US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Standard Operating Procedure for
Single Tube Method for Measuring Disinfectant Efficacy Against Biofilm Grown in the CDC Biofilm Reactor

SOP Number: MB-20-01

Date Pulished: 08-06-13

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Title	Single Tube Method for Measuring Disinfectant Efficacy Against Biofilm Grown in the CDC Biofilm Reactor
Scope	Describes the Single Tube Method (see 15.1) used to determine the efficacy of disinfectants against *Pseudomonas aeruginosa* biofilm grown in the CDC biofilm reactor.
Application	This SOP may be used for additional organisms like *S. aureus*; however, the growth and recovery parameters may need to be adjusted.

	Approval Date
SOP Developer:	_____ Print Name: _____
SOP Reviewer	_____ Print Name: _____
Quality Assurance Unit	_____ Print Name: _____
Branch Chief	_____ Print Name: _____

Date SOP issued:	
Controlled copy number:	
Date SOP withdrawn:	

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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. CDC = Centers for Disease Control and Prevention 2. Biofilm = e.g., 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. Coupon = materials used to support the growth of biofilm (e.g., polycarbonate, borosilicate, stainless steel, etc.) 4. Residence Time = the time that it takes for the entire volume of the reactor to exchange once (during continuous flow operation) and is equal to the inverse of the dilution rate. For example: an operating volume of 325 mL with a flow rate of 10.8 mL/min has a residence time of 30 min. Residence time is proportional to the volume and inversely proportional to the flow rate. In addition, refer to section 12. 5. Continuous Flow Operation = continuously stirred tank reactor (CSTR) mode, where growth is broadly controlled by the dilution rate.
2. Health and Safety	Follow procedures specified in SOP MB-01, Laboratory Biosafety. The Study Director and/or lead analyst should consult the Safety Data Sheet for specific hazards associated with products.
3. Personnel Qualifications and Training	Refer to SOP ADM-04, OPP Microbiology Laboratory Training.
4. Instrument Calibration	1. Refer to SOPs EQ-01, EQ-02, EQ-03, EQ-04, EQ-05, and EQ-10 for details on method and frequency of calibration. 2. Refer to MB-19 section 4 to confirm the operating volume of the reactor and for pump calibration using Linkable Instrument Network software.
5. Sample Handling and Storage	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures.
6. Quality Control	For quality control purposes, the required information is documented on the appropriate form(s) (see section 14).
7. Interferences	1. The speed at which the baffled stir bar rotates directly determines the strength of the shear stress that the biofilm experiences. Biofilm accumulation on the coupons is sensitive to changes in the baffle's rotational speed. The baffle rotational speed is a critical factor that

	must be controlled. If baffle speed is not maintained correctly, it may impact the quality of the biofilm. 2. Due to the deterioration of the materials, it may be necessary to change the tubing and filters on the reactor and carboys after 5-6 autoclaving processes. 3. Inspect all parts of the reactor system frequently because reuse of worn parts may cause variability in the data. 4. Do not place any plastic or rubber pieces of the reactor system under UV light due to potential degradation of the material. 5. Overuse of carriers or carriers not prescreened adequately may cause variability in the results.
8. Non-conforming Data	1. Management of non-conforming data will be specified in the study protocol; procedures will be consistent with SOP ADM-07, Non-Conformance Reports. 2. The mean *TestLD* for carriers inoculated with ***P. aeruginosa*** must be at least 8.0 (corresponding to a geometric mean density of 1.0×10^8); a mean *TestLD* below 8.0 invalidates the test.
9. Data Management	1. Data will be archived consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. Use appropriately sized secondary containment for contaminated waste to prevent a biohazard spill.
11. Special Apparatus and Materials	1. *Dilution blanks*. Standard Method Dilution Water (SMDW). Method 9050 C.1*a* (0.0425 g/L KH_2PO_4 and 0.405 g/L $MgCl_2 \cdot 6H_2O$) steam-sterilized for 15 min at 120°C (see ref. 15.2). 2. *Vortex.* Any vortex that will ensure proper mixing of tubes. 3. *Micropipettes*. For making dilutions. 4. *Ultrasonic water bath*. Any bath capable of maintaining a homogeneous sound distribution of 45 kHz with a variable power setting and a volume large enough to accommodate 50 mL conical tubes in a wet environment. For removing biofilm from coupons. 5. *Reactor components, carboys, and other associated materials*. Refer to SOP MB-19, section 11. 6. *Recirculating chiller unit and water bath*. For maintaining specified temperature of the test chemical. 7. *Detergent*. Micro-90 Concentrated Cleaning Solution for Critical Cleaning; International Products Corporation. For cleaning coupons

	and reactor parts.
12. Procedure and Analysis	This method is used for evaluating the efficacy of liquid disinfectants against *Pseudomonas aeruginosa* biofilms. Three randomly selected coupons are evaluated for efficacy and three are evaluated as controls. In advance of testing, verify the performance of the neutralizer using the procedure in Attachment 1.
12.1 Test culture preparation	a. Prepare mature *P. aeruginosa* biofilm per SOP MB-19, sections 12.1 through 12.6.
12.2 Disinfectant sample preparation	a. Prepare disinfectant sample per SOP MB-22. b. Equilibrate the water bath and allow it to come to 20 ± 1°C or the temperature specified (±1°C). Use the disinfectant within three hours of preparation unless test parameters specify otherwise. Record the time of disinfectant preparation on the Biofilm Single Tube Method Processing Sheet (see section 14). c. Place prepared disinfectant in the equilibrated water bath for approximately 10 min to allow disinfectant to come to specified temperature. Record the temperature of the water bath and recirculating chiller before and after testing on the Biofilm Single Tube Method Test Information Sheet (see section 14).
12.3 Test procedure	a. Aseptically remove a randomly selected rod containing coupons with biofilm from the CDC Biofilm Reactor by firmly pulling it straight up out of the reactor. i. Rods are numbered clockwise from 1-8, beginning with the rod to the right of the bacteria air vent (located on the reactor top). b. Rinse the coupons to remove planktonic cells. i. Orient the rod in a vertical position directly over a 50 mL conical tube containing 30 mL SMDW. ii. Immerse the rod with a continuous motion into the SMDW with minimal to no splashing, then immediately remove. iii. Use a new 50 mL conical tube with 30 mL SMDW for each rod. c. Hold the rod with one of the randomly selected coupons centered over an empty, sterile 50 mL conical tube. d. Loosen the set screw using a flame-sterilized Allen wrench and

allow the coupon to drop directly to the bottom of the tube.

i. If the coupon does not freely drop, press directly in the center of the coupon with the Allen wrench used to loosen the set screw.

ii. For each treatment or control, repeat coupon removal twice more for a total of three tubes each containing one coupon.

iii. Record the rod number and coupon position on the Biofilm Single Tube Method Results Sheet for each coupon used in the study.

Note: Upon transfer, avoid contact of the coupon with the lip or inner sides of the tube. Discard any tubes where the coupon touched the inner side of the tube and replace them with new tubes and coupons.

e. After removing the appropriate number of coupons, slowly pipette 4 mL prepared and equilibrated disinfectant (treatment) or SMDW (untreated control) into the tubes containing the coupons, being careful to completely cover the coupons. Record the time on the Biofilm Single Tube Method Time Recording Sheet (see section 14).

i. For a 10 minute contact time, a 1 minute interval between coupons is recommended.

f. Gently tap each tube to release any air bubbles trapped below the coupon. Do not shake the tubes.

i. To ensure that the maximum biofilm surface area is in contact with the disinfectant, the coupon should be at an angle in the bottom of the tube. The gentle tap used to release any air bubbles can correct the orientation of the coupon to allow full exposure to the disinfectant.

g. Place the tubes at 20 ± 1°C or other specified temperature for the specified contact time.

h. At the end of the contact time, add 36 mL of the appropriate neutralizer (e.g., Dey/Engley (D/E) broth) to each tube. Replace the cap and mix thoroughly by vigorously shaking the tube several times. Allow the coupons to remain in the neutralized disinfectant at room temperature.

i. After all tubes have been neutralized, vortex each tube on the

	highest setting for 30 ± 5 s. j. Place all tubes into a 50 mL conical tube rack and suspend the rack in the ultrasonic water bath so that the liquid level in the tubes is even with the liquid level in the tank of the bath. Sonicate the tubes at 45 kHz for 30 ± 5 s. k. Vortex the tubes as described in 12.3i. l. Sonicate the tubes as described in 12.3j. m. Vortex the tubes as described in 12.3i. These tubes are the 10^0 dilution. n. Serially dilute each 10^0 dilution in 9 mL blanks of SMDW. o. Briefly vortex each serial dilution tube prior to plating. Plate 0.1 mL aliquots of appropriate dilutions in duplicate on R2A using spread plating. Plate appropriate dilutions to achieve colony counts in the range of 30-300 colony forming units (CFU) per plate. Spread inoculum evenly over the surface of the agar. Plates must be dry prior to incubation. p. Incubate plates (inverted) at 36 ± 1°C for 24-48 h.
12.4 Recording Results	a. Count colonies. Plates that have colony counts over 300 will be reported as too numerous to count (TNTC). Record counts on the Biofilm Single Tube Method Results Sheet (see section 14). b. Inspect the growth on the plates for purity and typical characteristics of the test microbe. Gram stain one representative colony per carrier set with growth for treated and controls. Record results on the Biofilm Test Microbe Confirmation Sheet. Isolation streaks may be performed for additional verification of the test organism. i. *P. aeruginosa* is a Gram negative rod. It may display three colony types: a) circular, undulate edge, convex, rough and opaque; b) circular, entire edge, convex, smooth and translucent; c) irregular, undulate edge, convex, rough, spreading, and translucent.
12.5 Coupon and reactor reuse	a. After use, remove the coupons from each rod and place in an autoclavable container. Steam-sterilize the reactor, coupons, and necessary tubing for 30 min. b. After sterilization, clean the reactor components with a 1:100 dilution of laboratory soap (e.g., Micro-90 Concentrated Cleaning Solution) and tap water. After washing, rinse all components

<table>
<tr><td></td><td>with deionized water.
c. Clean and rescreen the coupons per SOP MB-19, section 12.2.</td></tr>
<tr><td>13. Data Analysis/ Calculations</td><td>1. All colony counts are recorded and used in calculations to determine log reductions.
2. To calculate the CFU/carrier use the following equation: $\left(\frac{CFU\ \ for 10^{-x} + CFU\ \ for 10^{-y} + CFU\ \ for 10^{-z}}{10^{-x} + 10^{-y} + 10^{-z}}\right) \times 10 \times 40$, where 10^{-x}, 10^{-y} and 10^{-z} are the dilution tubes plated, “10” accounts for the volume plated (0.1 mL) , and “40” is the volume of medium originally in the tube with the carrier (40 mL).
3. Calculate the log density of each carrier by taking the $\log_{10}$ of the density (per carrier).
4. Calculate the mean $\log_{10}$ density across treated carriers.
5. Calculate the mean $\log_{10}$ density across control carriers.
6. Calculate the $\log_{10}$ reduction (LR) for treated carriers:
$\log_{10}$ reduction = mean $\log_{10}$ control – mean $\log_{10}$ treated</td></tr>
<tr><td>14. Forms and Data Sheets</td><td>Test Sheets. Test sheets are stored separately from the SOP under the following file names:
<table><tr><td>Biofilm Single Tube Method Test Information Sheet</td><td>MB-20-01_F1.docx</td></tr><tr><td>Biofilm Single Tube Method Timing/Dilution/Plating Form</td><td>MB-20-01_F2.docx</td></tr><tr><td>Biofilm Single Tube Method Results Sheet</td><td>MB-20-01_F3.docx</td></tr><tr><td>Biofilm Single Tube Method Processing Sheet</td><td>MB-20-01_F4.docx</td></tr><tr><td>Biofilm Test Microbe Confirmation Sheet</td><td>MB-20-01_F5.docx</td></tr><tr><td>Biofilm Neutralization Test Information Sheet</td><td>MB-20-01_F6.docx</td></tr><tr><td>Biofilm Neutralization Dilution/Plating Tracking Form</td><td>MB-20-01_F7.docx</td></tr><tr><td>Biofilm Neutralization Timing Sheet</td><td>MB-20-01_F8.docx</td></tr><tr><td>Biofilm Neutralization Results Sheet</td><td>MB-20-01_F9.docx</td></tr></table></td></tr>
<tr><td>15. References</td><td>1. ASTM International, 2012. E2871-12: Standard Test Method for Evaluating Disinfectant Efficacy against Pseudomonas aeruginosa Biofilm Grown in CDC Reactor using Single Tube Method.</td></tr>
</table>

	2. Standard Methods for the Examination of Water and Wastewater. 21st Edition. Eaton, A.D., Clesceri L.S., Rice E.W., Greenberg A.E. (Eds.) 2005. American Public Health Association, 1015 15th Street, NW, Washington, DC.

Attachment 1

Biofilm Neutralization Assay

A1. Culture preparation

a. Defrost a single cryovial at room temperature and briefly vortex to mix. Add 10 µL of the thawed frozen stock (single use) to a tube containing 10 mL of TSB (30 g/L), vortex, and incubate at 36 ± 1°C for 24 ± 2 h.

b. Prepare serial dilutions in 9 mL blanks of SMDW to achieve concentrations of approximately 10^6 and 10^5 CFU/mL per dilution tube; these concentrations are typically observed in the 10^{-2} and 10^{-3} dilution tubes, respectively. These dilutions should result in counts of 30-300 CFU/plate (refer to the Biofilm Neutralization Assay Flowchart).

A2. Neutralization confirmation assay

a. *Test Culture Titer (TCT)*. At timed intervals, add 0.1 mL of test organism diluted to 10^6 CFU/mL to 40 mL SMDW and vortex to mix thoroughly. Repeat with the test organism diluted to 10^5 CFU/mL. Proceed with section A2.e.

b. *Neutralizer Toxicity Treatment (NTT)*. At timed intervals, add 0.1 mL of the test organism diluted to 10^6 CFU/mL to 40 mL neutralizer and vortex to mix thoroughly. Repeat with the test organism diluted to 10^5 CFU/mL. Proceed with section A2.e.

c. *Neutralization Confirmation Treatment (NCT)*. At timed intervals, add 4 mL disinfectant to 36 mL neutralizer, briefly mix, add 0.1 mL of the test organism diluted to 10^6 CFU/mL, and vortex to mix thoroughly. Repeat with the test organism diluted to 10^5 CFU/mL. Proceed with section A2.e.

d. *Test Chemical Control (TCC)*. At timed intervals, add 0.1 mL of the test organism diluted to 10^6 CFU/mL to 4 mL disinfectant and vortex to mix thoroughly. Proceed with section A2.e.

e. Hold all treatments at room temperature for 10 minutes.

i. For the *Test Chemical Control* only: after the contact time, add 36 mL neutralizer to the *Test Chemical Control* tube and vortex to mix thoroughly.

f. After the contact time, vortex each tube thoroughly and prepare one 10-fold dilution in 9 mL SMDW.

g. Briefly vortex the dilution tube prior to plating. Plate 0.1 mL aliquots from each tube on R2A using spread plating. Spread inoculum evenly over the surface of the agar. Plates must be dry prior to incubation.

h. Incubate plates (inverted) at 36 ± 1°C for 24-48 h.

A3. Results

a. For calculation purposes, use the dilution that resulted in 30-300 CFU/plate.

b. For determining and verifying the effectiveness of the neutralizer, ensure that:

i. The recovered number of CFU in the *Neutralizer Toxicity Treatment* (see section A2.b) is within at least 0.5 logs of the *Test Culture Titer* (see section A2.a). A count lower than 0.5 logs indicates that the neutralizer is harmful to the test organism. Note: counts higher than the *Test Culture Titer* (e.g., 0.5 logs above the *Test Culture Titer*) are also deemed valid.

ii. The recovered number of CFU in the *Neutralizer Confirmation Treatment* (see section 12.4c) is within 0.5 logs of the *Test Culture Titer*; this verifies effective neutralization. Note: counts higher than the *Test Culture Titer* (e.g., 0.5 logs above the *Test Culture Titer*) are also deemed valid.

Biofilm Neutralization Assay Flowchart