiological Methods*, Vol 70, 2007, pp. 236–244.

[6] The sole source of supply of the drip flow biofilm reactor apparatus known to the committee at this time is BioSurface Technologies, Corp., Bozeman, MT, www.imt.net/~mitbst. 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, which you may attend. Alternatively, the user may build the DFR apparatus.

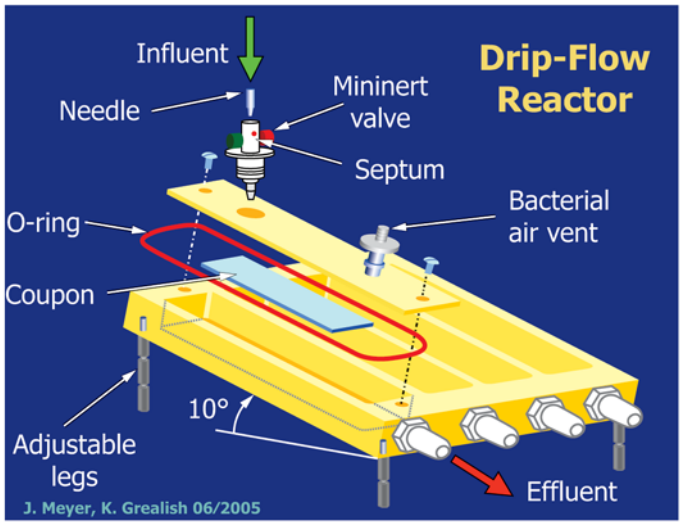

FIG. 1 Expanded View of the Drip Flow Reactor

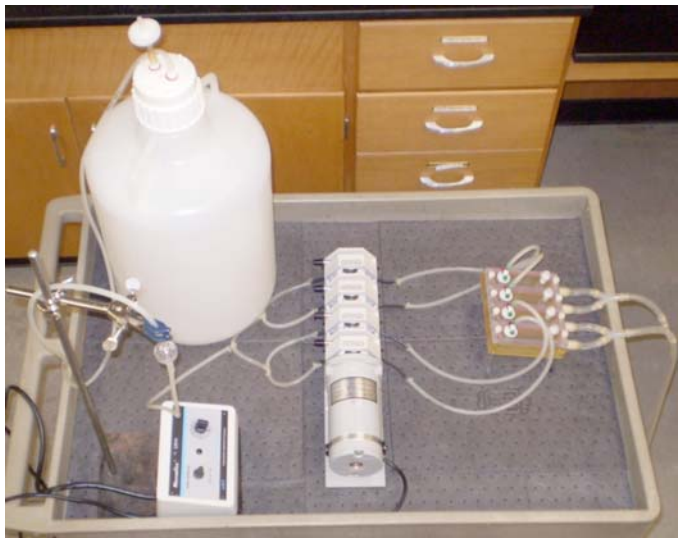

FIG. 2 Drip Flow Reactor Laboratory Set-Up in Continuous Flow Operation

6.21.2 *Top*—Four O-ring fitted polycarbonate tops each with two threaded holes for nylon screws to secure to reactor chamber (base). Two ports, one for Mininert valve and another for bacterial air vent attachment.

6.21.3 *Mininert Valves*—Fit into each top as influent ports to allow inoculation and media line attachment.

6.21.4 *Luer Lock Connectors with 3.1 mm Hose Barb*—Used to connect needles to the tubing.

6.21.5 *Needle*—1 in., 21 gauge, to fit into Mininert port.

6.21.6 *Glass Coupons*—Four new rectangular glass microscope slides (or other similar shaped material) with a top surface area of 18.75 cm^2 (25 by 75 by 1 mm).

6.21.7 *TFE Thread Seal Tape*—To prevent leakage from effluent port connector.

6.22 *Carboys*—Two 10 to 20 L autoclavable carboys for waste and nutrients.

6.22.1 *Carboy Lids*—(Note 1)—One carboy lid with at least two 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 1—Carboy tops can be purchased with fittings.

6.23 *Bacterial Air Vent (Filter)*—Autoclavable 0.2 µm pore size, to be attached into tubing on waste and nutrient carboy (recommended diameter is 37 mm) and each reactor channel top (recommended diameter is 15 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*—Tryptic Soy Broth (TSB)[7] is recommended.

[7] Atlas, R. M., Parks, L. C., Eds., *Handbook of Microbiological Media,* 2nd ed., CRC Press, Boca Raton, FL, 1997.

NOTE 2—Two different TSB concentrations are used in the test method, 3000 mg/L for the inoculum and batch reactor operation and 270 mg/L for the continuous flow reactor operation.

7.2.2 *Bacterial Plating Medium*—R2A agar[7] is recommended.

7.3 *Buffered Water*—0.0425 g/L KH_2PO_4 distilled water, filter sterilized and 0.405 g/L $MgCl{\cdot}6H_2O$ distilled water, filter sterilized (prepared according to Method 9050 C.1a).

8. Culture/Inoculum Preparation

8.1 *Pseudomonas aeruginosa* (ATCC 700888) is the organism used in this test. Aseptically remove an isolated colony from an R2A plate and inoculate into 100 mL of sterile bacterial liquid growth broth (3000 mg TSB/L). Incubate bacterial suspension in an environmental shaker at 35 ± 2°C for 20 to 24 h. Viable bacterial density should equal 10^8 CFU/mL and may be checked by serial dilution and plating.

9. Preparation of Apparatus

9.1 *Reactor Set-Up:*

9.1.1 Use new coupons (glass microscope slides) for every experiment.

9.1.2 Insert a coupon into each reactor channel by positioning the top of the slide directly under the influent media port and allowing the end of the slide to rest on the pegs at the bottom of the channel.

9.1.3 Place channel lids onto base and loosely screw in nylon screws.

9.1.4 Attach a bacterial air vent (15 mm diameter) to each channel lid near the effluent port.

9.1.5 Splice the glass flow break into the media tubing line that will be positioned near the carboy top.

9.1.6 Configure the media tubing so that four individual lines result. Feed each of the four lines through a pump head and attach to each channel as shown in Fig. 2.

9.1.7 Wrap each effluent barbed fitting with TFE tape and insert into end port. Tightly clamp each effluent line using metal screw clamps.

9.2 *Sterilizing the Reactor System:*

9.2.1 Remove the adjustable legs from the reactor chamber. Wrap all exposed tubing ends and openings with aluminum foil and place assembled reactor into an autoclave tray. Cover entire tray with aluminum foil.

9.2.2 Sterilize the reactor system for 20 min on liquid cycle.

NOTE 3—Tightening the screws prior to autoclaving may result in the lid cracking and/or the screws being stripped. Instead, tape down the lid when autoclaving to maintain sterility.

10. Procedure

10.1 *The Batch Phase:*

10.1.1 Place the cooled reactor in a level position on the bench top.

10.1.2 Aseptically add 15 mL of sterile 3000 mg TSB/L (see 7.2.1) and 1 mL of inoculum (see 8.1) to each channel. Tighten each channel lid securely with nylon screws.

10.1.3 Incubate the reactor system in batch mode at room temperature (21 ± 2°C) for 6 h, in the level position.

10.1.4 Remove foil from the effluent tubing and attach end into a waste carboy. Do not unclamp until continuous flow phase.

10.2 *Preparation for Continuous Flow Phase:*

10.2.1 Prepare continuous flow nutrient broth by adding sterilized bacterial liquid growth medium to 20 L sterile reagent grade water so that final concentration is equal to 270 mg TSB/L (see 7.2.1). That is, dissolve and sterilize a broth concentrate in a smaller volume of water to prevent caramelization that can occur under the lengthy sterilization times required for large volumes. Aseptically pour the concentrated medium into the carboy of sterile water to make a total of 20 L.

10.3 *Adjust Reactor Angle* (Fig. 3):

10.3.1 Measure a, the length of the reactor base, in cm.

10.3.2 Angle (x) is 10°.

10.3.3 Calculate y using the following equation:

$$y = a[\sin\ (x)] \tag{1}$$

where:

a = measured length of reactor base in cm,
x = angle (10°), and
y = difference between length of b and length of c in cm.

10.3.4 Decide upon the lengths of b and c to obtain the required difference (y). Use the following equation:

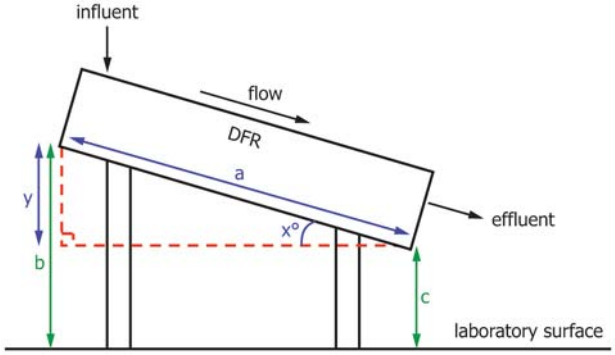

FIG. 3 Side View of the DFR Used for Calculating the Reactor Angle

$$y = b - c \quad (2)$$

where:

b = measured length (cm) from bottom corner of influent end of reactor base to laboratory surface,
c = measured length (cm) from bottom corner of effluent end of reactor base to laboratory surface, and
y = difference between length of b and length of c in cm.

NOTE 4—Determine the necessary lengths of b and c before starting an experiment. To do this, first calculate y using $y = a$ [sin(x)]. Set length c by tightening the legs near the effluent port until hand tight. Using the equation $y = b - c$, determine length b and adjust legs near influent port accordingly.

10.3.5 Unclamp the effluent tubing and attach the legs to the DFR.

10.3.6 Adjust the legs of the reactor chamber until the required lengths (b and c) are achieved so that it slopes downward 10°.

10.4 *Continuous Flow Phase:*

10.4.1 Aseptically connect the influent nutrient tubing line to the carboy containing the continuous flow nutrient broth. Feed each line through a pump head and connect a sterile needle on the end of each line.

10.4.2 Aseptically attach the influent tubing by inserting the sterile needle through the Mininert valves in the channel lids.

10.4.3 Turn on the pump, allowing media to slowly drip onto the bacterial cells attached to the coupon. A continuous flow of nutrients is pumped into the reactor through a pump set at a flow rate equal to 200 mL/h (50mL/h per channel).

10.4.4 The media should flow downward from the influent port to the effluent port. Periodically check the reactor for proper drainage and leaks. If problems occur, visually inspect the influent port and the tubing for bacterial plugging.

10.4.5 The reactor is operated in CF mode for 48 h.

10.5 *Sampling the Biofilm:*

10.5.1 Prepare sampling materials: vortex, homogenizer, sterile beakers, sterile centrifuge tubes, culture tubes, pipettes, empty sterile petri dish, sterile spatulas, and flame sterilized stainless steel hemostat or forceps.

10.5.2 Loosen channel lid screws with gloved hands and lift channel lid up. Aseptically remove one of the coupons by gently lifting up the coupon with sterile hemostat. Hold coupon over a sterile petri dish while carrying to the sampling area.

10.5.3 Hold the coupon with flame sterilized hemostat being careful not to disturb the attached biofilm.

10.5.4 Rinse the coupon to remove planktonic cells: Gently immerse coupon into the centrifuge tube containing 45 mL sterile buffered water with a fluid motion until slide is completely covered. Immediately reverse motion to remove the slide, being careful not to agitate liquid and biofilm.

10.5.5 Remove the biofilm from the coupon: Scrape biofilm-covered coupon surface in a downward direction for approximately 15 s, using the flat end of a sterile spatula or scraper, into the beaker containing 45 mL of sterile dilution buffer. Rinse the spatula or scraper by stirring it in the beaker. Repeat the scraping and rinsing process 3 to 4 times, ensuring full coverage of the coupon surface.

10.5.6 Hold the coupon at a 60° angle over the sterile beaker and pipette 1 mL of sterile buffered water over the top surface of the coupon. Repeat for a total of 5 rinses. The final volume in the beaker is 50 mL.

10.6 *Analyze the Biofilm Sample:*

10.6.1 Homogenize the scraped biofilm sample in the beaker at 20 500 ± 5000 r/min for 30 s. If more than one biofilm sample is taken, rinse the homogenizer probe between each new sample as follows: Homogenize a dilution blank for 30 s at 20 500 ± 5000 r/min, homogenize a tube containing 70 % ethanol for 15 s, then remove the probe and let the probe sit in the ethanol tube for 1 min. Shake any remaining ethanol off the probe, reattach probe, and homogenize a dilution blank for 30 s. Homogenize a third dilution blank and then homogenize the next sample beaker.

NOTE 5—Homogenizing the sample disaggregates the biofilm clumps to form a homogeneous cell suspension. Improper disaggregation could result in an underestimation of the viable cells present in the sample.

10.6.2 Perform serial 10-fold dilutions on the sample using sterile culture tubes.

10.6.3 Plate each dilution in duplicate for colony growth using an accepted plating technique such as spread or spiral plating (Practice D5465).

10.6.4 Incubate the plates for 17 to 20 h at 35 ± 2°C.

10.7 *Cell Enumeration:*

10.7.1 Count the appropriate number of colonies according to the plating method used.

10.7.2 Calculate the arithmetic mean of the colonies counted on the duplicate plates.

10.8 The log density for one coupon is calculated as follows:

$$\mathrm{LOG_{10}\,(CFU/cm^2)} = \mathrm{LOG_{10}}\left[(X/B)(V/A)(D)\right] \quad (3)$$

where:

X = mean CFU,
B = volume plated,
V = volume scraped into,
A = surface area scraped, and
D = dilution.

NOTE 6—The equation in 10.8 is accurate only if the beaker the biofilm was scraped into is referred to as the 10^0 dilution.

10.9 Calculate the overall biofilm accumulation by calculating the mean of the log densities calculated in 10.8.

11. Precision and Bias

11.1 Randomization is used whenever possible to reduce the potential for systematic bias.

11.2 The internal reproducibility standard deviation for this protocol, when the biofilm density is based on a single coupon, was calculated to equal 0.28. The sources of variability were: 65 % attributable to within experiment sources, and 35 % attributable to between experiments sources.

12. Keywords

12.1 biofilm; coupons; growth reactor; *Pseudomonas aeruginosa*; reactors; sampling; shear

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