

1 **Interim Quantitative Method for Evaluating the Efficacy of Antimicrobial Test Substances**
2 **on Porous Surfaces Against Bacteria**

3 (12/12/2022)
4

5 **Scope**

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

11 This method provides log reduction (LR) as the quantitative measure of efficacy for disinfectants
12 against the test microbes on a soft-porous surface.
13

14 **Method Overview**

15 In brief, the method uses 1 cm diameter discs (carriers) of a set of representative soft-porous
16 surface materials. Each disc receives 10 µL of microbial inoculum (with a three-part organic and
17 inorganic soil load) deposited in the center of each carrier. The inoculum is allowed to dry and is
18 then exposed to 50 µL of the antimicrobial treatment; control carriers receive an equivalent
19 volume of an innocuous fluid (e.g., phosphate buffered saline). The exposure time is allowed to
20 elapse; a liquid neutralizer is then added to the vial to halt the antimicrobial action. Each vial
21 with the carrier is vortexed, serially diluted, and the contents are filtered to recover viable
22 microorganisms. Based on the difference between the mean log₁₀ density values of the untreated
23 control and treated carriers, a mean log₁₀ reduction (LR) in viable bacteria is calculated. The LR
24 value is used as the measure of product effectiveness.

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

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34 **1) Special Apparatus and Materials**

- 35 a. Test microbes: *Pseudomonas aeruginosa* (ATCC #15442) and *Staphylococcus aureus*
36 (ATCC #6538).
- 37 i. Additional bacteria may be tested (for an additional label claim) per the Agency's
38 guidance.
- 39 b. Culture media
- 40 i. *Tryptic Soy Broth (TSB)*. Use to rehydrate lyophilized cultures. Purchase broth
41 from a reputable source or prepare according to manufacturer's instructions.
- 42 ii. *Synthetic broth (SB)*. Growth medium for test cultures. Commercial media
43 (HIMEDIA, Synthetic Broth, AOAC, #M334-500G). Suspend 16.9 g in 1000 mL
44 DI water. Heat if necessary, to dissolve the medium completely. Final pH at 25°C
45 should be 7.1±0.2. Medium may be dispensed in 10 mL amounts in 20×150 mm
46 culture tubes or alternatively in 500 mL volumes in a 1 L bottle; steam sterilize at
47 15 lbs pressure (121°C) for 15 minutes. Cool to room temperature and just before
48 use, aseptically add 0.1 mL of 10% sterile dextrose solution. Store prepared SB at
49 2-8°C.
- 50 1. Alternatively, SB made in-house per the recipe provided in AOAC
51 Methods 955.15, 964.02, and 955.14 may be substituted.
- 52 iii. *10% dextrose solution*. Add 5.0 g dextrose to 50 mL de-ionized water and mix by
53 stirring. Filter sterilize the solution using a 0.2 µm filter. Store the sterile solution
54 at 2-5°C for up to 30 days.
- 55 iv. *TSB with 15% (v/v) glycerol*. Use as a cryoprotectant. Suspend 7.5 g tryptic soy
56 broth in 212.5 mL de-ionized water. Add 37.5 mL glycerol and stir, warm slightly
57 to dissolve. Dispense into bottles and steam sterilize for 15 min at 121°C.
- 58 v. *Tryptic soy agar (TSA)* and *TSA with 5% sheep blood*. Use for culturing,
59 isolation, and characterization of the test microbes. Purchase plates from a
60 reputable source or prepare according to manufacturer's instructions.
- 61 vi. *Selective media. (optional)* Mannitol salt agar and Cetrimide agar. Use for quality
62 control of test microbes listed in this procedure. Purchase plates or prepare
63 according to manufacturer's instructions.
- 64 c. Reagents
- 65 i. *Neutralizer*. A liquid reagent used to inactivate and/or dilute the antimicrobial
66 treatment to end the contact time.
- 67 ii. *Phosphate buffered saline stock solution (e.g., 10X)*. To prepare 1X phosphate
68 buffered saline. The stock solution has a pH of approximately 7.2±0.2.
- 69 iii. *Phosphate buffered saline (PBS), 1X*. Dilution blanks and filtration. PBS with a
70 pH of approximately 7.0±0.5 is desirable.
- 71 iv. *Soil load, 3-part*. Use as the soiling agent. Add to the test suspension in the
72 following manner:

- 73 1. BSA: Add 0.5 g bovine serum albumin (BSA, radio immunoassay (RIA)
74 grade or equivalent, CAS# 9048-46-8) to 10 mL of PBS, mix and pass
75 through a 0.2 μm pore diameter (polyethersulfone) membrane filter,
76 aliquot (e.g., a minimum of 50 μL), and store at $-20\pm 2^\circ\text{C}$.
- 77 2. Yeast Extract: Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass
78 through a 0.2 μm pore diameter (polyethersulfone) membrane filter,
79 aliquot (e.g., a minimum of 70 μL), and store at $-20\pm 2^\circ\text{C}$.
- 80 3. Mucin: Add 0.04 g mucin (from bovine submaxillary gland, CAS #
81 84195-52-8) to 10 mL of PBS, stir or vortex-mix until thoroughly
82 dissolved, and pass through a 0.2 μm pore diameter (polyethersulfone)
83 membrane filter, aliquot, and store at $-20\pm 2^\circ\text{C}$.
- 84 4. The three stock solutions of the soil load are single use only. Do not
85 refreeze; store up to one year at $-20\pm 2^\circ\text{C}$.
- 86 v. *Antimicrobial Test substance.* Ready-to-use, activated, or concentrated
87 antimicrobial. If the antimicrobial test substance is prepared by diluting a
88 concentrate, adequately mix antimicrobial test substance with the appropriate
89 diluent (e.g., hard water), then use prepared test substance within 3 hours of
90 preparation or as otherwise instructed by the manufacturer. Measuring error
91 increases as delivery volume decreases. To minimize variability due to measuring
92 error, a minimum of 1.0 mL or 1.0 g of concentrated antimicrobial test substance
93 should be used when preparing use-dilutions for testing. Use v/v dilutions for
94 liquids antimicrobial test substances and w/v dilutions for solid antimicrobial test
95 substances. The use of a positive displacement pipette is recommended for
96 viscous liquids.
- 97 vi. *1 N NaOH and 1 N HCl.* Used for pH adjustment of media/reagents.
- 98 vii. *Water.* De-ionized (DI), distilled water or water with equivalent quality for
99 making reagent solutions and culture media.
- 100 viii. *Tween-80* (polysorbate 80). Used to prepare PBS-T.
- 101 ix. *Gram stain.* Used for diagnostic staining.
- 102 d. Apparatus
- 103 i. *Carriers:* Discs (1 cm in diameter) cut from porous material. Carriers are single
104 use only. See Section 2 for carrier specifications.
- 105 ii. *Hole punch:* If necessary, for use in the preparation of 1 cm disc from material.
106 Model number: SKU# HP-MEI448R or equivalent
- 107 iii. *Calibrated 10 μL positive displacement pipette* with corresponding 10 μL tips, for
108 carrier inoculation.
- 109 iv. *Filter paper.* Whatman No. 2, to line glass Petri plates.
- 110 v. *Calibrated micropipettes* (e.g., 200 μL , 1 mL) with appropriate corresponding

tips, for deposition of test substance on carriers and preparing dilutions.

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- 112 vi. *Bottle-top dispensers, squirt bottles, pre-measured volumes in tubes, or pipettes,*
113 *bottles, etc.* For rinsing vials and filters.
- 114 vii. *Forceps*, straight or curved, non-magnetic, disposable with smooth flat tips to
115 handle membrane filters, appropriate to pick up the carriers for placement in vials.
- 116 viii. *Polyethersulfone (PES) membranes.* Use for recovery of test microbe, 47 mm
117 diameter and 0.2 μm pore size.
- 118 1. Use filter membranes in either a reusable or disposable filtration unit.
- 119 ix. *Filter Sterilization Unit (with PES, 0.2 μm pore size).* Use to filter sterilize soil
120 components.
- 121 x. *20 x 150 m glass culture tubes* with Morton closures for test culture preparation.
- 122 xi. *Spectrophotometer.* For culture standardization (if deemed necessary)
- 123 xii. *Vials with lids (plastic or comparable).* Sterile, flat-bottomed, wide-mouthed (at
124 least 25 mm diameter), approximately 20 mL capacity, for holding inoculated
125 carriers to be exposed to the test chemical and for accommodating neutralizer.
- 126 1. Transparent vials are more desirable to facilitate application of 50 μL test
127 substance or control substance to inoculated carrier.
- 128 xiii. *Certified timer.* Readable in minutes and seconds, for tracking of timed events and
129 intervals.
- 130 xiv. *Desiccation unit* (with gauge to measure vacuum level) with fresh desiccant (e.g.,
131 anhydrous CaCO_3). For drying inoculated carriers.
- 132 xv. *Vacuum source.* In-house line or suitable vacuum pump capable of achieving
133 0.068 to 0.085 MPa, for drying inoculated carriers in desiccation unit and to
134 perform membrane filtration.
- 135 xvi. *Titration kit.* (i.e., Hach digital titrator) Used for measuring water hardness.
- 136 xvii. *Vortex-style mixer.* Used for vortex-mixing of various solutions.
- 137 xviii. *15 mL conical centrifuge tubes.* Used for centrifugation of test cultures.
- 138 xix. *Centrifuge* (with rotor capable of achieving 5,000g). Used for test culture
139 preparation.

140 2) **Carriers**

141 a. Carrier Materials

- 142 i. Privacy Curtain Fabric (PCF-03): 54% Polyester, 46% Fire Resistant (FR)
143 Polyester. CF Stinson, LLC. Mambo MAM34 Nights.
- 144 ii. Non-PVC Fabric (NVF-01): Polyurethane Face made with Polycarbonate and
145 Polyether Resins, Polyester Backing. CF Stinson, LLC. Kid BlueSky KID17.
- 146 iii. Vinyl Seating Fabric (VF-01): Vinyl Face, Polyester Backing. CF Stinson, LLC.
147 Hopsack - HOP24 Fjord.



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

150 b. Carrier Preparation

- 151 i. Punch, or obtain, 1 cm round carriers or use comparable cutting procedure from
 152 fabric.
- 153 ii. Visually screen carriers to ensure consistent surface characteristics; trim any
 154 jagged edges or loose fabric.
- 155 iii. No pre-cleaning of carriers is necessary. To sterilize carriers, sterilize using a
 156 gravity cycle, 121°C for 20 minutes; ensure carriers are dry following
 157 sterilization. Test sterility of carriers prior to testing.
- 158 1. Carriers may not be entirely flat after autoclaving; however, minor
 159 distortion of carriers is acceptable for testing.
 - 160 2. Prior to use in testing, document the condition of the screened and sterile
 161 carriers (e.g., digital photographs).

162 **3) Preparation of Test Culture and Carrier Inoculation**

- 163 a. Refer to Attachment A for preparation of the frozen stock cultures.
- 164 b. Defrost a cryovial; defrost rapidly to avoid loss in the viability of the preserved cells.
 165 Each cryovial is single use only.
- 166 c. Within 15 minutes prior to inoculation, using a calibrated pipette to aseptically add 0.1
 167 mL of 10% sterile dextrose (w/v) solution to each 10 mL tube of SB.
- 168 d. Using a calibrated micropipette, add 100 µL of defrosted stock culture to 10 mL SB with
 169 dextrose, briefly vortex-mix and incubate for 24±2 h at 36±1°C.
- 170 i. Incubate without disrupting the culture.
 - 171 ii. In addition, inoculate an agar plate (e.g., TSA or TSA with 5% sheep blood) from
 172 the inoculated tube and streak for isolation. Incubate plate with the test culture.
- 173 e. Following incubation, use the SB cultures to prepare a test suspension for each organism.
- 174 i. The 24±2 h culture should exhibit a titer of at least 10⁸ CFU/mL.

- 175 f. For *P. aeruginosa*, inspect culture prior to harvest; visible pellicle on the surface of the
176 culture is expected to form during incubation (record its presence). Discard the culture if
177 pellicle has been disrupted (fragments in culture).
- 178 i. Remove visible pellicle on surface of medium and around associated interior
179 edges of the tube by pipetting or with vacuum suction.
- 180 ii. Using a serological pipette, withdraw the remaining broth culture (at least 5 mL)
181 avoiding any sediment on the bottom of the tube and transfer it into a 15 mL
182 conical centrifuge tube.
- 183 iii. Record approximate volume harvested and transferred to 15 mL conical tube.
- 184 g. For *S. aureus*, briefly vortex-mix the 24±2 h culture and transfer to a 15 mL conical
185 centrifuge tube.
- 186 h. Within 15 min, centrifuge the 24±2 h harvested broth cultures at 5,000g_N for 20±5 min.
- 187 i. Remove the supernatant without disrupting the pellet. Re-suspend the pellet in 5-10 mL
188 PBS. Record resuspension volume.
- 189 i. Prepare the final test suspension within 30 min of resuspending the culture.
- 190 ii. If necessary, disrupt the pellet using vortex-mixing or repetitive tapping/striking
191 against a hard surface to disaggregate the pellet completely prior to re-suspending
192 it in 10 mL. If necessary, add 1 mL of PBS to the pellet to aid in the
193 disaggregation.
- 194 j. If needed, dilute the 5-10 mL of resuspended culture in PBS to achieve a mean control
195 carrier count level of 4.0-5.5 logs CFU/carrier for *S. aureus* and *P. aeruginosa*.
- 196 i. Optical density/absorbance (e.g., 650 nm) may be used as a tool to monitor/adjust
197 the diluted test suspension.
- 198 k. Use the resuspended or diluted culture to prepare the final test suspension with the
199 addition of the soil load.
- 200 l. To obtain 500 µL of the final test suspension with the 3-part soil load, vortex-mix each
201 component and combine in the following order using a calibrated micropipette:
- 202 i. 25 µL BSA stock
- 203 ii. 35 µL yeast extract stock
- 204 iii. 100 µL mucin stock
- 205 iv. Vortex soil suspension for 10s prior to adding microbial test suspension.
- 206 v. 340 µL microbial test suspension
- 207 m. Briefly vortex the final test suspension with 3-part soil load (at room temperature,
208 21±3°C) and use to inoculate carriers within 30 min of preparation.
- 209 i. Streak inoculate an agar plate with a loopful of the final test suspension. Incubate

210
211

plate with the treated and control carrier plates and examine for purity after incubation at $36\pm 1^{\circ}\text{C}$ for 72 ± 4 h.

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- 212 n. It is advisable to briefly rescreen each sterilized carrier for abnormalities prior to
213 inoculation. Place carriers screened side up inside an empty, sterile plastic Petri dish (no
214 more than 20 carriers/dish).
- 215 i. Privacy curtain carriers have no backing material and may be inoculated on either
216 side.
- 217 ii. Non-PVC and vinyl carriers are layered materials comprised of a smooth, colored
218 top surface and a white fabric bottom; only the top surface will be inoculated.
- 219 o. Vortex-mix the final test suspension for 10 s following the addition of the soil load and
220 immediately prior to use.
- 221 p. Inoculate the number of carriers required for the evaluation of the test substance (3
222 controls and 5 treated) along with a few extra carriers.
- 223 q. Using a calibrated positive displacement pipette with a 10 μ L tip, withdraw 10 μ L of the
224 final test suspension and deposit it at the center of each carrier (clean, screened and
225 sterile); avoid contact of pipette tip with carrier and do not spread the final test
226 suspension with the pipette tip.
- 227 i. For consistency, vortex-mix the inoculum frequently during inoculation of the
228 carrier set.
- 229 ii. The same pipette tip may be used to inoculate all carriers (unless the tip is
230 compromised).
- 231 iii. Discard any inoculated carrier where the final test suspension has run over the
232 edge.
- 233 r. Transfer the Petri dish(es) with the inoculated carriers into a desiccation unit (with
234 desiccant) and completely remove the lid of the Petri dish. Close the desiccation unit
235 door (or lid) and seal the unit. Apply vacuum to evacuate the desiccation unit.
- 236 i. Note: do not exceed 40 inoculated carriers per desiccator to ensure carriers dry
237 within the prescribed time.
- 238 s. Maintain and monitor the vacuum level using a gauge. Achieve and maintain consistent
239 level of vacuum (at 20-25 in of mercury, 508-635 torr, 677-847 mbar, or 68000-85000
240 Pascal) by leaving the vacuum on during the drying period with the desiccator stopcock
241 opened or closed as necessary.
- 242 t. Hold the inoculated carriers in the evacuated desiccation unit at $21\pm 3^{\circ}\text{C}$ for 45 to 60 min.
243 Visually inspect inoculated carriers to verify that they have completely dried and remove
244 from desiccation unit. Do not use carriers that are visibly wet for testing.
- 245 i. Record the time for all timed events.
- 246 ii. Depressurize the desiccator slowly to avoid the potential for carriers to move or
247 flip.
- 248 u. Use dried inoculated carriers for testing within 30 min following removal from
249 desiccation unit; hold carriers in closed Petri dish at room temperature ($21\pm 3^{\circ}\text{C}$) until
250 use.

251 **4) Performance Assessment – Efficacy**

- 252 a. Evaluate 3 control carriers and 5 treated carriers for each test substance tested (one test
253 organism and contact time /carrier type combination) unless specified otherwise.
- 254 i. One set of control carriers per carrier type may be used for evaluating multiple
255 test substances against one organism on one test day (assuming the carrier
256 material, neutralizer, and soil load are the same).
- 257 b. Using sterile forceps, transfer each dried carrier with the inoculated side up to a flat-
258 bottom vial and cap the vial. Repeat until all carriers are transferred.
- 259 c. Prepare the antimicrobial test substance. Use antimicrobial test substance within 3 hours
260 of preparation or as specified by the manufacturer.
- 261 d. In a timed fashion with appropriate intervals, sequentially deposit 50 μL of the test
262 substance (equilibrated to $21\pm 3^\circ\text{C}$) with a calibrated micropipette over the dried
263 inoculum on each test carrier, ensuring complete coverage.
- 264 i. Note: Gently apply the antimicrobial test substance at a perpendicular angle to the
265 inoculated carrier; do not forcefully deposit the disinfectant.
- 266 e. Use a new tip for each carrier; do not touch the carrier surface with a pipette tip during
267 the application of the test substance or the control substance; replace with new carrier(s)
268 and vial(s) if this occurs. Do not cap the vials.
- 269 i. For non-foaming aerosols and pump/trigger spray products, obtain the test
270 substance by dispensing the product into a sterile vessel for collection. Cap the
271 vessel and use dispensed product within 30 min.
- 272 ii. For foaming spray formulations, allow the foam to break down for at least 5-10
273 minutes for the generation of a 1-2 mL liquid sample. Cap the vessel and use
274 dispensed product within 30 min.
- 275 f. Do not process carriers where the test substance runs off the carrier or does not
276 completely cover the inoculum spot; replace with new carrier(s) and vial(s) if this occurs.
- 277 g. Conduct the test at room temperature ($21\pm 3^\circ\text{C}$) for the selected contact time. Use a
278 certified timer to ensure that each carrier receives the required contact time.
- 279 h. Process control carriers last. Each control carrier receives 50 μL PBS, equilibrated to
280 $21\pm 3^\circ\text{C}$, instead of the test substance. Hold the control carriers for the same contact time
281 as used for the test substance.
- 282 i. Within ± 5 s of the end of the contact period, add 10 mL of neutralizer equilibrated to
283 $21\pm 3^\circ\text{C}$ to each vial in the specified order according to the predetermined schedule.
284 Briefly vortex-mix (2-3 s) each vial following the addition of the neutralizer.
- 285 i. For calculation purposes, the solution in the neutralized vial with carrier is
286 considered to be 10^0 dilution.
- 287 ii. The neutralizer for the control carriers is the same as that for the treated carriers.
- 288 j. Immediately following the addition of the neutralizer and briefly (2-3 s) vortex, allow

carriers to sit in the vials for 5 minutes undisturbed then proceed as follows:

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- 290 i. Vortex-mix vials at high speed for 30 s (vortex-mix #1).
- 291 ii. Allow carriers to sit undisturbed in the vials for 5 minutes.
- 292 iii. Vortex-mix vials at high speed for 30 s (vortex-mix #2).
- 293 iv. Allow carriers to sit undisturbed in the vials for 5 minutes.
- 294 v. Vortex-mix vials at high speed for 30 s (vortex-mix #3).
- 295 k. Initiate dilutions within 30 min after neutralization and vortex-mixing. Initiate filtration
296 within 30 min of preparing the dilutions.
- 297 l. Dilute and filter samples from the treated and control carriers; process treated carriers
298 first.
- 299 m. Serially dilute the eluate from the 10⁰ dilution prior to filtration by transferring 1 mL into
300 9 mL PBS in a dilution tube.
- 301 n. Turn on vacuum and leave on for the duration of the filtration process.
- 302 o. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS.
- 303 p. Use separate membrane filters for each eluate (neutralized solution); however, the same
304 filtration unit may be used for processing eluates from a given carrier set starting with the
305 most dilute sample first.
- 306 q. Filter each sample through a separate 0.2 µm PES membrane filter.
- 307 r. For eluates from treated carriers remaining in the vial (10⁰ dilution), vortex-mix the vial
308 for ~5 s, carefully pour the eluate into the filter unit.
- 309 i. If a carrier falls onto the filter membrane, aseptically remove it using sterile
310 forceps.
- 311 s. Rinse the treated vial with ~20 mL PBS, vortex-mix for ~5 s, pour the wash into the same
312 filter unit. For dilution tubes, rinse tube once with ~10 mL PBS, briefly vortex-mix, and
313 pour into filter unit.
- 314 t. Swirl the contents of the filter unit and quickly filter with limited pooling of liquid in the
315 filter apparatus.
- 316 u. Rinse the inside surface of the funnel unit with at least 20 mL PBS and filter the contents.
- 317 v. Aseptically remove the membrane filter and place on the appropriate recovery medium.
318 Avoid trapping any air bubbles between the filter and the agar surface.
- 319 w. Sterility controls.
- 320 i. On the day of the test, filter ~20 mL of neutralizer and ~20 mL of the PBS used in
321 the test using two separate membrane filters and place on TSA.
- 322 ii. Incubate these filters along with a plate of recovery medium (e.g., TSA) for 72±4
323 h at 36±1°C, record sterility results.
- 324 x. Incubate plates at 36±1°C for 48±4 h for control carriers and for a minimum of 72±4 h
325 for treated carriers.

- 326 i. Monitor filters daily to optimize counting of colonies. CFUs may be counted
327 daily. Record controls after 48±4 h and treated carriers after 72±4 h.
- 328 y. Count colonies and record results.
- 329 i. Any level of contamination which interferes with the recording and interpretation
330 of results will result in invalid data.
- 331 ii. For example, contamination occurring on multiple filters within one set of serial
332 dilutions and/or across multiple carriers is considered systemic and the test is
333 deemed invalid.
- 334 z. For colony counts on filters in excess of 200 record as Too Numerous to Count (TNTC).
- 335 aa. If no colonies are present, record as zero.
- 336 bb. Report non-conforming data (e.g., systemic contamination and atypical serial dilution
337 results) and repeat tests as necessary.
- 338 i. Systemic contamination
- 339 ii. Atypical serial dilution results (e.g., higher CFUs at more dilute levels).
- 340 cc. Inspect the growth on the filters for purity and typical characteristics of the test microbe,
341 see Attachment A, Table 1.
- 342 dd. If isolated colonies are present, assess one representative colony per 5-carrier set (treated)
343 or 3-carrier set (controls) using a Gram stain.
- 344 i. If confluent growth is present, perform a streak isolation on TSA or TSA with 5%
345 sheep blood on growth taken from at least 1 carrier incubate at 36±1°C for 24-48
346 h.
- 347 ee. If additional verification of the test organism is required, perform further confirmatory
348 analyses (e.g., Vitek and biochemical analyses) and isolation streaks on selective media.

349 5) Data Requirements

- 350 a. Per test, use colony counts to determine log reduction.
- 351 b. For an acceptable test, each of the three control carriers must exhibit counts between 4.0-
352 5.5 logs CFU/carrier.
- 353 c. Use values with at least three significant figures when performing calculations (e.g., log
354 density, mean log density). Report the final mean log reduction value with two
355 significant figures (e.g., round up to the nearest tenth).
- 356 d. Calculate the Colony Forming Units (CFU)/carrier using the following equation:

$$357 \log_{10} \left(\frac{\sum_{i=1}^n (r_i)}{\sum_{i=1}^n (C_{ii} \times D_{ii})} \right) \times V$$

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).

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

372 **Attachment A**

373 **Preparation of Frozen Stock Culture**

374

- 375 1) Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* and
376 *Staphylococcus aureus* from a reputable vender at least every 18 months.
- 377 a. New frozen stock culture may be initiated one time using an existing,
378 unexpired frozen stock culture as the source. Begin process at step 3 below,
379 by streaking a loopful of the frozen stock culture onto 2 TSA plates.
- 380 2) Open ampule of freeze-dried organism per manufacturer's instructions. Using a tube
381 containing 5-6 mL of TSB, aseptically withdraw 0.5 to 1.0 mL and rehydrate the
382 lyophilized culture. Aseptically transfer the entire rehydrated pellet back into the
383 original tube of broth. Mix thoroughly. Incubate broth culture at $36\pm 1^{\circ}\text{C}$ for 24 ± 2 h.
- 384 3) At the end of the incubation timeframe, streak a loopful of the broth culture onto 2 TSA
385 plates to obtain isolated colonies. Perform a streak isolation of the broth culture onto BAP as
386 a purity check and streak the broth culture onto the appropriate selective media. Refer to
387 appropriate selective media in Table 1. Incubate all plates for 24 ± 2 h at $36\pm 1^{\circ}\text{C}$.
- 388 a. Record results at the end of the incubation timeframe. Refer to Table 1 for results on
389 selective media and diagnostic characteristics of the test microbes.
- 390 4) From the TSA plates, select 3-5 isolated colonies of the test organism and re-suspend in 1
391 mL of TSB. For *S. aureus*, select only golden yellow colonies. For *P. aeruginosa*, select
392 colonies from each of the two possible phenotypes present. Spread plate 0.1 mL of the
393 suspension onto each of 6-10 TSA plates. Incubate the plates for 24 ± 2 h at $36\pm 1^{\circ}\text{C}$. If
394 necessary, to obtain more frozen stock cultures, a larger suspension (e.g., 2 mL) may be
395 prepared using the same ratio of TSB (1 mL) to number of colonies (3-5 colonies).
- 396 a. Using the TSB suspension, perform a streak isolation of the suspension onto a BAP as a
397 purity check, and streak on the appropriate selective media (refer to Table 1).
- 398 b. Incubate all plates for 24 ± 2 h at $36\pm 1^{\circ}\text{C}$. Record results. Refer to Table 1 for results on
399 selective media and diagnostic characteristics of the test microbes.
- 400 5) After the incubation period, harvest growth from TSA plates by adding approximately 5 mL
401 sterile cryoprotectant solution (TSB with 15% (v/v) glycerol) on the surface of each plate.
402 Re-suspend the growth in the cryoprotectant solution using a sterile spreader without
403 damaging the agar surface. Aspirate the suspension from the plate with a pipette and place it
404 in a sterile vessel large enough to hold about 30 mL.
- 405 6) Repeat the growth harvesting procedure with the remaining plates and continue adding the
406 suspension to the vessel (more than 1 vessel may be used if necessary). Mix the contents of
407 the vessel(s) thoroughly; if more than 1 vessel is used, pool the vessels prior to aliquoting
408 culture.
- 409 7) Immediately after mixing, dispense 0.5-1.0 mL aliquots of the harvested suspension into
410 cryovials; these represent the frozen stock cultures.