

# **Quantitative Method for Evaluating the Efficacy of Antimicrobial Test Substances on Soft Surface Textiles Against Bacteria**

**(08/11/2023)**

## **Scope**

The Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) recommends that applicants utilize this method to support efficacy criteria for the registration of products bearing claims for use on soft surface textiles. The method provides a quantitative assessment of the performance of antimicrobial substances against *Pseudomonas aeruginosa* and *Staphylococcus aureus* on soft surface textiles.

This method provides log reduction (LR) as the quantitative measure of efficacy for disinfectants against the test microbes on a soft surface textile.

## **Method Overview**

In brief, the method uses 1 cm diameter discs or 1 cm<sup>2</sup> squares (carriers) of a set of representative soft surface textiles. Each carrier receives 10 µL of microbial inoculum (with a three-part soil load) deposited in the center of each carrier. The inoculum is allowed to dry and is then exposed to 50 µL of the antimicrobial treatment; control carriers receive an equivalent volume of an innocuous fluid (e.g., phosphate buffered saline). The exposure time is allowed to elapse; a liquid neutralizer is then added to the vial to halt the antimicrobial action. Each vial with the carrier is vortexed, serially diluted, and the contents are filtered to recover viable microorganisms. Based on the difference between the mean log<sub>10</sub> density values of the untreated control and treated carriers, a mean log<sub>10</sub> reduction (LR) in viable bacteria is calculated. The LR value is used as the measure of product effectiveness.

Appropriate safety procedures should always be used when working with laboratory test systems which include human pathogenic microorganisms. Laboratory safety is discussed in the current edition of “Biosafety in Microbiological and Biomedical Laboratories (BMBL)” 6<sup>th</sup> edition, from the subject matter experts within the U.S. Department of Health and Human Services (HHS), including experts from the Centers for Disease Control and Prevention (CDC) and National Institutes of Health (NIH).

## TABLE OF CONTENTS

1) Special Apparatus and Materials.....	3
2) Carriers.....	5
3) Preparation of Test Cultures and Carrier Inoculation .....	6
4) Performance Assessment – Efficacy.....	9
5) Data Requirements.....	12
Appendix 1: Preparation of Frozen Stock Culture.....	14
Appendix 2: Neutralization Assay and Flowcharts.....	16
Appendix 3: Carrier Examples.....	19

## 1) Special Apparatus and Materials

- a. Test microbes: *Pseudomonas aeruginosa* (ATCC #15442) and *Staphylococcus aureus* (ATCC #6538).
  - i. Additional bacteria may be tested (for an additional label claim) per the Agency's guidance.
- b. Culture media
  - i. *Tryptic Soy Broth (TSB)*. Use to rehydrate lyophilized cultures. Purchase broth from a reputable source or prepare according to manufacturer's instructions.
  - ii. *Synthetic broth (SB)*. Growth medium for test cultures. Commercial media (HIMEDIA, Synthetic Broth, AOAC, #M334-500G). Suspend 16.9 g in 1000 mL DI water. Heat if necessary, to dissolve the medium completely. Final pH at 25°C should be 7.1±0.2. Medium may be dispensed in 10 mL amounts in 20×150 mm culture tubes or alternatively in 500 mL volumes in a 1 L bottle; steam sterilize at 121°C for 15 minutes. Store prepared SB at 2-8°C.
    1. Alternatively, SB made in-house per the recipe provided in AOAC Methods 955.15, 964.02, and 955.14 may be substituted.
  - iii. *10% dextrose solution*. Add 5.0 g dextrose to 50 mL de-ionized water and mix by stirring. Filter sterilize the solution using a 0.2 µm filter. Store the sterile solution at 2-5°C for up to 30 days.
  - iv. *TSB with 15% (v/v) glycerol*. Use as a cryoprotectant. Suspend 7.5 g tryptic soy broth in 212.5 mL de-ionized water. Add 37.5 mL glycerol and stir, warm slightly to dissolve. Dispense into bottles and steam sterilize for 15 min at 121°C.
  - v. *Tryptic soy agar (TSA)* and *TSA with 5% sheep blood*. Use for culturing, isolation, and characterization of the test microbes. Purchase plates from a reputable source or prepare according to manufacturer's instructions.
  - vi. *Selective media (optional)*. Mannitol salt agar and Cetrimide agar. Use for quality control of test microbes listed in this procedure. Purchase plates or prepare according to manufacturer's instructions.
- c. Reagents
  - i. *Neutralizer*. A liquid reagent used to inactivate and/or dilute the antimicrobial treatment to end the contact time.
  - ii. *Phosphate buffered saline stock solution (e.g., 10X)*. To prepare 1X phosphate buffered saline. The stock solution has a pH of approximately 7.2±0.2.
  - iii. *Phosphate buffered saline (PBS), 1X*. Dilution blanks and filtration. PBS with a pH of approximately 7.0±0.5 is desirable.
  - iv. *Soil load, 3-part*. Use as the soiling agent.
    1. BSA: Add 0.5 g bovine serum albumin (BSA, radio immunoassay (RIA) grade or equivalent, CAS# 9048-46-8) to 10 mL of PBS, mix and pass

- through a 0.2  $\mu\text{m}$  pore diameter (polyethersulfone) membrane filter, aliquot (e.g., a minimum of 50  $\mu\text{L}$ ), and store at  $-20\pm 2^\circ\text{C}$ .
2. Yeast Extract: Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2  $\mu\text{m}$  pore diameter (polyethersulfone) membrane filter, aliquot (e.g., a minimum of 70  $\mu\text{L}$ ), and store at  $-20\pm 2^\circ\text{C}$ .
  3. Mucin: Add 0.04 g mucin (from bovine submaxillary gland, CAS # 84195-52-8) to 10 mL of PBS, stir or vortex-mix until thoroughly dissolved, and pass through a 0.2  $\mu\text{m}$  pore diameter (polyethersulfone) membrane filter, aliquot, and store at  $-20\pm 2^\circ\text{C}$ .
  4. The three stock solutions of the soil load are single use only. Do not refreeze; store up to one year at  $-20\pm 2^\circ\text{C}$ .
- v. *Antimicrobial Test substance.* Ready-to-use, activated, or concentrated antimicrobial. If the antimicrobial test substance is prepared by diluting a concentrate in hard water, adequately mix antimicrobial test substance with the appropriate diluent (e.g., hard water), then use prepared test substance within 3 hours of preparation or as otherwise instructed by the manufacturer. Measuring error increases as delivery volume decreases. To minimize variability due to measuring error, a minimum of 1.0 mL or 1.0 g of concentrated antimicrobial test substance should be used when preparing use-dilutions for testing. Use v/v dilutions for liquids antimicrobial test substances and w/v dilutions for solid antimicrobial test substances. The use of a positive displacement pipette is recommended for viscous liquids.
  - vi. *1 N NaOH and 1 N HCl.* Used for pH adjustment of media/reagents.
  - vii. *Water.* De-ionized (DI), distilled water or water with equivalent quality for making reagent solutions and culture media.
  - viii. *Gram stain.* Used for diagnostic staining.
- d. Apparatus
- i. *Carriers:* Discs (1 cm in diameter) or 1  $\text{cm}^2$  squares cut from soft surface textile. Carriers are single use only. See Section 2 for carrier specifications.
  - ii. *Hole punch:* If necessary, for use in the preparation of 1 cm disc from material. Model number: SKU# HP-MEI448R or equivalent
  - iii. *Calibrated 10  $\mu\text{L}$  positive displacement pipette* with corresponding 10  $\mu\text{L}$  tips, for carrier inoculation.
  - iv. *Filter paper.* Whatman No. 2, to line glass Petri plates.
  - v. *Calibrated micropipettes* (e.g., 200  $\mu\text{L}$ , 1 mL) with appropriate corresponding tips, for deposition of test substance on carriers and preparing dilutions.
  - vi. *Bottle-top dispensers, squirt bottles, pre-measured volumes in tubes, or pipettes, bottles, etc.* For rinsing vials and filters.

- vii. *Forceps*, straight or curved, non-magnetic, disposable with smooth flat tips to handle membrane filters, appropriate to pick up the carriers for placement in vials.
- viii. *Polyethersulfone (PES) membranes*. Use for recovery of test microbe, 47 mm diameter and 0.2  $\mu\text{m}$  pore size.
  - 1. Use filter membranes in either a reusable or disposable filtration unit.
- ix. *Filter Sterilization Unit (with PES, 0.2  $\mu\text{m}$  pore size)*. Use to filter sterilize soil components.
- x. *20 x 150 m glass culture tubes* with Morton closures for test culture preparation.
- xi. *Spectrophotometer*. For culture standardization (if deemed necessary)
- xii. *Vials with lids (plastic or comparable)*. Sterile, flat-bottomed, wide-mouthed (at least 25 mm diameter), approximately 20 mL capacity, for holding inoculated carriers to be exposed to the test chemical and for accommodating neutralizer (e.g., Thermofisher #2116-0015).
  - 1. Transparent vials are more desirable to facilitate application of 50  $\mu\text{L}$  test substance or control substance to inoculated carrier.
- xiii. *Certified timer*. Readable in minutes and seconds, for tracking of timed events and intervals.
- xiv. *Desiccation unit* (with gauge to measure vacuum level) with fresh desiccant (e.g., anhydrous  $\text{CaCO}_3$ ). For drying inoculated carriers.
- xv. *Vacuum source*. In-house line or suitable vacuum pump capable of achieving 0.068 to 0.085 MPa, for drying inoculated carriers in desiccation unit and to perform membrane filtration.
- xvi. *Titration kit*. (i.e., Hach digital titrator) Used for measuring water hardness.
- xvii. *Vortex-style mixer*. Used for vortex-mixing of various solutions.
- xviii. *Conical centrifuge tubes (e.g., 15 mL)*. Used for centrifugation of test cultures.
- xix. *Centrifuge (with rotor capable of achieving 5,000g)*. Used for test culture preparation.

## 2) Carriers

- a. Carrier Materials (see Figure 1)<sup>1</sup>
  - i. Privacy Curtain Fabric (PCF-03): 56% Polyester, 44% Fire Resistant (FR) Polyester, 12.93 oz./lin. yd.  $\pm$  1.0 oz. CF Stinson, LLC. Mambo MAM34.

---

<sup>1</sup>EPA and its employees do not endorse the products, services, or enterprises of nonfederal entities. These carriers were chosen because they demonstrated consistent recovery and repeatability but may not be the only soft surface textiles capable of achieving these results. Use of these carriers does not constitute an endorsement by EPA or its employees.

- ii. Non-PVC Fabric (NVF-01): Polyurethane Face made with Polycarbonate and Polyether Resins, Polyester Backing, 20.3 oz./lin. yd.  $\pm$  1.0 oz. CF Stinson, LLC. KID17.
- iii. Vinyl Seating Fabric (VF-01): Non-Phthalate Vinyl Surface with Polyester Backing, 30.0 oz./lin. yd.  $\pm$  1.0 oz. CF Stinson, LLC. Hopsack HOP24.

**Figure 1:** Examples of carrier materials cut into 1 cm discs; materials 2.a.i, 2.a.ii, and 2.a.iii (from left to right)



#### b. Carrier Preparation

- i. Punch or obtain approximately 1 cm round carriers or use comparable cutting procedure from fabric. Alternatively, 1 cm<sup>2</sup> square carriers can be utilized if punching is unavailable or too difficult.
  1. Note: punch can be sharpened by punching aluminium foil.
- ii. Visually screen carriers to ensure consistent surface characteristics; trim any jagged edges or loose fabric.
  1. Carriers that demonstrate excessive fraying, ripping, backing separation, discoloration, etc., should not be used.
- iii. No pre-cleaning of carriers is necessary. Sterilize carriers using a gravity cycle at 121°C for 20 minutes; ensure carriers are dry following sterilization. Test sterility of carriers prior to or concurrently with efficacy testing.
  1. Carriers may not be entirely flat after autoclaving; however, minor distortion, such as cupping or doming, of carriers is acceptable for testing if the test substance and inoculum are not affected when applied to the surface of the carrier.
  2. Prior to use in testing, document the condition of the screened and sterile carriers (e.g., digital photographs or documentation on the paperwork).
  3. To assess carrier sterility, place a carrier in tryptic soy broth (TSB) and incubate at 36 $\pm$ 1°C for 3-10 days.
  4. Use sterilized carriers for up to 6 months. After 6 months, re-sterilize any remaining carriers.

### 3) Preparation of Test Culture and Carrier Inoculation

- a. Refer to Appendix 1 for preparation of the frozen stock cultures.
- b. Defrost a cryovial; defrost rapidly to avoid loss in the viability of the preserved cells. Each cryovial is single use only.
- c. Prior to inoculation, on the day inoculated, use a calibrated pipette to aseptically add 0.1 mL of 10% sterile dextrose (w/v) solution to each 10 mL tube of SB.
- d. Using a calibrated micropipette, add 100  $\mu$ L of defrosted stock culture to 10 mL SB with dextrose, briefly vortex-mix and incubate for 24 $\pm$ 2 h at 36 $\pm$ 1 $^{\circ}$ C.
  - i. Incubate without disrupting the culture.
  - ii. To assess culture purity, inoculate an agar plate (e.g., TSA or TSA with 5% sheep blood) from the inoculated tube and streak for isolation. Incubate plate with the test culture.
- e. Following incubation, use the SB cultures to prepare a test suspension for each organism.
  - i. The 24 $\pm$ 2 h culture should exhibit a titer of at least 10<sup>8</sup> CFU/mL.
- f. For *P. aeruginosa*, inspect culture prior to harvest; visible pellicle on the surface of the culture is expected to form during incubation (record its presence). Discard the culture if pellicle has been disrupted (fragments in culture).
  - i. Remove visible pellicle on surface of medium and around associated interior edges of the tube with vacuum suction.
  - ii. Using a serological pipette, withdraw the remaining broth culture (at least 5 mL) avoiding any sediment on the bottom of the tube and transfer it into a conical centrifuge tube.
    1. If necessary, culture may be harvested from two 10 mL 24 $\pm$ 2 h broth cultures to centrifuge a maximum of 10 mL *P. aeruginosa*; record the associated information.
- g. For *S. aureus*, briefly vortex-mix the 24 $\pm$ 2 h culture and transfer to a conical centrifuge tube.
- h. Within 15 min, centrifuge the 24 $\pm$ 2 h harvested broth cultures at 5,000g for 20 min.
- i. Remove the supernatant without disrupting the pellet. Re-suspend the pellet in 5-10 mL PBS. Record resuspension volume.
  - i. Prepare the final test suspension within 30 min of resuspending the culture.
  - ii. If necessary, disrupt the pellet using vortex-mixing or repetitive tapping/striking against a hard surface to disaggregate the pellet completely prior to re-suspending it in 10 mL. If necessary, add 1 mL of PBS to the pellet to aid in the disaggregation.
- j. If needed, dilute the 5-10 mL of resuspended culture in PBS to achieve a mean control carrier count level of 4.0-5.5 logs CFU/carrier for *S. aureus* and *P. aeruginosa*.
  - i. Optical density/absorbance (e.g., 650 nm) may be used as a tool to monitor/adjust the diluted test suspension.

- k. Use the resuspended or diluted culture to prepare the final test suspension with the addition of the soil load.
- l. To obtain 500  $\mu\text{L}$  of the final test suspension with the 3-part soil load, vortex-mix each component and combine in the following order using a calibrated micropipette:
  - i. 25  $\mu\text{L}$  BSA stock
  - ii. 35  $\mu\text{L}$  yeast extract stock
  - iii. 100  $\mu\text{L}$  mucin stock
  - iv. Vortex soil suspension for 10 s prior to adding microbial test suspension.
  - v. 340  $\mu\text{L}$  microbial test suspension
- m. Briefly vortex the final test suspension with 3-part soil load (at room temperature,  $21\pm 3^\circ\text{C}$ ) and use to inoculate carriers within 30 min of preparation.
  - i. Streak inoculate an agar plate with a loopful of the final test suspension. Incubate plate with the treated and control carrier plates and examine for purity after incubation at  $36\pm 1^\circ\text{C}$  for  $72\pm 4$  h.
- n. It is advisable to briefly rescreen each sterilized carrier for abnormalities prior to inoculation. Place carriers screened side up inside an empty, sterile Petri dish (no more than 20 carriers/dish).
  - i. Privacy curtain carriers have no backing material and may be inoculated on either side.
  - ii. Non-PVC and vinyl carriers are layered materials comprised of a smooth, colored top surface and a white fabric bottom; only inoculate the top surface.
- o. Vortex-mix the final test suspension for 10 s following the addition of the soil load and immediately prior to use.
- p. Inoculate the number of carriers required for the evaluation of the test substance (3 controls and 5 treated) along with a few extra carriers.
- q. Using a calibrated positive displacement pipette with a 10  $\mu\text{L}$  tip, withdraw 10  $\mu\text{L}$  of the final test suspension and deposit it at the center of each carrier (screened and sterile), keeping the pipette perpendicular to the carrier during deposition of the final test suspension. Avoid contact of pipette tip with carrier and do not spread the final test suspension with the pipette tip.
  - i. For consistency, vortex-mix the inoculum frequently during inoculation of the carrier set.
  - ii. The same pipette tip may be used to inoculate all carriers (unless the tip is compromised).
  - iii. Discard any inoculated carrier where the final test suspension has run over the edge.
  - iv. Discard any inoculated carriers that flip over.



- v. Note: If inoculum soaks/absorbs into the carrier and is no longer visible, carriers are still acceptable to use. Refer to Appendix 3 for examples of inoculated carriers and carriers with 50  $\mu\text{L}$  of test substance added.
- r. Transfer the Petri dish(es) with the inoculated carriers into a desiccation unit (with desiccant) and completely remove the lid of the Petri dish(es). Close the desiccation unit door (or lid) and seal the unit. Apply vacuum to evacuate the desiccation unit.
  - i. Pressurize the desiccator slowly to lessen the potential for carriers to move or flip.
  - ii. Note: do not exceed 40 inoculated carriers per desiccator to ensure carriers dry within the prescribed time.
- s. Maintain and monitor the vacuum level using a gauge. Achieve and maintain consistent level of vacuum (at 20-25 in of mercury, 508-635 torr, 677-847 mbar, or 68000-85000 Pascal) by leaving the vacuum on during the drying period with the desiccator stopcock opened or turning the vacuum off with the stopcock closed.
- t. Hold the inoculated carriers in the evacuated desiccation unit at  $21\pm 3^\circ\text{C}$  for 45 to 60 min. Visually inspect inoculated carriers to verify that they have completely dried and remove from desiccation unit. Do not use carriers that are visibly wet for testing.
  - i. Record the time for all timed events.
  - ii. Depressurize the desiccator slowly to lessen the potential for carriers to move or flip.
  - iii. Carriers that become stuck or flip during desiccation should be discarded.
- u. Use dried inoculated carriers for testing within 30 min following removal from desiccation unit; hold carriers in closed Petri dish(es) at room temperature ( $21\pm 3^\circ\text{C}$ ) until use.

#### 4) **Performance Assessment – Efficacy**

- a. Evaluate 3 control carriers and 5 treated carriers for each test substance tested (one test organism and contact time/carrier type combination) unless specified otherwise.
  - i. One set of control carriers per carrier type may be used for evaluating multiple test substances against one organism on one test day (assuming the carrier material, neutralizer, and soil load are the same).
- b. Using sterile forceps, transfer each dried carrier with the inoculated side up to a flat-bottom vial and cap the vial. Repeat until all carriers are transferred.
  - i. Note: An anti-static gun may be aseptically used to reduce the carriers clinging to the side of the vial.
- c. Prepare the antimicrobial test substance. Use antimicrobial test substance within 3 hours of preparation or as specified by the manufacturer.

- d. In a timed fashion with appropriate intervals, sequentially deposit 50  $\mu\text{L}$  of the test substance (equilibrated to  $21\pm 3^\circ\text{C}$ ) with a calibrated micropipette over the dried inoculum on each test carrier, ensuring complete coverage.
  - i. Note: Gently apply the antimicrobial test substance at a perpendicular angle to the inoculated carrier; do not forcefully deposit the disinfectant.
- e. Use a new tip for each carrier; do not touch the carrier surface with a pipette tip during the application of the test substance or the control substance; replace with new carrier(s) and vial(s) if this occurs. Do not cap the vials.
  - i. For non-foaming aerosols and pump/trigger spray products, obtain the test substance by dispensing the product into a sterile vessel for collection. Cap the vessel and use dispensed product within 30 min.
  - ii. For foaming spray formulations, allow the foam to break down for at least 5-10 minutes for the generation of a 1-2 mL liquid sample. Cap the vessel and use dispensed product within 30 min.
- f. For NFV-01 and VF-01 carriers, do not process carriers where the test substance runs off the carrier or does not completely cover the inoculum spot; replace with new carrier(s) and vial(s) if this occurs. When test substance is applied to the PCF-03 carriers it is anticipated to soak into and through the carrier (e.g., the test substance may be observed to pool around the carrier).
  - i. If the inoculum spot is no longer observable, place the test substance in the area where the inoculum spot was placed (e.g., the center of the carrier).
- g. Conduct the test at room temperature ( $21\pm 3^\circ\text{C}$ ) for the selected contact time. Use a certified timer to ensure that each carrier receives the required contact time.
- h. Process control carriers last. Each control carrier receives 50  $\mu\text{L}$  PBS, equilibrated to  $21\pm 3^\circ\text{C}$ , instead of the test substance. Hold the control carriers for the same contact time as used for the test substance.
- i. Within  $\pm 5$  s of the end of the contact period, add 10 mL of neutralizer equilibrated to  $21\pm 3^\circ\text{C}$  to each vial in the specified order according to the predetermined schedule. Briefly vortex-mix (2-3 s) each vial following the addition of the neutralizer.
  - i. For calculation purposes, the solution in the neutralized vial with carrier is considered to be  $10^0$  dilution.
  - ii. The neutralizer for the control carriers is the same as that for the treated carriers.
  - iii. Ensure that carrier's treated surface comes into contact with the neutralizer by swirling the vial to submerge the carrier prior to briefly vortex-mixing.
    1. Note: The carrier may float back to the surface after vortex-mixing.
- j. Following the neutralization of the entire set of carriers, vortex-mix vials at high speed for  $30\pm 5$  s then proceed as follows:
  - i. Allow carriers to sit undisturbed in the vials for approximately 5 minutes.
  - ii. Vortex-mix vials at high speed for  $30\pm 5$  s (vortex-mix #2).

1. Ensure that the carriers are submerged in the neutralizer during this step.
  2. Ensure that the liquid and carrier are both spinning in the vial during the vortex-mix.
- iii. Allow carriers to sit undisturbed in the vials for approximately 5 minutes.
- iv. Vortex-mix vials at high speed for  $30 \pm 5$  s (vortex-mix #3).
1. Ensure that the carriers are submerged in the neutralizer during this step.
  2. Ensure that the liquid and carrier are both spinning in the vial during the vortex-mix.
- k. Initiate dilutions within 30 min after completion of the vortex-mixing. Initiate filtration within 30 min of preparing the dilutions.
- l. Dilute and filter samples from the treated and control carriers; process treated carriers first.
- m. Serially dilute the eluate from the  $10^0$  dilution prior to filtration by transferring 1 mL into 9 mL PBS in a dilution tube.
- n. Turn on vacuum and leave on for the duration of the filtration process.
- o. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS.
- p. Use separate membrane filters for each eluate (neutralized solution); however, the same filtration unit may be used for processing eluates from a given carrier set starting with the most dilute sample first.
- q. Filter each sample through a separate  $0.2 \mu\text{m}$  PES membrane filter.
- r. For eluates from treated carriers remaining in the vial ( $10^0$  dilution), vortex-mix the vial for ~5 s, carefully pour the eluate into the filter unit.
- i. If a carrier falls onto the filter membrane, aseptically remove it using sterile forceps.
- s. Rinse the treated vial with ~20 mL PBS, vortex-mix for ~5 s, pour the wash into the same filter unit. For dilution tubes, rinse tube once with ~10 mL PBS, briefly vortex-mix, and pour into filter unit.
- t. Swirl the contents of the filter unit and quickly filter with limited pooling of liquid in the filter apparatus.
- u. Rinse the inside surface of the funnel unit with at least 20 mL PBS and filter the contents.
- v. Aseptically remove the membrane filter and place on the appropriate recovery medium. Avoid trapping any air bubbles between the filter and the agar surface.
- w. Sterility controls.
- i. On the day of the test, filter ~20 mL of neutralizer and ~20 mL of the PBS used in the test using two separate membrane filters and place on individual TSA plates.
  - ii. Incubate these filters along with a plate of recovery medium (e.g., TSA) for  $72 \pm 4$

- h at  $36\pm 1^{\circ}\text{C}$ , record sterility results.
- x. Incubate plates at  $36\pm 1^{\circ}\text{C}$  for  $48\pm 4$  h for control carriers and  $72\pm 4$  h for treated carriers.
    - i. It is recommended to monitor filters daily to optimize counting of colonies. CFUs may be counted daily. Record controls after  $48\pm 4$  h and treated carriers after  $72\pm 4$  h.
  - y. Count colonies and record results.
    - i. Any level of contamination which interferes with the recording and interpretation of results will result in invalid data.
    - ii. For example, contamination occurring on multiple filters within one set of serial dilutions and/or across multiple carriers is considered systemic and the test is deemed invalid.
  - z. For colony counts on filters in excess of 200 record as Too Numerous to Count (TNTC).
  - aa. If no colonies are present, record as zero.
  - bb. Report non-conforming data (e.g., systemic contamination and atypical serial dilution results) and repeat tests as necessary.
    - i. Systemic contamination
    - ii. Atypical serial dilution results (e.g., higher CFUs at more dilute levels).
  - cc. Inspect the growth on the filters for purity and typical characteristics of the test microbe, see Appendix 1, Table 1.
  - dd. If isolated colonies are present, assess one representative colony per 5-carrier set (treated) or 3-carrier set (controls) using a Gram stain.
    - i. If confluent growth is present, perform a streak isolation on TSA or TSA with 5% sheep blood on growth taken from at least 1 carrier incubate at  $36\pm 1^{\circ}\text{C}$  for 24-48 h.
  - ee. If additional verification of the test organism is required due to observation of atypical morphology, perform further confirmatory analyses (e.g., Vitek, biochemical analyses) and isolation streaks on selective media.

## 5) Data Requirements

- a. Per test, use colony counts to determine log reduction.
- b. For an acceptable test, each of the three control carriers must exhibit counts between 4.0-5.5 logs CFU/carrier.
- c. Use values with at least three significant figures when performing calculations (e.g., log density, mean log density). Report the final mean log reduction value with two significant figures (e.g., round up to the nearest tenth).

- d. Calculate the Colony Forming Units (CFU)/carrier using the following equation:

$$\text{Log}_{10} \left\{ \left[ \frac{\sum_{i=1}^n (Y_i)}{\sum_{i=1}^n (C_i \times D_i)} \right] \times V \right\},$$

where:

Y = CFU per filter,

C = volume filtered,

V = total volume of neutralizer,

D =  $10^{-k}$ ,

k = dilution,

n = number of dilutions, and

i = lower limit of summation (the fewest number of dilutions).

- e. When TNTC (Too Numerous To Count) values are observed for each dilution filtered, substitute 200 for the TNTC at the highest (most dilute) dilution and account for the dilution factor in the calculations.
- f. Calculate the log density of each carrier by taking the  $\log_{10}$  of the density per carrier.
- g. Calculate the mean  $\log_{10}$  density across treated carriers.
- h. Calculate the mean  $\log_{10}$  density across control carriers.
- i. Calculate the mean  $\log_{10}$  reduction (LR) for treated carriers:
- Mean  $\log_{10}$  reduction = the mean  $\log_{10}$  density for control carriers minus the mean  $\log_{10}$  density for treated carriers.
- j. For a set of treated carriers: when the  $10^0$  dilution (the contents of the vial with the carrier) is filtered either by itself or in addition to other dilutions and the data for each carrier result in zeros for each dilution filtered, report the mean LR as greater than or equal to the mean  $\log_{10}$  density for the control carriers.
- k. Log reduction data based on estimates due to the occurrence of TNTC outcomes at each dilution in a dilution series for control and treated carriers is deemed unacceptable.

## Appendix 1

### Preparation of Frozen Stock Culture

- 1) Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from a reputable vendor at least every 18 months.
  - a. New frozen stock culture may be initiated one time using an existing, unexpired frozen stock culture as the source. Begin process at step 3 below, by streaking a loopful of the frozen stock culture onto 2 TSA plates.
- 2) Rehydrate lyophilized culture.
  - a. For lyophilized cultures received as ampules, 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  $36\pm 1^{\circ}\text{C}$  for  $24\pm 2$  h.
  - b. For lyophilized cultures received on loops/swabs, as pellets, etc., open and rehydrate the lyophilized organism as indicated by the manufacturer. Transfer the rehydrated culture to a tube of 5-6 mL TSB (e.g., swish inoculated swab/loop in the TSB tube or transfer the rehydrated pellet to the TSB tube). Mix thoroughly. Incubate broth culture at  $36\pm 1^{\circ}\text{C}$  for  $24\pm 2$  h.
- 3) At the end of the incubation timeframe, streak a loopful of the broth culture onto 2 TSA plates to obtain isolated colonies. Perform a streak isolation of the broth culture onto BAP as a purity check and streak the broth culture onto the appropriate selective media. Refer to appropriate selective media in Table 1. Incubate all plates for  $24\pm 2$  h at  $36\pm 1^{\circ}\text{C}$ .
  - a. Record results at the end of the incubation timeframe. Refer to Table 1 for results on selective media and diagnostic characteristics of the test microbes.
- 4) From the TSA plates, 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. For *P. aeruginosa*, select colonies from each of the two possible phenotypes present. Spread plate 0.1 mL of the suspension onto each of 6-10 TSA plates. Incubate the plates for  $24\pm 2$  h at  $36\pm 1^{\circ}\text{C}$ . If necessary, to obtain more frozen stock cultures, a larger suspension (e.g., 2 mL) may be prepared using the same ratio of TSB (1 mL) to number of colonies (3-5 colonies).
  - a. Using the TSB suspension, perform a streak isolation of the suspension onto a BAP as a purity check, and streak on the appropriate selective media (refer to Table 1).
  - b. Incubate all plates for  $24\pm 2$  h at  $36\pm 1^{\circ}\text{C}$ . Record results. Refer to Table 1 for results on selective media and diagnostic characteristics of the test microbes.
- 5) After the incubation period, harvest growth from TSA plates by adding approximately 5 mL sterile cryoprotectant solution (TSB with 15% (v/v) glycerol) 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.

- 6) Repeat the growth harvesting procedure with the remaining plates and continue adding the suspension to the vessel (more than 1 vessel 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.
- 7) Immediately after mixing, dispense 0.5-1.0 mL aliquots of the harvested suspension into cryovials; these represent the frozen stock cultures.
  - a. For QC purposes, perform a streak isolation of the pooled culture onto a BAP as a purity check and streak on appropriate selective media (refer to Table 1).
  - b. Incubate all plates for 24±2 h at 36±1°C.
  - c. Record results. Refer to Table 1 for results on selective media and diagnostic characteristics of the test microbes.
  - d. After incubation, perform a Gram stain on growth from the BAP; observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion).
  - e. Conduct confirmation using an automated identification system (e.g., Vitek) or biochemical and antigenic analyses from growth taken from the BAP according to the manufacturer's instructions.
- 8) Store the cryovials at approximately -80°C for a maximum of 18 months. These cultures are single use only.
- 9) If the characteristics of the organism are not consistent with the information in Table 1 at any step in the process, or the Vitek profile is inconsistent with the organism, discard the cultures and re-initiate the process.

**Table 1.** Selective media and diagnostic characteristics for *P. aeruginosa* and *S. aureus*

Aspect	<i>P. aeruginosa</i> *	<i>S. aureus</i>
Gram stain reaction	Negative	Positive
Mannitol Salt Agar (Selective medium)	N/A	Circular, small, yellow colonies, agar turning fluorescent yellow
Cetrimide Agar (Selective medium)	Circular, small, initially opaque, turning fluorescent green over time; agar fluorescent yellowish green	N/A
Blood agar (BAP)	Flat, opaque to off-white, round spreading (1), metallic sheen, slightly beta hemolytic	Small, circular, yellow or white, glistening, beta hemolytic
<b>Typical Microscopic Characteristics</b>		
Cell appearance	Straight or slightly curved rods, single polar flagella, rods formed in chains; 0.5-1.0 µm in diameter x 1.5-5.0 µm in length	Spherical, occurring singly, in pairs and tetrads, sometimes forming irregular clusters; 0.5-1.0 µm in diameter

\*After 24±2 h (1) *P. aeruginosa* may display two phenotypes.

## Appendix 2

### Neutralization Assay

The purpose of this section is to assess the effectiveness of the neutralization processes associated with this method. Perform the neutralization assay with both microbes for each carrier type prior to or concurrently with testing to demonstrate the neutralizer's ability to inactivate the chemical and determine if there is interference from the carrier itself. Differences in performance (quality) between batches of media may lead to misleading neutralization results.

Select a neutralizing medium that is not inhibitory to the test microbe. The acceptance criteria for acceptable neutralization is  $\leq 50\%$  difference in colony counts between the neutralization effectiveness, neutralization toxicity control, titer control, carrier control, and test interference control.

- 1) Refer to Section 3 in the preceding method for preparation of the test cultures. Conduct preliminary tests as necessary to determine appropriate dilution(s) of *Test Suspension A* (used to prepare *Test Suspension B*) to achieve the target challenge of 20-200 CFU per 10  $\mu\text{L}$  or per carrier.
  - a. Prepare *Test Suspension A (without soil load)*. Serially dilute the microbial test suspension with PBS (e.g., through  $10^{-4}$  or  $10^{-5}$ ). Select appropriate dilutions of *Test Suspension A* so that after the addition of the soil load, the *Test Suspension B* will achieve an average challenge of 20-200 CFU per 10  $\mu\text{L}$ . Use *Test Suspension A* within 30 min of preparation.
  - b. Prepare *Test Suspension B (with soil load)*. Prepare the soil load: using a vortex, mix each component and combine 25  $\mu\text{L}$  BSA, 35  $\mu\text{L}$  yeast extract, and 100  $\mu\text{L}$  of mucin; then vortex-mix the solution. Combine 340  $\mu\text{L}$  of diluted *Test Suspension A* and the 160  $\mu\text{L}$  of the soil load (SL) and vortex-mix for 10 seconds. Use *Test Suspension B* within 30 minutes of preparation.
  - c. Ensure *Test Suspension B* provides an average challenge of 20-200 CFU per 10  $\mu\text{L}$ .
  - d. Two separate serial dilutions of *Test Suspension A* may be used to prepare two different concentrations of *Test Suspension B* to ensure at least one dilution with an average challenge of 20-200 CFU per 10  $\mu\text{L}$ .
  - e. A calibration curve (OD @ 650nm) may be used to estimate the number of viable organisms in *Test Suspension A*.
- 2) Neutralization Treatments (see Appendix 2, Figure 2)
  - a. **Treatment 1: Neutralizer Effectiveness.** Add 50  $\mu\text{L}$  of the test substance to each of three reaction vessels. At timed intervals, add 10 mL neutralizer to each vessel and briefly swirl (by hand). After 10 s, gently add 10  $\mu\text{L}$  of neutralizer test suspension using a micropipette to each vessel and briefly vortex. Proceed with section 4.
  - b. **Treatment 2: Neutralizer Toxicity Control.** Add 10 mL neutralizer to each of three reaction vessels. At timed intervals, add 10  $\mu\text{L}$  of *Test Suspension B* using a micropipette to each vessel and briefly vortex. Proceed with section 4.

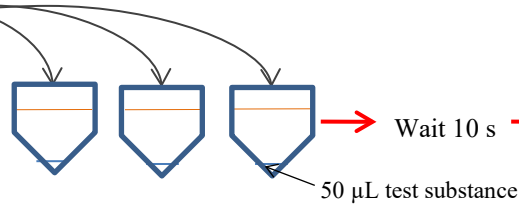


- c. **Treatment 3: Titer Control.** Add 10 mL PBS to each of three reaction vessels. At timed intervals, add 10  $\mu$ L of *Test Suspension B* using a micropipette to each vessel and briefly vortex. Proceed with section 4.
  - d. **Treatment 4: Carrier Interference Control.** Add one carrier to each of three reaction vessels. At timed intervals, add 10 mL neutralizer to each vessel and briefly swirl (by hand). After 10 s. gently add 10  $\mu$ L of *Test Suspension B* using a micropipette to each vessel and briefly vortex. Proceed with section 4. Assess one set of three carriers for each carrier type.
  - e. **Treatment 5: Test Interference Control.** Add one carrier to each of three reaction vessels. At timed intervals, add 50  $\mu$ L of test substance and 10 mL neutralizer to each vessel and briefly swirl (by hand). After 10 s gently add 10  $\mu$ L of *Test Suspension B* using a micropipette to each vessel and briefly vortex. Proceed with section 4. Assess one set of three carriers for each carrier type.
- 3) Hold the neutralization treatments for  $10 \pm 1$  minutes at room temperature ( $21 \pm 3^\circ\text{C}$ ).
  - 4) At the conclusion of the holding period, vortex each reaction and filter each mixture through a separate, pre-wetted 0.2  $\mu\text{m}$  PES membrane filter. Initiate filtration as soon as possible.
  - 5) Wash each reaction vessel with  $\sim 20$  mL PBS and vortex; filter the wash through the same filter membrane. Finish the filtering process by rinsing the inside of the funnel unit with  $\sim 20$  mL PBS and filter the rinsing liquid through the same filter membrane.
  - 6) Remove the membrane aseptically with sterile forceps and place it carefully over the surface of the recovery medium. Avoid trapping air bubbles between the filter and the agar surface.
  - 7) Incubate plates at  $36 \pm 1^\circ\text{C}$  for  $72 \pm 4$  h.
  - 8) It is recommended to monitor filters daily to optimize counting of colonies.

**Figure 2: Neutralization Schematic**

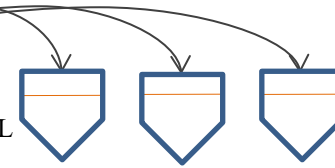
**Treatment 1**

Add 50  $\mu\text{L}$  of test substance to each vessel. At timed intervals add 10 mL neutralizer and swirl by hand.



Wait 10 s

Add 10  $\mu\text{L}$  of *Test Suspension B* to each vessel containing 50  $\mu\text{L}$  test substance and 10 mL neutralizer.

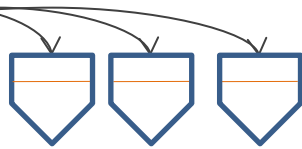


Vortex and hold for 10 min at  $21\pm 3^\circ\text{C}$ . Proceed to vortexing/filtering.

**Neutralizer Effectiveness**

**Treatment 2**

Add 10  $\mu\text{L}$  of *Test Suspension B* to each vessel containing 10 mL neutralizer.

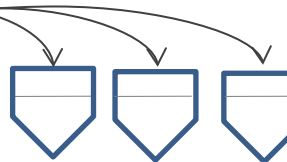


Vortex and hold for 10 min at  $21\pm 3^\circ\text{C}$ . Proceed to vortexing/filtering.

**Neutralizer Toxicity Control**

**Treatment 3**

Add 10  $\mu\text{L}$  of *Test Suspension B* to each vessel containing 10 mL PBS.

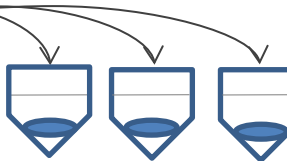


Vortex and hold for 10 min at  $21\pm 3^\circ\text{C}$ . Proceed to vortexing/filtering.

**Titer Control**

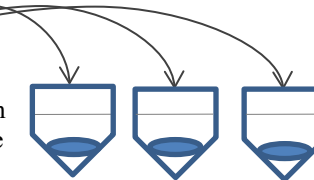
**Treatment 4**

Add 1 sterile carrier to each vessel. At timed intervals, add 10 mL neutralizer and swirl by hand.



Wait 10 s

Add 10  $\mu\text{L}$  of *Test Suspension B* to each vessel containing the carrier and 10 mL neutralizer.

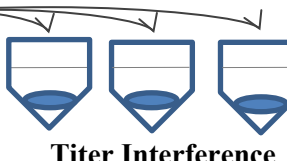


Vortex and hold for 10 min at  $21\pm 3^\circ\text{C}$ . Proceed to vortexing/filtering.

**Titer Interference Control**

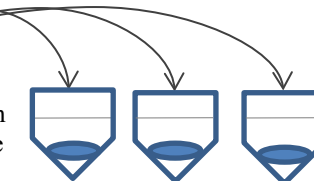
**Treatment 5**

Add 1 sterile carrier to each vessel. At timed intervals, add 50  $\mu\text{L}$  of test substance and 10 mL neutralizer and swirl by hand.



Wait 10 s

Add 10  $\mu\text{L}$  of *Test Suspension B* to each vessel containing the carrier, 50  $\mu\text{L}$  test substance, and 10 mL neutralizer.



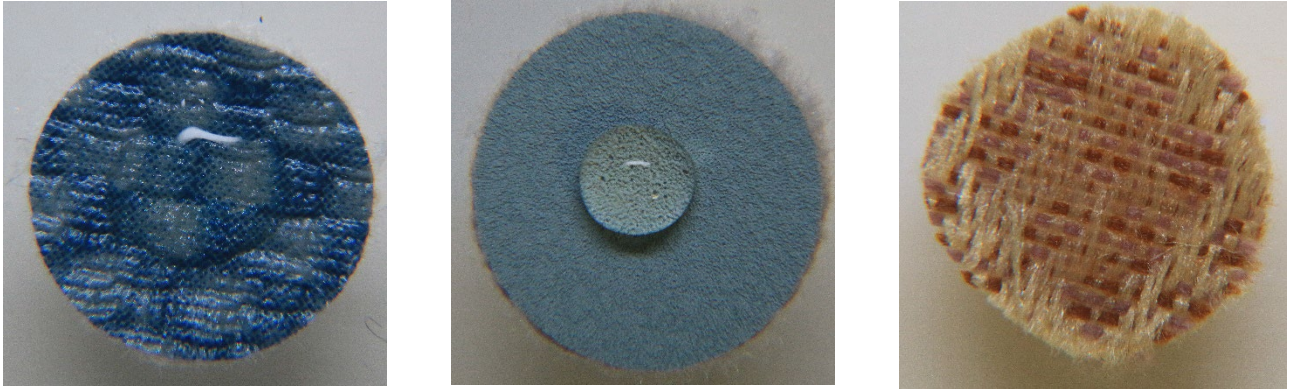
Vortex and hold for 10 min at  $21\pm 3^\circ\text{C}$ . Proceed to vortexing/filtering.

**Titer Interference Control**

## Appendix 3

### Carrier Examples

**Figure 3:** Examples of Inoculated Carriers



**Figure 4:** Examples of Dried Inoculated Carriers with 50  $\mu\text{L}$  of Test Substance Added

