US Environmental Protection Agency
Office of Pesticide Programs

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Proposed Procedure: EPA MLB SOP MB-19: Growing a Biofilm using the CDC Biofilm Reactor

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Growing a Biofilm using the CDC Biofilm Reactor

I. Overview

A. This document describes the methodology intended for use for growing a *Pseudomonas aeruginosa* or *Staphylococcus aureus* biofilm in the CDC biofilm reactor for the purpose of product efficacy testing.

B. The method is based on ASTM E2562-12, however, it has been modified to support biofilm claims for antimicrobial products tested using the Single Tube Method.

C. Growing the organism in the reactor is partitioned into 2 phases. The biofilm is established by operating the reactor in batch phase (i.e., no flow) for 24 hours followed by 24 hours with continuous flow of growth medium to form biofilm on coupons (CSTR phase). The coupons are harvested to conduct the efficacy test. The main components of this procedure are as follows:

1. Verify reactor operating volume (once per reactor) and periodically calibrate the pump
2. Clean and screen coupons
3. Prepare the reactor and steam-sterilize the assembled reactor
4. Sterilize the 20 L carboy containing 19-20 L de-ionized water
5. Prepare the inoculum
6. Initiate batch phase
7. Initiate CSTR phase
8. Harvest coupons for efficacy testing (refer to "Single Tube Method for Determining the Efficacy of Disinfectants against Bacterial Biofilm," dated 08/09/16)

II. Data Requirements

A. None

III. Special Apparatus and Materials

A. Test organisms.

1. *Pseudomonas aeruginosa* (ATCC No. 15442) obtained directly from ATCC.
2. *Staphylococcus aureus* (ATCC No. 6538) obtained directly from ATCC.

B. *Growth media.* Various concentrations of TSB for inoculum production, batch phase, and CSTR phase.

C. *Recovery media.*

1. R2A: for *P. aeruginosa*
2. Tryptic soy agar (TSA): for *S. aureus*

D. *Calibrated micropipettes*. For performing culture transfers.

E. *Ultrasonic water bath*. For cleaning the CDC Reactor coupons. Use any bath capable of maintaining a homogeneous sound distribution of 45±5 kHz and which has a volume large enough to accommodate 50 mL or 250 mL conical tubes.

F. *Peristaltic pump*. Example: Masterflex L/S Computerized Drive Model 755-50 with Easy-Load II pump head, model 77201-60, or equivalent. The pump head is capable of holding tubing with inner diameter (ID) 3.1 mm and outer diameter (OD) 3.2 mm. Use Masterflex Norprene tubing (Cole Palmer 06404-16).

G. *Magnetic stir plate*. Top plate 10.16×10.16 cm, capable of rotating at 60-125 rpm.

H. *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.

I. *Glass flow break.* Any that will connect with tubing of ID 3.1 mm and withstand sterilization.

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

K. *Clamp stand*. Height no less than 76.2 cm, used with clamp to suspend glass flow break vertically and stabilize tubing above reactor.

L. *Reactor Components*. For schematic illustrations of CDC reactor components, see ref. VII.A. Reactor components available from BioSurface Technologies, Inc.

1. *Berzelius borosilicate glass tall beaker*. 1000 mL without pour spout, 9.5±0.5 cm diameter. Barbed outlet spout added at 400±50 mL mark. Spout angled to 30-45° to ensure drainage. Spout to accommodate flexible tubing with an ID of 8-11 mm. NOTE: The rods and baffle described in III.L.3 and III.L.6, respectively, will displace approximately 50 mL of liquid when the system is completely assembled. Therefore, an outlet spout at the 400 mL mark will result in a reactor operating volume of approximately 350 mL.

2. *Reactor top*. UHMW (ultra-high molecular weight) polyethylene top (10.1 cm diameter tapering to 8.33 cm) equipped with 3 holes accommodating 6-8 cm long pieces of stainless steel or other rigid autoclavable tubing with OD of 5-8 mm for media inlet, air exchange and

inoculation port. Center hole, 1.27 cm diameter, to accommodate the glass rod used to support the baffle assembly. Eight rod holes, 1.905 cm diameter, notched to accommodate stainless steel rod alignment spike (0.236 cm OD).

3. *Polypropylene rods*. Eight polypropylene rods, 21.08 cm long, machined to hold three coupons at the immersed end. 316 stainless steel set screws imbedded in side to hold coupons in place. Rods to fit into holes in reactor top and lock into preformed notches.

4. *Coupons*. Twenty-four cylindrical coupons (i.e., borosilicate glass) with a diameter of 1.27±0.013 cm, thickness of approximately 3.0 mm.

5. *Small Allen wrench*. For loosening set screws.

6. *Stir blade assembly (baffled stir bar)*. Teflon blade (5.61 cm) fitted into cylindrical Teflon holder (8.13 cm) and held in place with a magnetic stir bar (2.54 cm). Teflon holder fits onto a glass rod (15.8 cm), fitted into the reactor top. The glass rod is held in place with a Swagelock fitting and acts as a support for the moving blade assembly.

M. *Carboys*. Two 20 L autoclavable carboys, one used for waste and one for the growth medium.

1. *Carboy lids*. Two carboy lids. One carboy lid with at least 3 barbed fittings to accommodate tubing ID 3.1 mm (one for the growth medium, one for bacterial air vent (filter), and one for injecting TSB medium concentrate). 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).

N. *Bacterial air vent (filter)*. Autoclavable 0.2 µm pore size, spliced into tubing on waste carboy, carboy with growth medium, and reactor top; recommended diameter 37 mm.

O. *Detergent*. Micro-90 Concentrated Cleaning Solution for Critical Cleaning; International Products Corporation. For cleaning coupons and reactor parts.

IV. Procedure and Analysis

A. Coupon preparation

1. Coupons may be used repeatedly with proper cleaning and screening between each use (see below). Check each coupon under 20X magnification for scratches, chips, other damage or accumulated debris before each use. Discard those with visible damage to surface topography.

i. After use in the reactor, place contaminated coupons in an

appropriate vessel, cover with liquid, and autoclave with the other parts of the contaminated reactor system for 30 min.

ii. For glass coupons, sonicate coupons individually (e.g., in plastic 50 mL conical tubes or in a 24 well plate) for 5 min in a 1:100 dilution of detergent and tap water. Cover the coupons completely with soapy water. Process coupons individually to minimize damage to the coupons.

iii. Rinse coupons with reagent grade water and sonicate for approximately 1 min in reagent grade water. Repeat rinsing and sonication with reagent grade water until no soap is left on the coupons.

iv. Once the coupons are clean, prevent oils and other residue from contaminating the surface. Store screened and cleaned coupons in a Petri dish.

B. Preparation of reactor

1. Place a cleaned and screened coupon into each hole in the reactor rods, leaving the coupon flush with the inside rod surface. Tighten the set screw.

2. Place rods loosely into reactor top (not yet fitted into notches).

3. Invert the reactor top and place baffled stir bar onto glass rod positioned in the center of the reactor top.

4. Place assembled top into the reactor beaker.

5. Connect the bacterial air vent by fitting the vent to a small section of appropriately sized tubing and attach to one of the rigid tubes on the reactor top.

6. Splice the glass flow break into the growth medium tubing line near the reactor top.

C. Sterilization of reactor system

1. Cover the end of the injection ports, the growth medium tubing that connects to the growth medium carboy, and the overflow (waste) tubing with aluminum foil. Cover any extra openings on the reactor top with aluminum foil or plastic caps.

2. Steam-sterilize the empty reactor system for 20 min.

D. Culture preparation

1. Refer to Attachment 1 for stock culture generation and QC.

2. For *P. aeruginosa*, defrost a single cryovial and briefly vortex to mix. Add 10 µL of the thawed frozen stock (single use) to a tube containing 10 mL of TSB (300 mg/L), vortex, and incubate at 36±1°C for 24±2 h.

3. For *S. aureus*, defrost a single cryovial 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.

4. Inoculate an agar plate (e.g., TSA with 5% sheep blood, BAP) with a loopful from the inoculated tube and streak for isolation. Incubate plate and examine for purity.

5. If desired, verify that the concentration of the 24±2 h culture is at least 10^7 CFU/mL.

E. Growth of biofilm in CDC reactor – batch phase

1. Make sure that the overflow (waste) line is clamped and aseptically add 500 mL of the batch culture medium to the cooled reactor (e.g., carefully remove one rod, pour the medium into the reactor through the rod opening, and re-insert the rod).

   i. For *P. aeruginosa*, the batch culture medium is 300 mg/L TSB.

   ii. For *S. aureus*, the batch culture medium is 3 g/L TSB.

2. Secure the rod alignment pins into the reactor top notches.

3. Place prepared reactor on a stir plate.

4. Clamp the flow break in an upright position.

5. Vortex the 10 mL tube of culture and use 1 mL to inoculate the reactor (see I.D.2-3) through one of the available rigid stainless steel tubes in the reactor top.

6. Turn on the magnetic stir plate.

   i. For *P. aeruginosa*, the rotational speed of the baffle is 125±5 rpm. Run the reactor system in batch phase at room temperature (e.g., 21±2°C) for 24±2 h. Record ambient temperature.

   ii. For *S. aureus*, the rotational speed of the baffle is 60±5 rpm. Incubate the reactor system in batch phase at 36±1°C for 24±2 h.

F. CSTR phase for *P. aeruginosa*

1. For *P. aeruginosa* biofilm, run the CSTR phase at room temperature.

2. Add 50 mL of sterile 40 g/L TSB to 20 L sterile water (in carboy) to achieve a final growth medium concentration of 100 mg/L TSB. Shake the carboy to thoroughly mix the contents. Other concentrations of TSB may be used to achieve a final concentration of 100 mg/L TSB.

3. Aseptically connect the tubing from the reactor to the carboy containing the CSTR growth medium (100 mg/L TSB). Record ambient temperature.

G. Preparation of CSTR medium for *S. aureus*

1. For *S. aureus* biofilm, run the CSTR phase at 36±1°C. Preheat the CSTR phase growth medium prior to it entering the reactor in order to maintain 36±1°C during CSTR phase.

2. Add 0.5 L of sterile 40 g/L TSB to 19 L sterile water (in carboy), then fill to 20 L with sterile water to achieve a final growth medium concentration of 1 g/L TSB. Shake the carboy to thoroughly mix the contents. Other concentrations of TSB may be used to achieve a final concentration of 1 g/L TSB.

3. Aseptically connect the tubing from the reactor to the carboy containing the CSTR growth medium. At least one day prior to CSTR phase, preheat a portion of the CSTR growth medium from the carboy (assuming the carboy is outside the incubator). To preheat, loosely coil ~12-15 feet of tubing (ID 3.1 mm) inside the incubator with the reactor. Prime the tubing with CSTR growth medium (1 g/L TSB).

4. Refer to Attachment 3 for the reactor/tubing setup.

H. Growth of biofilm in CDC reactor – CSTR mode

1. Pump a continuous flow of growth medium into the reactor to achieve a 30±2 min residence time based on the reactor's operating volume (e.g., 10.8 mL/min). Connect the end of the reactor drain to the waste carboy and remove the clamp.

2. Allow the reactor to run in CSTR mode for 24±2 h.

3. For conducting disinfectant efficacy or control coupon count evaluations, proceed to "Single Tube Method for Determining the Efficacy of Disinfectants against Bacterial Biofilm," dated 08/09/16.

V. Data Analysis and Calculations

A. None

VI. Attachments

A. Attachment 1: Procedures for Maintenance of Vegetative Bacterial Cultures –

Preparation of Frozen Stock Cultures

B. Attachment 2: Typical Growth Characteristics of strains of *P. aeruginosa* and *S. aureus*

C. Attachment 3: Reactor/Tubing Setup for Generation of *S. aureus* Biofilm

VII. References

A. ASTM International, 2012. E-2562-12: Standard Test Method for Quantification of *Pseudomonas aeruginosa* Biofilm Grown with High Shear and Continuous Flow Using CDC Biofilm Reactor.

B. ASTM International, 2013. E-2871-13: Standard Test Method for Evaluating Disinfectant Efficacy Against *Pseudomonas aeruginosa* Biofilm Grown in CDC Biofilm Reactor Using Single Tube Method

C. Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD. *P. aeruginosa* p. 164.

D. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD. *S. aureus* p. 1015.

E. Package Insert – Gram Stain Kit and Reagents. Becton, Dickinson and Company. Part no. 882020191JAA. Revision 07/2011.

F. Package Insert – Oxidase Reagent Droppers. Becton, Dickinson and Company. Part no. L001133. Revision 06/2010.

G. Package Insert – Catalase Reagent Droppers. Becton, Dickinson and Company. Part no. L001237. Revision 06/2010.

H. Package Insert – Staphaurex Plus. Remel. Part no. R30950102. Revised 11/23/07.

Attachment 1

Procedures for Maintenance of Vegetative Bacterial Cultures – Preparation of Frozen Stock Cultures

I. Preparation of Frozen Stock Cultures.

A. Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from ATCC at least every 18 months.

B. Open ampule of freeze dried organism per manufacturer's instructions. Using a tube containing 5-6 mL of TSB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the original tube of broth. Mix thoroughly. Incubate broth culture at 36±1ºC for 24±2 h.

C. After incubation, streak a loopful of the suspension on TSA to obtain isolated colonies. Incubate the plates for 18-24 h at 36±1°C. Refer to section II for QC of stock cultures.

D. Select 3-5 isolated colonies of the test organism and re-suspend in 1 mL of TSB. For *S. aureus*, select only golden yellow colonies. Multiple phenotypes are present for *P. aeruginosa* – the stock culture should be representative of all phenotypes present on the streak isolation plate. Spread plate 0.1 mL of the suspension on each of 6-10 TSA plates. Incubate the plates for 18-24 h at 36±1°C.

E. Following the incubation of the agar plates from I.D, place approximately 5 mL sterile cryoprotectant solution on the surface of each plate. Re-suspend the growth in the cryoprotectant solution using a sterile spreader without damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it in a sterile vessel large enough to hold about 30 mL. Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 tube may be used if necessary). Mix the contents of the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting culture.

F. Immediately after mixing, dispense 0.5-1 mL aliquots of the harvested suspension into cryovials; these represent the frozen stock cultures.

G. Store the cryovials at -70ºC or lower for a maximum 18 months then reinitiate with a new lyophilized culture.

II. QC of Stock Cultures.

A. Conduct a purity check (isolation streak) throughout each step of the frozen stock culture generation process prior to freezing the culture.

1. For section I.B, conduct a streak isolation onto BAP from the rehydrated

lyophilized culture. In addition, streak a loopful onto both MSA and Cetrimide.

2. For section I.C, conduct a streak isolation onto BAP from the TSA or NA 18-24 hour plate. In addition, streak a loopful onto both MSA and Cetrimide.

3. For section I.D, conduct a streak isolation onto BAP from the resuspended culture tube. In addition, streak a loopful onto both MSA and Cetrimide.

B. Conduct QC of the pooled culture concurrently with freezing (section I.E). Streak a loopful on a plate of BAP. In addition, streak a loopful onto both MSA and Cetrimide. Incubate all plates at 36±1ºC for 24±2 hours.

C. Following the incubation period, record the colony morphology as observed on the BAPs and selective media plates (including the absence of growth) and Gram stain. See Attachment 2 for details on cell and colony morphology, colony characteristics on selective media, and stain reactions.

D. For each organism, perform a Gram stain (refer to VII.E) from growth taken from the BAPs according to the manufacturer's instructions. Observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion).

E. Conduct additional biochemical and antigenic analyses; refer to sections VII.G-VII.H for *S. aureus* and VII.F for *P. aeruginosa* for instructions. Alternatively, Vitek 2 Compact may be used.

F. Record all confirmation results.

Attachment 2

Typical Growth Characteristics of strains of *P. aeruginosa* (see ref. VII.C) and *S. aureus* (see ref. VII.D).

	*P. aeruginosa**	*S. aureus**
Gram stain reaction	(-)	(+)
Typical Growth Characteristics on Solid Media		
Mannitol Salt	No Growth	circular, small, yellow colonies, agar turning fluorescent yellow
Cetrimide	circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	No Growth
Blood agar (BAP)	flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic	small, circular, yellow or white, glistening, beta hemolytic
Typical Microscopic Characteristics		
Cell dimensions	0.5-1.0 µm in diameter by 1.5-5.0 µm in length*	0.5-1.5 µm in diameter*
Cell appearance	straight or slightly curved rods, single polar flagella, rods formed in chains	spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters

*After 24±2 hours

(1) *P. aeruginosa* 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. Pyocyanin is not produced.

Attachment 3

Reactor/Tubing Setup for Generation of *S. aureus* Biofilm