US Environmental Protection Agency
Office of Pesticide Programs

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Proposed Procedure: EPA MLB SOP MB-20: Single Tube Method for Determining the Efficacy of Disinfectants against Bacterial Biofilm

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Single Tube Method for Determining the Efficacy of Disinfectants against Bacterial Biofilm

I. Overview

A. This document describes the Single Tube Method intended for use to determine the efficacy of disinfectants against biofilm grown in the CDC biofilm reactor. This method is available for use for evaluating the efficacy of aqueous disinfectants against biofilm grown on borosilicate glass coupons.

B. Five randomly selected coupons are evaluated for efficacy and three are evaluated as controls.

C. In advance of testing, verify the performance of the neutralizer using the procedure in Attachment 1.

D. The method is based on ASTM E2871-13.

II. Data Requirements

A. For the purpose of conducting the Single Tube Method, the mean log density for coupons (colony forming units (CFU) per coupon) inoculated with *P. aeruginosa* or *S. aureus* should be 8.0-9.5 (corresponding to a geometric mean density of 1.0×10^8 to 3.2×10^9), based on data collected from multiple collaborative studies.

III. Special Apparatus and Materials

A. *Dilution buffer*. Prepare stock phosphate buffer solution by dissolving 34.0 g KH_2PO_4 in 500 mL reagent-grade water, adjust to pH 7.2±0.5 with 1 N NaOH, and dilute to 1 L with reagent-grade water. Prepare stock magnesium chloride solution: 81.1 g $MgCl_2{\cdot}6H_2O$/L reagent-grade water. Filter sterilize both stock solutions. Prepare buffered dilution water by combining 1.25 mL KH_2PO_4 stock solution and 5.0 mL $MgCl_2{\cdot}6H_2O$, and dilute to 1 L with reagent-grade water (for final concentrations of 0.0425 g/L KH_2PO_4 and 0.405 g/L $MgCl_2{\cdot}6H_2O$) and sterilize appropriately (see ref. VII.B).

1. Alternatively, phosphate buffered dilution water (PBDW) or phosphate buffered saline (PBS) may be used for rinse tubes (with 30 mL), control coupon exposure fluid, dilution blanks, and filtration fluid, provided that the same buffer is used for each step.

B. *Vortex.* Any variable speed vortex that will ensure proper mixing of tubes. A platform adapter may be used to vortex more than one conical tube at a time.

C. *Calibrated micropipettes.* For making dilutions.

D. *Ultrasonic water bath*. Any bath capable of maintaining a homogeneous sound distribution of 45±5 kHz and a volume large enough to accommodate 50 mL or 250 mL conical tubes. For removing biofilm from coupons.

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

F. *Conical tubes.* 50 mL or 250 mL polypropylene sterile screw cap centrifuge tubes (e.g., Corning brand). Used as the reaction tube for the coupon/test substance or control fluid/neutralizer combination. 250 mL tubes are used to accommodate an increased volume of neutralizer (e.g., 196 mL). Use tubes that properly accommodate the splashguard insert (i.e., appropriate interior diameter and length).

G. *Filter membranes.* 47 mm diameter and 0.45 µm polyethersulfone (PES) pore size. Filtration units (reusable or disposable) may be used. For microbe recovery from treated coupons.

H. *Splashguard inserts*. BioSurface Technologies. Used during coupon deposition. Two sizes are available for both the 50 mL and 250 mL conical tubes.

IV. Procedure and Analysis

A. Test culture preparation

1. Prepare biofilm per "Growing a Biofilm using the CDC Biofilm Reactor" dated 07/20/16.

2. Once the flow of nutrients has stopped, harvest coupons for testing with one hour.

B. Reaction tube preparation

1. Refer to attachment 2 for pictures of technique sensitive steps.

2. Prior to sterilization, verify that the splashguards will sit properly in the conical tubes so that the end of the splashguard sits at the straight/conical interface of the tube.

3. Splashguards may be sterilized separately and then placed into sterile conical tubes.

   i. Prior to sterilization, separate the flared top and the cylindrical bottom of the splashguard and place the cylindrical bottom piece into an empty conical tube with the etched side up. Ensure the bottom of the splashguard sits properly in the conical tube (see IV.B.2).

   ii. Place the flared top of the splashguard onto the cylindrical bottom piece and press down. Remove the assembled unit from the conical tube. Repeat the process for the remaining splashguards, wrap the assembled units in foil or place in a sterilization pouch, and sterilize for at least 25 min on a gravity cycle.

4. Alternatively, splashguards may be sterilized inside the conical tubes.

   i. Remove the lids from a rack of conical tubes and place the lids into a sterilization pouch or wrap with foil.

   ii. Place a splashguard into each conical tube, ensuring proper fit (see IV.B.2). Cover the conical tubes containing the splashguards with foil and sterilize along with the lids as in IV.B.3.ii.

5. Splashguards are only needed for reaction tubes with coupons treated with test substances.

6. For test substances requiring larger neutralizer volumes, use 250 mL conical tubes with corresponding splashguards.

C. Disinfectant sample preparation

1. Use the test substance within three hours of preparation unless test parameters specify otherwise. Record the time of test substance preparation.

2. Evaluate the test substance at 21±2°C. If necessary, place test substance in water bath prior to use to achieve the appropriate temperature.

3. Bring the neutralizer to room temperature prior to use.

D. Test procedure

1. 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.

2. 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 dilution buffer.

   ii. Immerse the rod with a continuous motion into the dilution buffer with minimal to no splashing, then immediately remove.

   iii. Use a new 50 mL conical tube with 30 mL dilution buffer for each rod.

3. Hold the rod with one of the randomly selected coupons centered over an empty, sterile 50 mL or 250 mL conical tube containing a splashguard (for coupons exposed to test substance).

4. During coupon deposition, do not allow the rod to contact the tube or splashguard for treated or control samples. If contact occurs, replace the coupon and associated tube and/or splashguard. Refer to Attachment 2 for

a picture of proper rod orientation.

5. 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 treated coupon, repeat coupon removal four more times for a total of five tubes, each containing one coupon.

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

6. After depositing the coupons in the tubes, gently remove the splashguards from each tube using sterile forceps.

7. To reduce the risk of cross contamination, process coupons treated with test substance first.

8. Apply 4 mL prepared test substance (antimicrobial treatment) or control substance (dilution buffer) down the side of the conical tubes containing the coupons, avoiding direct contact with the coupon during application and being careful to completely cover the coupons. Refer to Attachment 2 for a picture of proper treatment application.

   i. For a 10 min contact time, a 30 s interval between coupons is recommended. Track contact time.

9. Immediately after deposition of test substance or control substance, gently swirl the tube 1-2 times to fully expose the biofilm on the coupon to the liquid, ensuring there are no air bubbles trapped beneath the coupon.

10. Allow tubes to remain at room temperature for the duration of the contact time.

11. 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.

    i. Some test substances (e.g., highly acidic products) may need additional neutralizer volume (e.g., 196 mL). In these instances, use 250 mL conical tubes.

12. After neutralization, vortex the contents of each tube on the highest setting for 30±5 s.

13. After the first vortex, place all tubes into a wire or plastic conical tube rack and suspend the rack in the ultrasonic water bath (previously degassed for ~5 min) so that the liquid level in the tubes is even with the liquid level in the bath. Sonicate the tubes at 45±5 kHz for 30±5 s without sweep function. Do not allow the tubes or the rack to touch the bottom or sides of the ultrasonic water bath.

14. After the first sonication, vortex the contents of each tube on the highest setting for 30±5 s.

15. After the second vortex, place all tubes into a wire or plastic 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 bath. Sonicate the tubes at 45±5 kHz for 30±5 s without sweep function. Do not allow the tubes or the rack to touch the bottom or sides of the ultrasonic water bath.

16. After the second sonication, vortex the contents of each tube on the highest setting for 30±5 s.

17. For calculation purposes, tubes containing the coupon are referred to as the 10^0 dilution.

18. Serially dilute each 10^0 dilution (by removing 1 mL) for treated and control coupons in 9 mL blanks of dilution buffer.

    i. For treated coupons, filter a minimum of 10 mL from the 10^0 dilution and the entire contents of the 10^{-1} dilution tube (10 mL) through a 0.45 µm PES filter membrane.

    ii. Pass liquid from the 10^0 tube through the filter within 1 min with limited pooling of liquid in the filter apparatus.

19. For test substances that require additional neutralizer volume, filter a minimum of 25% of the total volume of neutralizer + test substance. If necessary, multiple filters may be used to assay these larger volumes.

20. To filter, pre-wet the membrane with ~20 mL dilution buffer then filter the appropriate volume from the appropriate tube.

21. If filtering the entire contents of a tube, rinse the tube with ~10 mL dilution buffer and filter the rinsate.

22. Rinse the sides of the filter funnel with additional dilution buffer and place the filter membrane on R2A (for *P. aeruginosa*) or TSA (for *S. aureus*). Gently roll the filter onto the surface of the agar to remove any air bubbles that may be trapped between the agar and the membrane.

23. For spread plating (control coupons), briefly vortex each serial dilution tube prior to plating. Plate 0.1 mL aliquots of appropriate dilutions in duplicate on R2A (for *P. aeruginosa*) or TSA (for *S. aureus*) using spread plating. Spread inoculum evenly over the surface of the agar. Dry plates prior to incubation.

    i. Alternatively, 1 mL aliquots may be plated on Petrifilm.

24. For control coupons, plate appropriate dilutions to achieve colony counts in the range of 30-300 colony forming units (CFU) per plate.

25. Incubate all filters, plates and/or Petrifilm at 36±1°C for 48±4 h.

E. Recording results

1. Count colonies. Spread plates and Petrifilm that have colony counts over 300 will be reported as too numerous to count (TNTC); filter membranes that have colony counts over 200 will be reported as TNTC.

2. Inspect the growth on the plates and filters for purity and typical characteristics of the test microbe. Gram stain one representative colony per coupon set with growth for treated and controls. Isolation streaks, biochemical and antigenic analyses, and/or Vitek 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.

    ii. *S. aureus* is a Gram positive cocci.

F. Coupon and reactor reuse

1. 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 (including splashguards) for 30 min.

2. After sterilization, clean the reactor components with a 1:100 dilution of detergent and tap water. After washing, rinse all components with deionized water.

3. Clean and rescreen the coupons per "Growing a Biofilm using the CDC Biofilm Reactor" dated 07/20/16, section IV.A.

V. Data Analysis and Calculations

A. Record all colony counts and use in calculations to determine log reductions.

B. To calculate the CFU/coupon for control coupons, use the following equation: $\left[\frac{\left(\frac{Mean\ CFU\ for\ 10^{w} + Mean\ CFU\ for\ 10^{x}}{10^{w}+10^{x}}\right)}{Y}\right] \times Z$; where 10^{w} and 10^{x} are the dilution tubes plated, *Y* accounts for the volume plated (mL), and *Z* is the volume of liquid (disinfectant + neutralizer) in the tube with the coupon.

C. To calculate the CFU/coupon for treated coupons, use the following equation: $\left(\frac{CFU\ per\ filter\ for\ 10^{w} + CFU\ per\ filter\ for\ 10^{x}}{(a \times 10^{w}) + (b \times 10^{x})}\right) \times Z$, where "a" and "b" are the volumes filtered at each dilution, and *Z* is the volume of liquid (disinfectant + neutralizer) in the tube with the coupon.

D. For example, when 2 filters are used to assay the reaction tube (10^0 dilution), proceed as follows: $\left(\frac{(CFU_1 + CFU_2\ for\ 10^{w}) + CFU\ for\ 10^{x}}{(a \times 10^{w}) + (b \times 10^{x})}\right) \times Z$, where CFU_1+CFU_2 is the sum of the CFU/filter for a given dilution (10^{w}),"a" and "b" are the total volumes filtered for each dilution, and *Z* is the volume of liquid (disinfectant + neutralizer) in the tube with the coupon.

E. Calculate the $\log_{10}$ density of the CFU/coupon of each treated and control coupon.

F. Calculate the mean $\log_{10}$ density across treated coupons.

G. Calculate the mean $\log_{10}$ density across control coupons.

H. Calculate the $\log_{10}$ reduction (LR) for treated coupons:
$\log_{10}$ reduction = mean $\log_{10}$ control – mean $\log_{10}$ treated

I. For cases where there is no recovery for the treated coupons and only a sample of the 10^0 tube is filtered, substitute 0.5 CFU at the 10^0 dilution and scale up accordingly.

J. For cases where there is no recovery for the treated coupons and the entire contents of the 10^0 tube is filtered, the LR is greater than or equal to the mean control counts.

VI. Attachments

A. Attachment 1: Neutralization Assay

B. Attachment 2: Method Photographs

VII. References

A. ASTM International, 2013. E2871-13: Standard Test Method for Evaluating Disinfectant Efficacy against *Pseudomonas aeruginosa* Biofilm Grown in CDC Reactor using Single Tube Method.

B. 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

I. 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 dilution buffer 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. At least one of these dilutions when diluted and plated should result in counts of 30-300 CFU/plate (refer to the Biofilm Neutralization Assay Flowchart).

II. Neutralization confirmation assay

A. *Neutralization Confirmation Treatment (NCT).* At timed intervals, add 4 mL disinfectant to 36 mL neutralizer (in triplicate), briefly mix, within 10 s add 0.1 mL of the test organism diluted to 10^5 CFU/mL, and vortex to mix thoroughly. Repeat with the test organism diluted to 10^6 CFU/mL if desired. Proceed with section II.D.

B. *Neutralizer Toxicity Treatment (NTT).* At timed intervals, add 0.1 mL of the test organism diluted to 10^5 CFU/mL to 40 mL neutralizer (in triplicate) and vortex to mix thoroughly. Repeat with the test organism diluted to 10^6 CFU/mL if desired. Proceed with section II.D.

C. *Test Culture Titer (TCT).* At timed intervals, add 0.1 mL of test organism diluted to 10^5 CFU/mL to 40 mL dilution buffer (in triplicate) and vortex to mix thoroughly. Repeat with the test organism diluted to 10^6 CFU/mL if desired. Proceed with section II.D.

D. Hold all treatments at room temperature (e.g., 21±2°C) for 10 min±30 s.

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

F. Briefly vortex the dilution tube prior to plating; initiate plating within 30 min of making dilutions. Plate 0.1 mL aliquots from each tube in duplicate on R2A (for *P. aeruginosa*) or TSA (for *S. aureus*) using spread plating. Spread inoculum evenly over the surface of the agar. Plates must be dry prior to incubation.

G. Alternatively, 10 mL from each of the NCT, NTT, and TCT treatment tubes may be filtered through individual 0.45 µm polyethersulfone membranes; no additional dilution is necessary.

1. Make adjustments to the initial dilution series in advance to achieve a target of 20-200 CFU per filter.

2. For test substances that require additional neutralizer volume, filter a minimum of 20% of the total volume of neutralizer + test substance. If necessary, multiple filters may be used to assay these larger volumes.

3. To filter, pre-wet the membrane with ~20 mL dilution buffer then add the appropriate volume from the treatment tube. Rinse the sides of the filter funnel with additional dilution buffer and place the filter membrane on R2A (for *P. aeruginosa*) or TSA (for *S. aureus*). Gently roll the filter onto the surface of the agar to remove any air bubbles that may be trapped between the agar and the membrane.

H. Incubate plates (inverted) at 36±1°C for 48±4 h.

III. Results

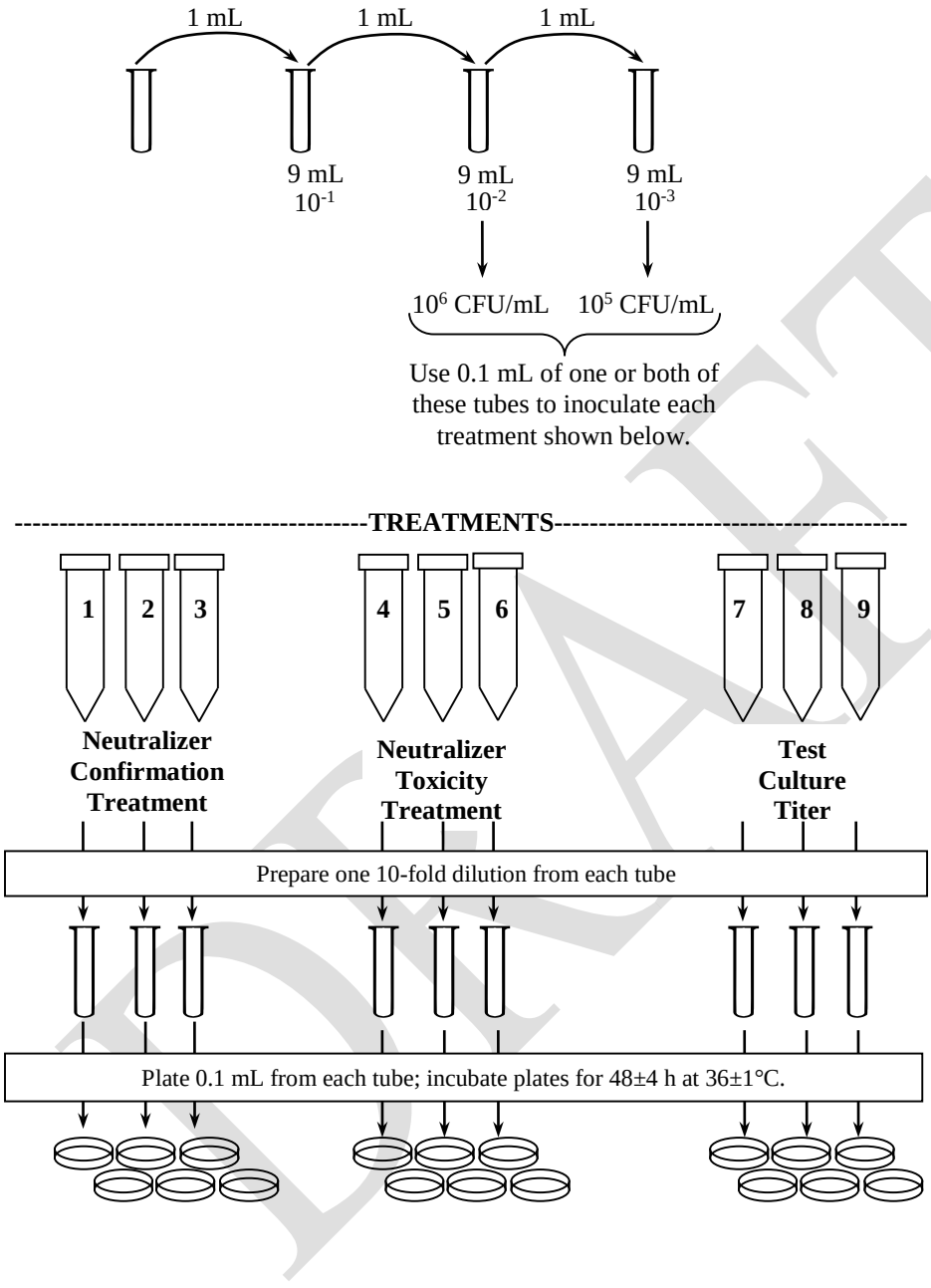
A. For calculation purposes, use the dilution that resulted in 30-300 CFU/plate (or 20-200 CFU/filter). Average between spread plates for a given tube (if using), then average results from the three tubes per treatment.

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

1. The recovered number of CFU in the *Neutralizer Toxicity Treatment* (see section II.B) is within 50% of the *Test Culture Titer* (see section II.C). A count less than 50% indicates that the neutralizer is harmful to the test organism. Note: counts higher than the *Test Culture Titer* (e.g., 120% of the *Test Culture Titer*) are also deemed valid.

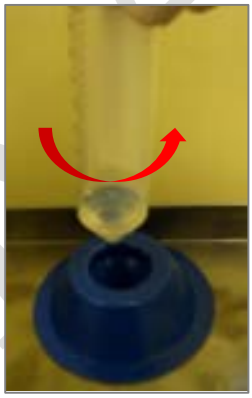
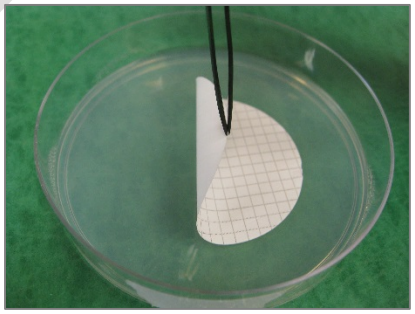
2. The recovered number of CFU in the *Neutralizer Confirmation Treatment* (see section II.A) is within 50% of the *Test Culture Titer*; this verifies effective neutralization. Note: counts higher than the *Test Culture Titer* (e.g., 120% of the *Test Culture Titer*) are also deemed valid.

Biofilm Neutralization Assay Flowchart (for one dilution of the test organism)



Attachment 2

Method Photographs

 Splashguard insert in 50 mL conical tube	 Appropriate location of splashguard insert (arrow indicates appropriate position of bottom of insert in conical tube)	 Desirable rod positioning.
 Addition of test substance down the side of the reaction tube (10^0 tube).	 Gentle swirl of tube with carrier after addition of 4 mL test substance/control substance.	 Application of filter to agar surface (rolling filter onto agar plate).