



**US Environmental Protection Agency  
Office of Pesticide Programs**

**Office of Pesticide Programs**

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

**Date Revised: 08/09/16**

DRAFT

# 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  $1.0 \times 10^8$  to  $3.2 \times 10^9$ ), 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  $\text{KH}_2\text{PO}_4$  in 500 mL reagent-grade water, adjust to pH  $7.2 \pm 0.5$  with 1 N NaOH, and dilute to 1 L with reagent-grade water. Prepare stock magnesium chloride solution: 81.1 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ /L reagent-grade water. Filter sterilize both stock solutions. Prepare buffered dilution water by combining 1.25 mL  $\text{KH}_2\text{PO}_4$  stock solution and 5.0 mL  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , and dilute to 1 L with reagent-grade water (for final concentrations of 0.0425 g/L  $\text{KH}_2\text{PO}_4$  and 0.405 g/L  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ ) 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  $45 \pm 5$  kHz and a volume large enough to accommodate 50 mL or 250 mL conical tubes. For removing biofilm from coupons.

- 37 E. *Detergent*. Micro-90 Concentrated Cleaning Solution for Critical Cleaning;  
38 International Products Corporation. For cleaning coupons and reactor parts.
- 39 F. *Conical tubes*. 50 mL or 250 mL polypropylene sterile screw cap centrifuge tubes  
40 (e.g., Corning brand). Used as the reaction tube for the coupon/test substance or  
41 control fluid/neutralizer combination. 250 mL tubes are used to accommodate an  
42 increased volume of neutralizer (e.g., 196 mL). Use tubes that properly  
43 accommodate the splashguard insert (i.e., appropriate interior diameter and  
44 length).
- 45 G. *Filter membranes*. 47 mm diameter and 0.45  $\mu$ m polyethersulfone (PES) pore  
46 size. Filtration units (reusable or disposable) may be used. For microbe recovery  
47 from treated coupons.
- 48 H. *Splashguard inserts*. BioSurface Technologies. Used during coupon deposition.  
49 Two sizes are available for both the 50 mL and 250 mL conical tubes.

#### 50 IV. Procedure and Analysis

- 51 A. Test culture preparation
- 52 1. Prepare biofilm per “Growing a Biofilm using the CDC Biofilm Reactor”  
53 dated 07/20/16.
- 54 2. Once the flow of nutrients has stopped, harvest coupons for testing with  
55 one hour.
- 56 B. Reaction tube preparation
- 57 1. Refer to attachment 2 for pictures of technique sensitive steps.
- 58 2. Prior to sterilization, verify that the splashguards will sit properly in the  
59 conical tubes so that the end of the splashguard sits at the straight/conical  
60 interface of the tube.
- 61 3. Splashguards may be sterilized separately and then placed into sterile  
62 conical tubes.
- 63 i. Prior to sterilization, separate the flared top and the cylindrical  
64 bottom of the splashguard and place the cylindrical bottom piece  
65 into an empty conical tube with the etched side up. Ensure the  
66 bottom of the splashguard sits properly in the conical tube (see  
67 IV.B.2).
- 68 ii. Place the flared top of the splashguard onto the cylindrical bottom  
69 piece and press down. Remove the assembled unit from the  
70 conical tube. Repeat the process for the remaining splashguards,  
71 wrap the assembled units in foil or place in a sterilization pouch,  
72 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  $21 \pm 2^\circ\text{C}$ . 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

- 107 a picture of proper rod orientation.
- 108 5. Loosen the set screw using a flame-sterilized Allen wrench and allow the  
109 coupon to drop directly to the bottom of the tube.
- 110 i. If the coupon does not freely drop, press directly in the center of  
111 the coupon with the Allen wrench used to loosen the set screw.
- 112 ii. For each treated coupon, repeat coupon removal four more times  
113 for a total of five tubes, each containing one coupon.
- 114 iii. For each control coupon, repeat coupon removal twice more for a  
115 total of three tubes, each containing one coupon.
- 116 6. After depositing the coupons in the tubes, gently remove the splashguards  
117 from each tube using sterile forceps.
- 118 7. To reduce the risk of cross contamination, process coupons treated with  
119 test substance first.
- 120 8. Apply 4 mL prepared test substance (antimicrobial treatment) or control  
121 substance (dilution buffer) down the side of the conical tubes containing  
122 the coupons, avoiding direct contact with the coupon during application  
123 and being careful to completely cover the coupons. Refer to Attachment 2  
124 for a picture of proper treatment application.
- 125 i. For a 10 min contact time, a 30 s interval between coupons is  
126 recommended. Track contact time.
- 127 9. Immediately after deposition of test substance or control substance, gently  
128 swirl the tube 1-2 times to fully expose the biofilm on the coupon to the  
129 liquid, ensuring there are no air bubbles trapped beneath the coupon.
- 130 10. Allow tubes to remain at room temperature for the duration of the contact  
131 time.
- 132 11. At the end of the contact time, add 36 mL of the appropriate neutralizer  
133 (e.g., Dey/Engley (D/E) broth) to each tube. Replace the cap and mix  
134 thoroughly by vigorously shaking the tube several times.
- 135 i. Some test substances (e.g., highly acidic products) may need  
136 additional neutralizer volume (e.g., 196 mL). In these instances,  
137 use 250 mL conical tubes.
- 138 12. After neutralization, vortex the contents of each tube on the highest setting  
139 for  $30 \pm 5$  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  $45 \pm 5$  kHz for  $30 \pm 5$  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  $30 \pm 5$  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  $45 \pm 5$  kHz for  $30 \pm 5$  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  $30 \pm 5$  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.
- 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  $0.45 \mu\text{m}$  PES filter membrane.
  - 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  $36\pm 1^{\circ}\text{C}$  for  $48\pm 4$  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:

$$\left[ \frac{\left( \frac{\text{Mean CFU for } 10^w + \text{Mean CFU for } 10^x}{10^w + 10^x} \right)}{Y} \right] \times Z; \text{ where } 10^w \text{ and } 10^x \text{ are the dilution}$$

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:

$$\left( \frac{\text{CFU per filter for } 10^w + \text{CFU per filter for } 10^x}{(a \times 10^w) + (b \times 10^x)} \right) \times Z, \text{ where "a" and "b" are the}$$

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:  $\left( \frac{(\text{CFU}_1 + \text{CFU}_2 \text{ for } 10^w) + \text{CFU for } 10^x}{(a \times 10^w) + (b \times 10^x)} \right) \times Z$ , where  $\text{CFU}_1 + \text{CFU}_2$

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:

$\log_{10} \text{ reduction} = \text{mean } \log_{10} \text{ control} - \text{mean } \log_{10} \text{ treated}$

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. 21<sup>st</sup> 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.

244 Attachment 1

245 Biofilm Neutralization Assay

246 I. Culture Preparation

247 A. Defrost a single cryovial at room temperature and briefly vortex to mix. Add 10  
248  $\mu\text{L}$  of the thawed frozen stock (single use) to a tube containing 10 mL of TSB (30  
249 g/L), vortex, and incubate at  $36\pm 1^\circ\text{C}$  for  $24\pm 2$  h.

250 B. Prepare serial dilutions in 9 mL blanks of dilution buffer to achieve  
251 concentrations of approximately  $10^6$  and  $10^5$  CFU/mL per dilution tube; these  
252 concentrations are typically observed in the  $10^{-2}$  and  $10^{-3}$  dilution tubes,  
253 respectively. At least one of these dilutions when diluted and plated should result  
254 in counts of 30-300 CFU/plate (refer to the Biofilm Neutralization Assay  
255 Flowchart).

256 II. Neutralization confirmation assay

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

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

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

270 D. Hold all treatments at room temperature (e.g.,  $21\pm 2^\circ\text{C}$ ) for  $10\text{ min}\pm 30\text{ s}$ .

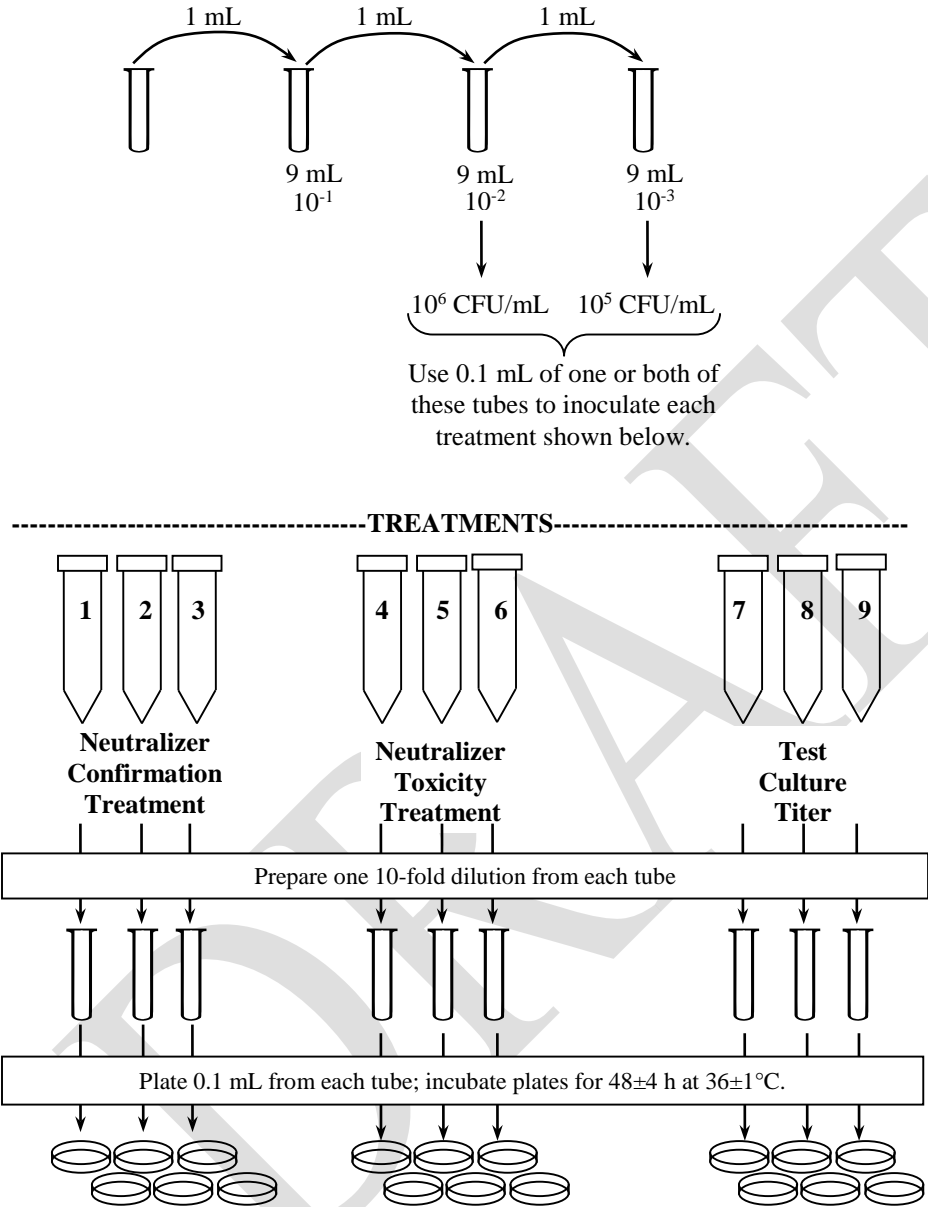
271 E. After the contact time, vortex each tube thoroughly and prepare one 10-fold  
272 dilution in 9 mL dilution buffer.

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





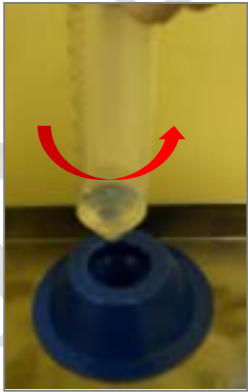
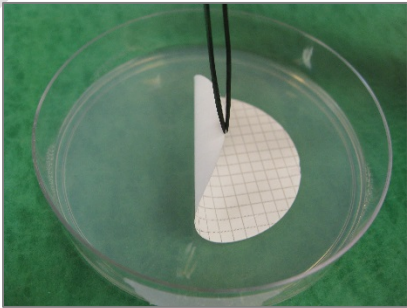
277 G. Alternatively, 10 mL from each of the NCT, NTT, and TCT treatment tubes may  
278 be filtered through individual  $0.45\text{ }\mu\text{m}$  polyethersulfone membranes; no additional  
279 dilution is necessary.

- 280 1. Make adjustments to the initial dilution series in advance to achieve a  
281 target of 20-200 CFU per filter.
- 282 2. For test substances that require additional neutralizer volume, filter a  
283 minimum of 20% of the total volume of neutralizer + test substance. If  
284 necessary, multiple filters may be used to assay these larger volumes.
- 285 3. To filter, pre-wet the membrane with ~20 mL dilution buffer then add the  
286 appropriate volume from the treatment tube. Rinse the sides of the filter  
287 funnel with additional dilution buffer and place the filter membrane on  
288 R2A (for *P. aeruginosa*) or TSA (for *S. aureus*). Gently roll the filter onto  
289 the surface of the agar to remove any air bubbles that may be trapped  
290 between the agar and the membrane.
- 291 H. Incubate plates (inverted) at  $36\pm 1^{\circ}\text{C}$  for  $48\pm 4$  h.
- 292 III. Results
- 293 A. For calculation purposes, use the dilution that resulted in 30-300 CFU/plate (or  
294 20-200 CFU/filter). Average between spread plates for a given tube (if using),  
295 then average results from the three tubes per treatment.
- 296 B. For determining and verifying the effectiveness of the neutralizer, ensure that:
- 297 1. The recovered number of CFU in the *Neutralizer Toxicity Treatment* (see  
298 section II.B) is within 50% of the *Test Culture Titer* (see section II.C). A  
299 count less than 50% indicates that the neutralizer is harmful to the test  
300 organism. Note: counts higher than the *Test Culture Titer* (e.g., 120% of  
301 the *Test Culture Titer*) are also deemed valid.
- 302 2. The recovered number of CFU in the *Neutralizer Confirmation Treatment*  
303 (see section II.A) is within 50% of the *Test Culture Titer*; this verifies  
304 effective neutralization. Note: counts higher than the *Test Culture Titer*  
305 (e.g., 120% of the *Test Culture Titer*) are also deemed valid.

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



308 Attachment 2  
 309  
 310 Method Photographs  
 311

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

312