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 Determining the Efficacy of Disinfectants against Bacterial Biofilms

SOP Number: MB-20-04

Date Revised: 09-19-22

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Title	Single Tube Method for Determining the Efficacy of Disinfectants against Bacterial Biofilm
Revisions Made	• Minor editorial changes for clarification purposes. • Removed Dey Engley (D/E) Neutralizing broth as an example of neutralizer. • Took out footnotes that steps were not currently in ASTM E2871-19. • Updated ASTM International Standard reference from 2019 to 2021 and updated version number from E2871-19 to E2871-21.

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Title	Single Tube Method for Determining the Efficacy of Disinfectants against Bacterial Biofilm
Scope	Describes the operational parameters required to perform a quantitative liquid disinfectant efficacy test against bacterial biofilm grown in the CDC biofilm reactor.
Application	For use in determining the efficacy of aqueous disinfectants against biofilm grown on borosilicate glass coupons.

	Approval	Date
SOP Developer:	Rebecca Pines	09/19/22
	Print Name: Rebecca Pines	
SOP Reviewer	Lisa Smith	09/19/22
	Print Name: Lisa Smith	
Quality Assurance Unit	Michele Cottrill	09/19/22
	Print Name: Michele Cottrill	
Branch Chief	Rebecca Pines	09/19/22
	Print Name: Rebecca Pines	

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1. Definitions	Additional abbreviations/definitions are provided in the text. 1. CDC = Centers for Disease Control and Prevention 2. Biofilm = 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 = biofilm growth surface 4. Disinfectant = antimicrobial test substance applied to coupons 5. Control substance = innocuous liquid applied to coupons
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 (pH meters), EQ-02 (thermometers), EQ-03 (weigh balances), EQ-05 (timers), QC-19 (pipettes), and QC-22 (Vitek) for details on method and frequency of calibration. 2. Refer to MB-19 section 4 to confirm the operating volume of the reactor and residence time verification.
5. Sample Handling and Storage	Refer to SOP MB-22, Disinfectant Sample Preparation, and SOP COC-01, Chain of Custody Procedures as necessary.
6. Quality Control	1. For quality control purposes, the required information is documented on the appropriate form(s) (see section 14). 2. See MB-19 for purity check of test organism.
7. Interferences	1. Coupons must be screened prior to use; replace coupons that are compromised (presence of nicks or scratches). 2. Touching the rod to the top of the conical tube will cause contamination of the test system. 3. Improper use of the splashguard may cause erroneous data (e.g., unexposed inoculum).

	4. Improper placement of the conical tubes with the coupons in the sonicator during the recovery steps may interfere with removal of the biofilm from the coupon. 5. Conduct neutralizer confirmation in advance of the disinfectant assay to ensure validity of the efficacy 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. For the purpose of conducting the Single Tube Method, the mean *TestLD* for coupons inoculated with *P. aeruginosa* must be 8.0-9.5 CFU/coupon (corresponding to a geometric mean density of 1.0×10^8 to 3.2×10^9), with each coupon exhibiting a LD of 8.0 to 9.5. A mean *TestLD* below 8.0 or above 9.5 invalidates the test. 3. For the purpose of conducting the Single Tube Method, the mean *TestLD* for coupons inoculated with *S. aureus* must be 7.5-9.0 CFU/coupon (corresponding to a geometric mean density of 3.2×10^7 to 1.0×10^9), with each coupon exhibiting a LD of 7.5 to 9.0. A mean *TestLD* below 7.5 or above 9.0 invalidates the test.
9. Data Management	Archive the data consistent with SOP ADM-03, Records and Archives.
10. Cautions	1. Evaluate treated coupons prior to evaluating control coupons to mitigate cross-contamination.
11. Special Apparatus and Materials	1. *Test organisms.* a. Coupons with mature *Pseudomonas aeruginosa* ATCC 15442 or *Staphylococcus aureus* ATCC 6538 biofilm grown per MB-19. A mature biofilm of *P. aeruginosa* or *S. aureus* meets the criteria identified in 8.2 and 8.3, respectively. 2. *Bacterial plating medium.* R2A agar for *P. aeruginosa*, tryptic soy agar (TSA) for *S. aureus*. 3. *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 $MgCl_2\cdot6H_2O$/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 $MgCl_2\cdot6H_2O$, 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 $MgCl_2\cdot6H_2O$)

and sterilize appropriately (see ref. 15.2).

a. 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.

4. *Neutralizer.* One specific to the disinfectant being evaluated as determined for effectiveness and toxicity according to Attachment 1.

a. Some treatments may require additional neutralizer volume (for example, up to 196 mL). In these instances, use 250 mL conical tubes.

5. *Small Allen wrench (1.27 mm, hex)*. For loosening set screws and pushing coupons out of reactor rods.

6. *Vortex mixer.* Any vortex that will ensure proper mixing of tubes.

7. *Calibrated micropipettes*. Continuously adjustable pipettes with volume capacity of 100 µL and 1000 µL.

8. *Ultrasonic water bath*. Any 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 in a wet environment.

a. Prior to using the sonicating bath for the first time, verify that the sonicating bath does not kill viable cells by placing the standardized broth culture into the sonicator for 60 s, serially dilute, and plate. Compare sonicated counts to a non-sonicated control. The sonicated and non-sonicated counts should be comparable.

9. *Detergent*. Laboratory detergent for cleaning coupons and reactor parts (e.g., Micro-90 Concentrated Cleaning Solution for Critical Cleaning; International Products Corporation).

10. *Conical centrifuge tubes.* Sterile, any with 50 mL volume capacity and secure leak-proof lids.

a. For foaming disinfectants or for disinfectants requiring a larger volume of neutralizer, 250 mL conical tubes are used to preserve the required geometry and allow for greater neutralization capacity.

11. *Polyethersulfone (PES) filter membranes.* Sterile 47 mm diameter membranes with 0.45 µm pore size for microbe recovery from treated coupons. Filtration units (reusable or disposable) may be used.

	12. *Forceps*. Any appropriate for handling membrane filters. 13. *Splashguard inserts (from BioSurface Technologies)*. Used during coupon deposition. Two sizes to fit the 50 mL and 250 mL conical tubes used during coupon deposition. Equivalent splashguards (2.54 cm tapered to 2.39 cm (outer diameter) × 10.48 cm long for the 50 mL conical tubes and 2.50 cm (outer diameter) × 14.80 cm long for the 250 mL conical tubes) from other suppliers may also be used.
12. Procedure and Analysis	Each test includes three untreated control coupons (exposed to buffered dilution water) and five treated coupons (per disinfectant/concentration/contact time combination). In advance of testing, verify the performance of the neutralizer using the procedure in Attachment 1.
12.1 Test culture preparation	a. Prepare mature biofilm per SOP MB-19, section 12.
12.2 Reaction tube preparation	a. Prior to steam 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. b. Steam sterilize the splashguards appropriately (e.g., place splashguards into an autoclave pouch or wrap with aluminum foil and steam sterilize for at least 25 min). c. Splashguards are only required for reaction tubes with coupons treated with disinfectants. d. For disinfectants requiring larger neutralizer volumes, use 250 mL conical tubes with corresponding splashguards.
12.3 Disinfectant sample preparation	a. Prepare disinfectant per SOP MB-22. When preparing disinfectant, ensure that the disinfectant is adequately mixed. Use within 3 h of preparation or as specified in the manufacturer's instructions. Record the time of disinfectant preparation on the Biofilm Single Tube Method Processing Sheet (see section 14). b. Evaluate the disinfectant at room temperature (21±2°C). If necessary, place disinfectant in water bath prior to use to equilibrate to the appropriate temperature for 10-15 min. Record temperature on the Single Tube Method Processing Sheet (see section 14). c. Bring the neutralizer to room temperature prior to use.
12.4 Removal of coupons from	a. Prepare sampling materials: reaction tubes with splashguards, rinse tubes, and flame-sterilized Allen wrench.

the CDC biofilm reactor	b. Turn off growth medium flow and baffled stir bar. After growth medium flow and baffled stir bar have been turned off, use coupons for testing (that is, exposed to disinfectant/control) within 30 min. i. If necessary for experiments in which more than 8 coupons are evaluated or removed from the reactor and evaluated in batches over a period of time, the growth medium flow and baffled stir bar may remain on during coupon removal such that the total amount of time during the CSTR phase does not exceed 24±2 h. Insert sterile coupon holder blank in place of those rods removed for coupon evaluation to maintain the appropriate flow dynamics within the reactor. c. 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. d. Rinse the coupons to remove planktonic cells. Orient the rod in a vertical position directly over a 50 mL conical tube containing 30 mL sterile buffered water. With one continuous motion, immerse the rod into the buffered water with minimal to no splashing, then immediately remove. Use a new 50 mL conical tube with 30 mL sterile buffered water for each rod. e. 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 a disinfectant). i. During coupon deposition, do not allow the rod to contact the tube or splashguard for treated or control samples. Refer to Attachment 2 for proper rod orientation. If contact occurs, replace the coupon and associated tube and/or splashguard. f. 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 in the center of the coupon with the Allen wrench used to loosen the set screw. g. Remove an appropriate number of coupons for testing. Obtain a set of five coupons for each treatment and a set of three coupons for the controls (one set of control coupons per reactor run) as

	described in section 12.4c-f. h. After removing the coupons for testing, gently remove the splashguard from each tube using sterile forceps. Cap the reaction tube to mitigate dehydration. Splashguards are not required for control coupons.
12.5 Conduct efficacy evaluation	a. Slowly pipette 4 mL previously prepared disinfectant (treatment) or buffered dilution water (untreated control) down the side of each of the conical tubes containing the coupons, avoiding direct contact with the coupon during application and being careful to completely cover the coupon. Record the time of coupon exposure and the room temperature (21±2°C), see section 14. Refer to Attachment 2 for proper treatment application positioning. Process coupons treated with disinfectant first followed by controls. i. If necessary for experiments in which more than 8 coupons are evaluated, evaluate one control coupon prior to the start of the disinfectant treatment(s) and the remaining two control coupons after the disinfectant treatment(s). ii. For a 10 min contact time, a 1 min interval between coupons is recommended. b. Immediately after deposition of disinfectant or control substance, gently swirl the tube 1-2 times to fully expose the biofilm on the coupon to the liquid, ensuring the coupon is fully covered by the test substance and that there are no air bubbles trapped beneath the coupon. The coupon is invalid if it is not fully exposed to the test substance due to trapped air bubbles; replace with new coupon and tube if this occurs. For those test substances that cause effervescence, the presence of the effervescence does not invalidate the coupon. c. Allow tubes to remain at room temperature (21±2°C) for the duration of the contact time. d. At the end of the contact time, add the appropriate volume of neutralizer (for example, 36 mL or 196 mL) to each tube. Replace the cap and briefly vortex the tube.
12.6 Remove and disaggregate biofilm	a. Vortex each tube on the highest setting, ensuring a complete vortex for 30±5 s. b. 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 water level in the tank of the bath. Do not allow the tubes or the rack to touch the bottom or sides of the ultrasonic water bath. Within the conical tube rack, allow space between the tubes. c. Sonicate the tubes at 45±5 kHz for 30±5 s at room temperature (21±2°C) (use normal mode if sonicator has variable settings). d. Vortex the tubes a second time as described in 12.6a. e. Sonicate the tubes a second time as described in 12.6b and 12.6c. f. Vortex the tubes a third time as described in 12.6a. These tubes are the 10^0 dilution.
12.7 Dilute and recover disaggregated biofilm samples	a. Dilute and recover treated samples followed by controls samples. Initiate dilutions within 30 min of neutralization. b. Serially dilute the disaggregated biofilm from control samples in buffered water. Test coupons may be serially diluted, if necessary, to achieve countable filters in the target range of 20-200 CFU. Make serial ten-fold dilutions in 9 mL buffered water. c. For treated coupons, filter at least 25% of the total volume of neutralizer + disinfectant from the 10^0 reaction tube through 0.45 µm PES membrane filters. Initiate filtration within 30 min of making dilutions. i. Vortex the reaction or dilution tube prior to filtration. ii. Pre-wet the membrane filter with approximately 10 mL dilution buffer. iii. Filter the appropriate volume. Liquid should pass through the filter quickly (for example, within approximately 1 min of addition) with limited pooling of liquid in the filter apparatus. If necessary to lessen this occurrence, use multiple membrane filters per sample. iv. Separate filters but the same filtration unit can be used for a given coupon provided the dilutions are filtered in order starting with the most dilute. d. If filtering the entire contents of a tube, rinse the tube with approximately 10 mL dilution buffer, vortex, and filter the rinsate. e. Rinse the sides of the filter funnel with additional dilution buffer (for example, approximately 40 mL) and transfer the membrane

	filter to the recovery medium. Gently roll the filter onto the surface of the agar to prevent trapping air bubbles between the agar and the membrane; use sterile forceps to reposition the filter if necessary. f. For control coupons, briefly vortex each tube and spread-plate aliquots of the appropriate dilutions in duplicate on the recovery medium. i. Plate 0.1 mL aliquots of appropriate dilutions in duplicate on agar plates (R2A for *P. aeruginosa* or TSA for *S. aureus*) for spread plating. Spread inoculum evenly over the surface of the agar. Ensure plates are dry prior to incubation. ii. Alternatively, 1 mL aliquots may be plated on Petrifilm.[1] g. Incubate plates from control coupons at 36±2°C for 48±4 h. Incubate plates with filters from treated coupons for 72±4 h. h. If necessary, monitor recovery media for growth and assess the number of visible colonies beginning at 24 h. i. Count the appropriate number of CFU according to the recovery method used (for example, up to 200 CFU for filters and up to 300 CFU for plating). Record counts on the Biofilm Single Tube Method Results Sheet (see section 14). Use CFU to calculate log reduction. Log reduction is used to determine disinfectant effectiveness. j. Inspect the growth on the plates and filters for purity and typical characteristics of the test microbe. k. Gram stain one representative colony per coupon set with growth for treated and controls. Record results on the Biofilm Test Microbe Confirmation Sheet. i. *P. aeruginosa* is a Gram-negative rod. ii. *S. aureus* is a Gram-positive coccus. l. Isolation streaks, biochemical and antigenic analyses, and/or Vitek may be performed for additional verification of the test organism.
12.8 Coupon, reactor, and	a. After use in the reactor, place contaminated coupons in an appropriate vessel, cover with liquid (for example, water), and

[1] Option not provided in ASTM E2871-21.

splashguard reuse	autoclave with the other parts of the contaminated reactor system (including splashguards) for 30 min. b. After sterilization, clean the reactor components and splashguards with a 1:100 dilution of detergent and tap water. After washing, rinse all components with deionized water. c. Clean and rescreen the coupons per SOP MB-19, section 12.1.
13. Data Analysis/ Calculations	1. All colony counts are recorded and used in calculations to determine log reductions. 2. Calculate *X*, the mean CFU from the replicate samples plated. a. The $\log_{10}$ density (LD) for each control coupon is calculated as follows: $\mathrm{Log}_{10}\left\{\left[\frac{\sum_{i=1}^{n}(X_i)}{\sum_{i=1}^{n}(B\times D_i)}\right]\times V\right\}$ where: n = number of dilutions X = average CFU of the replicate sample plates, B = volume plated, V = total volume of buffered dilution water plus neutralizer, D = 10^{-k}, and k = dilution. 3. Calculate the mean LD for control coupons as follows: Mean LD = [Log_{10}(Coupon A) + Log_{10}(Coupon B) + Log_{10}(Coupon C)]/3 4. Calculate biofilm density for treated coupons. a. The LD for each treated coupon is calculated as follows: $\mathrm{Log}_{10}\left\{\left[\frac{\sum_{i=1}^{n}(Y_i)}{\sum_{i=1}^{n}(C_i\times D_i)}\right]\times V\right\}$ where: n = number of dilutions Y = CFU per filter, C = volume filtered, V = total volume of disinfectant plus neutralizer, D = 10^{-k}, and k = dilution. b. For the purpose of calculation, if no organism is recovered from a treated carrier, the log density for that coupon is 0 provided that the entire contents of the 10^0 reaction tube was filtered. c. If no organism is recovered from a treated carrier and only a

	fraction of the 10^0 reaction tube was filtered, substitute 0.5 CFU at the 10^0 dilution and scale up accordingly. 5. Calculate the mean LD for each set of treated coupons as follows: Mean LD = [Log_{10} (Coupon A) + Log_{10} (Coupon B) + Log_{10} (Coupon C) + Log_{10} (Coupon D) + Log_{10} (Coupon E)]/5 6. Calculate the log_{10} reduction (LR) for each disinfectant as follows: LR = Mean LD (Control Coupons) – Mean LD (Treated Coupons) a. If no organism is recovered from each of the five treated coupons and the entire contents of the 10^0 reaction tube was filtered, the log reduction is greater than or equal to the mean control carrier log density. 7. Use values with at least three significant figures when performing calculations. Report log reduction values with at least two significant figures.
14. Forms and Data Sheets	1. Attachment 1: Neutralization Assay 2. Attachment 2: Method Photographs 3. Test Sheets. Test sheets are stored separately from the SOP under the following file names: Biofilm Single Tube Method Test Information Sheet — MB-20-04_F1.docx Biofilm Single Tube Method Dilution/Plating Tracking Form — MB-20-04_F2.docx Biofilm Single Tube Method Results Sheet — MB-20-04_F3.docx Biofilm Single Tube Method Processing Sheet — MB-20-04_F4.docx Biofilm Test Microbe Confirmation Sheet — MB-20-04_F5.docx Biofilm Calculations Spreadsheet — MB-20-04_F6.xlsx Biofilm Neutralization Test Information Sheet — MB-20-04_F7.docx Biofilm Neutralization Dilution/Plating Tracking Form — MB-20-04_F8.docx Biofilm Neutralization Timing Sheet — MB-20-04_F9.docx Biofilm Neutralization Results Sheet — MB-20-04_F10.docx Biofilm Neutralization Processing Sheet — MB-20-04_F11.docx
15. References	1. ASTM International, 2021. E2871-21: Standard Test Method for Determining Disinfectant Efficacy Against Biofilm Grown in CDC

	Reactor Using the Single Tube Method.

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±2°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).

A2. Neutralization confirmation assay

a. *Neutralization Confirmation Treatment (NCT)*. At timed intervals, add 4 mL disinfectant to the appropriate volume of neutralizer (for example, 36 mL or 196 mL) in triplicate and 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 A2.d.

b. *Neutralizer Toxicity Treatment (NTT)*. At timed intervals, add 0.1 mL of the test organism diluted to 10^5 CFU/mL to the appropriate volume of neutralizer (for example, 40 mL or 200 mL) in triplicate and vortex to mix thoroughly. Repeat with the test organism diluted to 10^6 CFU/mL if desired. Proceed with section A2.d.

c. *Test Culture Titer (TCT)*. At timed intervals, add 0.1 mL of test organism diluted to 10^5 CFU/mL to the appropriate volume of dilution buffer (for example, 40 mL or 200 mL) in triplicate and vortex to mix thoroughly. Repeat with the test organism diluted to 10^6 CFU/mL if desired. Proceed with section A2.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, filter 10 mL from each of the NCT, NTT, and TCT treatment tubes through individual 0.45 µm polyethersulfone membranes; no additional dilution is necessary.

i. In advance of testing, make adjustments to the initial dilution series to achieve a target of 20-200 CFU per filter.

ii. For disinfectants that require additional neutralizer volume, filter a minimum of 25% of the total volume of neutralizer + disinfectant. If necessary, use multiple filters to assay these larger volumes.

iii. To filter, pre-wet the membrane with approximately 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 prevent trapping air bubbles between the agar and the membrane; use sterile forceps to reposition the filter if necessary.

h. Incubate plates (inverted) at 36±2°C for 48±4 h.

A3. Results

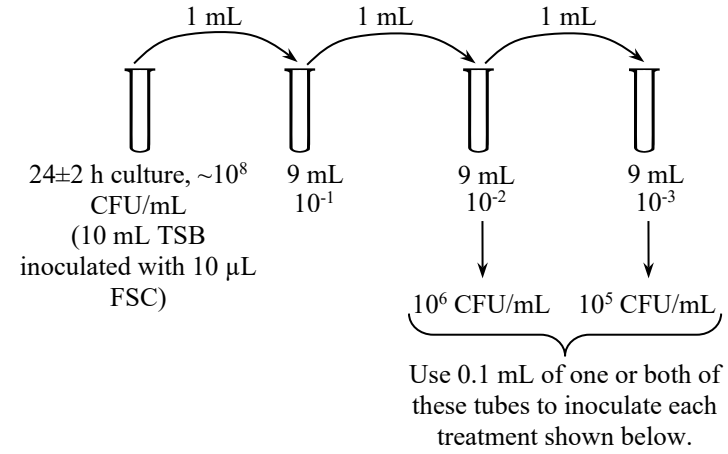
a. For calculation purposes, use the dilution that resulted in 30-300 CFU/plate (or 20-200 CFU/filter). Average between spread plates for a given dilution (if using), then average results from the three tubes per treatment.

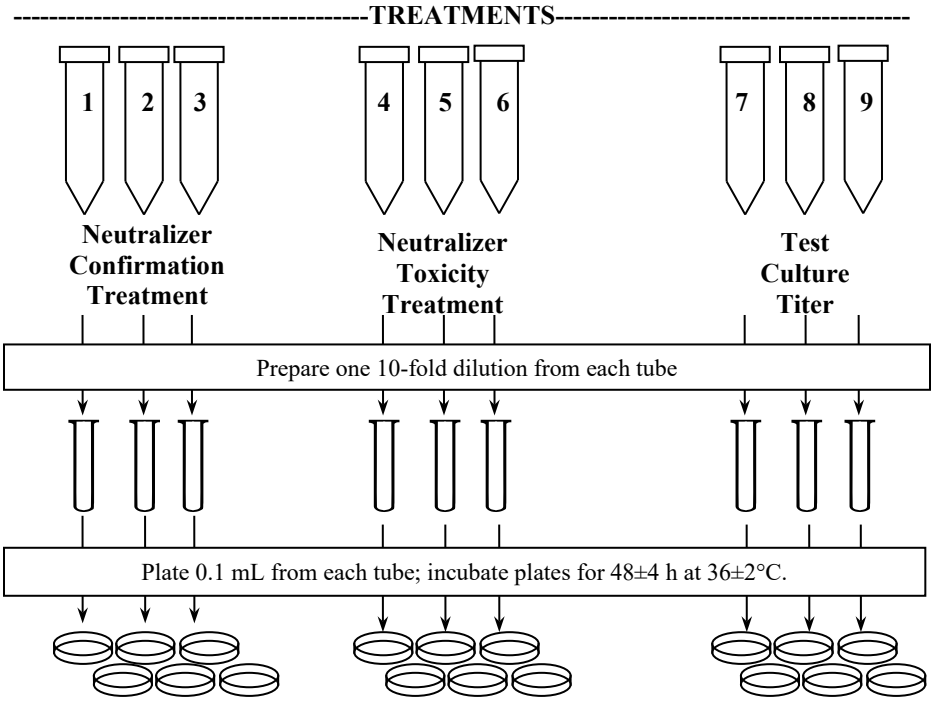
b. For determining and verifying the effectiveness of the neutralizer, ensure that:

i. The recovered number of CFU in the *Neutralizer Toxicity Treatment* (see section A2.b) is within 50% of the *Test Culture Titer* (see section A2.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.

ii. The recovered number of CFU in the *Neutralizer Confirmation Treatment* (see section A2.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 – Direct Plating[2] (for one dilution of the test organism)

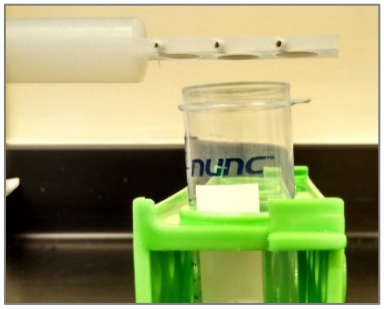
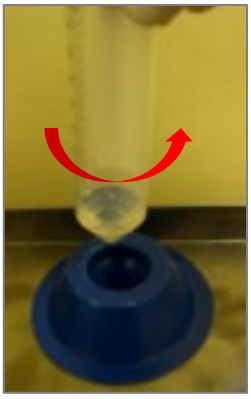
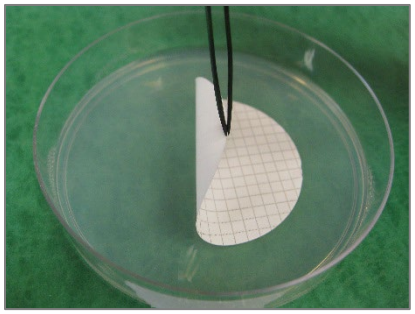




[2] For recovery via filtration (see section A2.g), make adjustments to the initial dilution series of the 24±2 h culture (~10^8/mL) to achieve a target of 20-200 CFU per filter.

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 disinfectant down the side of the reaction tube (10^0 tube).	 Gentle swirl of tube with carrier after addition of 4 mL disinfectant/control substance.	 Application of filter to agar surface (rolling filter onto agar plate).