



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

**Office of Pesticide Programs  
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**Interim Method for Evaluating the Efficacy of  
Antimicrobial Surface Coatings**

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# Interim Method for Evaluating the Efficacy of Antimicrobial Surface Coatings

10/02/2020

## Scope

The Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) recommends that applicants utilize this interim method to support efficacy requirements for the registration of coatings applied to surfaces that are intended to provide residual antimicrobial activity for a period of weeks and are designed to be supplements to standard disinfection practices. The interim method includes an efficacy assessment of the coated coupons following exposure to certain chemical solutions/mechanical abrasion. The test method provides for the evaluation of durability and the baseline efficacy of these treated surfaces against *Staphylococcus aureus* and *Pseudomonas aeruginosa*; the method can be adapted for additional microbes and viruses. A minimum 3 log reduction of test microbes within 1-2 hours is the required level of performance.

This interim method is based on the *Revised Method for the Evaluation of Bactericidal Activity of Hard, Non-porous Copper-Containing Surface Products (01/23/2020)*; refer to regulations.gov, docket number EPA-HQ-2016-0347.

## Method Overview

In brief, the test method is comprised of two parts: 1) chemical treatment and abrasion, and 2) product efficacy. The method uses 1" × 1" brushed stainless-steel carriers coated with the antimicrobial chemical and uncoated control carriers. Carriers are exposed to 10 cycles of chemical treatment/abrasion in order to support a 1-week duration label claim of residual activity. Testing can be scaled up to support longer claims up to 4 weeks. The chemical exposure and abrasion processes are intended to represent a degree of normal and relevant physical wear, as well as reproduce potential effects resulting from repeated exposure of antimicrobial coated surfaces to three different biocidal materials (chemical solutions) as well as the impact of dry abrasion. Under controlled environmental conditions, the carriers receive a 20 µL mixture of the test organism and soil load. Following a 1-2 hour contact time, the carriers are neutralized and the number of viable microorganisms is determined quantitatively. The log reduction (LR) in the viable test organisms on exposed carriers is calculated in relation to the viable test organisms on the unexposed control carriers. The impact of the chemical exposure and abrasion on product efficacy is also determined by comparing carriers with and without coating not exposed to chemical treatment and abrasion.

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)" 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).

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43 **1) Special Apparatus and Materials**

- 44 a. Test microbes: *Pseudomonas aeruginosa* (ATCC #15442) and *Staphylococcus aureus*  
45 (ATCC #6538)
- 46 b. Culture media
- 47 i. *Tryptic Soy Agar (TSA) and TSA with 5% sheep blood (BAP)*. Used for culturing,  
48 isolation, and characterization of the test microbes. Purchase plates from a  
49 reputable source or prepare according to manufacturer's instructions.
- 50 ii. *Tryptic Soy Broth (TSB)*. Used to rehydrate lyophilized cultures and grow  
51 overnight cultures. Purchase broth from a reputable source or prepare according to  
52 manufacturer's instructions.
- 53 iii. *TSB with 15% (v/v) glycerol*. Used as a cryoprotectant solution. Purchase broth  
54 from a reputable source or prepare according to manufacturer's instructions.
- 55 c. Reagents
- 56 i. *Ethanol (e.g., 70%)*. Used to treat abrasion platform.
- 57 ii. *De-ionized (DI) Water*. For preparing reagents and media; use sterile DI water for  
58 rinsing test solutions off carriers prior to efficacy testing.
- 59 iii. *Gram stain kit*. Used for diagnostic staining of *P. aeruginosa* and *S. aureus*.
- 60 iv. *Liquinox or equivalent non-ionic detergent solution*. To clean carriers.
- 61 v. *Neutralizer*. Various confirmed neutralizers may be used, including letheen broth.  
62 If necessary, other ingredients may be added to letheen broth. Purchase letheen  
63 broth from a reputable source or prepare according to manufacturer's instructions.
- 64 vi. *Phosphate buffered saline stock solution (e.g., 10X)*. Use to prepare 1X phosphate  
65 buffered saline. The stock solution has a pH of approximately  $7.2 \pm 0.2$ .
- 66 vii. *Phosphate buffered saline (PBS), 1X*. Use for dilution blanks and filtration. PBS  
67 with a pH of approximately  $7.0 \pm 0.5$  is desirable.
- 68 viii. *Soil Load*. The soil load to be incorporated in the test suspension is a mixture of  
69 the following stock solutions in PBS:
- 70 1. BSA: Add 0.5 g bovine serum albumin (BSA) to 10 mL of PBS, mix and  
71 pass through a 0.2  $\mu\text{m}$  pore diameter membrane filter, aliquot and store at  
72 approximately  $-20^\circ\text{C}$ .
- 73 2. Yeast extract: Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass  
74 through a 0.2  $\mu\text{m}$  pore diameter membrane filter, aliquot and store at  
75 approximately  $-20^\circ\text{C}$ .
- 76 3. Mucin: Add 0.04 g mucin (from bovine submaxillary gland or equivalent)  
77 to 10 mL of PBS, mix, and pass through a 0.2  $\mu\text{m}$  pore diameter PES  
78 membrane filter, aliquot and store at approximately  $-20^\circ\text{C}$ .
- 79 4. The stock solutions of the soil load solutions are single use only. Do not  
80 refreeze once thawed; store up to one year at  $-20 \pm 2^\circ\text{C}$ .
- 81 ix. Solutions used in chemical exposure of carriers:
- 82 1. *Solution A*. A  $2000 \pm 100$  ppm sodium hypochlorite ( $\text{NaOCl}$ ) solution (e.g.,  
83 Sigma-Aldrich reagent grade sodium hypochlorite) prepared in sterile  
84 deionized water. Verify the final concentration of the solution using a  
85 suitable titration method (e.g., Hach digital titrator).
- 86 2. *Solution B*. Use an EPA-registered antimicrobial pesticide product  
87 containing hydrogen peroxide (between 3.0% and 6.0%) and peracetic

acid as active ingredients that allows liquid application to hard, non-

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89 porous surfaces. The solution concentration for the peracetic acid  
 90 component is not limited to a defined range.  
 91 3. *Solution C*. Use an EPA-registered hospital disinfectant product with  
 92 quaternary ammonium compound as the active ingredient labeled as a one-  
 93 step cleaner/disinfectant that allows liquid application to hard, non-porous  
 94 surfaces.

95 d. Materials

- 96 i. *0.2 µm Polyethersulfone (PES) membrane filters*. For recovery of the test  
 97 microbe. Filtration units (reusable or disposable) may be used.
- 98 ii. *Carriers*: Die/machine cut 1 × 1 inch square made of AISI Type 304 stainless  
 99 steel with 150 grit unidirectional brushed finish on one side. Carriers are single  
 100 use. See Appendix B for carrier specifications.
- 101 iii. *Conical tubes*. (e.g., 15 mL, 50 mL) Capable of being centrifuged at 5,000 g.  
 102 Used for neutralization.
- 103 iv. *Cryovials*. For storage of frozen stock culture.
- 104 v. *Dilution tubes*. Glass/plastic tubes that are used for preparing dilutions.
- 105 vi. *Filter paper*. Whatman No. 2, used to line Petri plates.
- 106 vii. *Forceps*. Use appropriate tips (smooth or curved) to pick up carriers for  
 107 placement in conical tubes and for membrane filtration.
- 108 viii. *Microcentrifuge tubes*. For storage of soil single use aliquots.
- 109 ix. *Cleaning Sponge*. Example: Scotch Brite Non-Scratch Scrub Sponge, item  
 110 number C05068 or equivalent.
  - 111 1. Prior to sterilization, cut sponge to fit snugly into sponge boat, sponge-  
 112 side down.
- 113 x. *Spacer Material*. Use to ensure that the sponge extends a minimum of 5 mm  
 114 beyond the sponge boat (example: foam pad, additional sponge, etc.) so that the  
 115 sponge boat does not contact the carriers or abrasion unit plate.

1161 xi. *Petri dishes*. Multiple sizes

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- 117 1. 100 mm glass/plastic dishes used as a flat surface for inoculating and  
 118 incubating carriers. Also used with filter paper for carrier drying and  
 119 storage.
- 120 2. 150 mm glass/plastic dishes. Glass dishes used to sterilize sponges; plastic  
 121 or glass dishes used to hold moistened sponges during the test day.

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- S* *g* *ipettes*. (e.g., 10 mL, 25 mL) Used for removing/adding large volumes of  
*e* *i* liquid.  
*r* *c* *Sterile Squirt Bottle*. Used to rinse carriers after chemical treatments.  
*o* *a* *Neodymium Magnets (optional)*. Place magnets on bottom and top of abrasion  
*l* *l* platform to hold the carriers in place during abrasion process. Placed at the  
*o* *P* beginning and end of the abraded carriers.
- 128 e. Equipment  
129 i. *-20°C Freezer*. For storage of soil aliquots.  
130 ii. *-80°C Freezer*. For storage of frozen stock cultures.  
131 iii. *Calibrated 20 µL positive displacement pipette*. With corresponding tips for  
132 carrier inoculation.  
133 iv. *Calibrated micropipettes*. (e.g., 200 µL, 1 mL) With 20-200 and 100-1000 µL  
134 tips. For preparing dilutions.

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- 135 v. *Centrifuge* (with rotor capable of achieving 5,000 g). For test culture preparation.
- 136 vi. *Certified timer*. Readable in minutes and seconds, for tracking of timed events and
- 137 intervals.
- 138 vii. *Environmental chamber*. Used to hold carriers during microbe contact time at
- 139 22±2°C and 30-40% relative humidity.
- 140 viii. *Gardco Model D10V or comparable*. Abrasion instrument used to simulate wear
- 141 on carriers.
- 142 ix. *Sponge boat applicator with weight*. To achieve total weight of approximately
- 143 454 g (without sponge).
- 144 1. Use weight (approximately 230 g) for Treatments A, B, and C; do not add
- 145 weight to sponge boat for Treatment D.
- 146 x. *Hach Digital Titrator Kit*. For measuring total chlorine.
- 147 xi. *Incubator*. Used to incubate test cultures and growth medium plates at 36±1°C.
- 148 xii. *Microscope* (100X optics and 10X ocular). For observation of Gram stains.
- 149 xiii. *Refrigerator* (2-8°C). Storage of media and post-incubated plates.
- 150 xiv. *Sonicator capable of producing 45 Hz*. For removal of organism from carriers.
- 151 xv. *Vortex*. For vortex mixing of various solutions including carriers.
- 152

## 153 2) Carriers

154 Two market relevant lots of the test product should be used to evaluate efficacy. The test

155 product is the formulation used to coat the stainless-steel carriers. Lot 1 of test product is used

156 for chemical treatments/abrasion and efficacy testing on exposed and unexposed control and

157 coated carriers; two additional controls are included. Lot 2 is used to compare the unexposed

158 control carriers to the exposed coated carriers only. See Table 1 for a summary of carrier

159 distribution. “Exposed” refers to carriers subjected to the chemical treatment/physical

160 abrasion, while “unexposed” refers to those carriers not subjected to the chemical

161 treatment/physical abrasion. Test carriers are coated with the residual product while controls

162 are uncoated carriers.

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164 **Table 1.** Carrier distribution

	Carrier Type	# of carriers for <i>S. aureus</i>	# of carriers for <i>P. aeruginosa</i>
<b>Lot 1</b>	Control Set #1: Unexposed (no residual product applied)	3	3
	Control Set #2: Exposed (no residual product applied)	3 per exposure* (9 total)	3 per exposure* (9 total)
	Coated Set #1: Unexposed (residual product applied)	3	3
	Coated Set #2: Exposed (residual product applied)	5 per exposure** (20 total)	5 per exposure** (20 total)
	<b>Total Carriers for Lot #1</b>	<b>35</b>	<b>35</b>
<b>Lot 2</b>	Control Set #1: Unexposed (no residual product applied)	3	3
	Coated Set #2: Exposed (residual product applied)	5 per exposure** (20 total)	5 per exposure** (20 total)
	<b>Total Carriers for Lot #2</b>	<b>23</b>	<b>23</b>

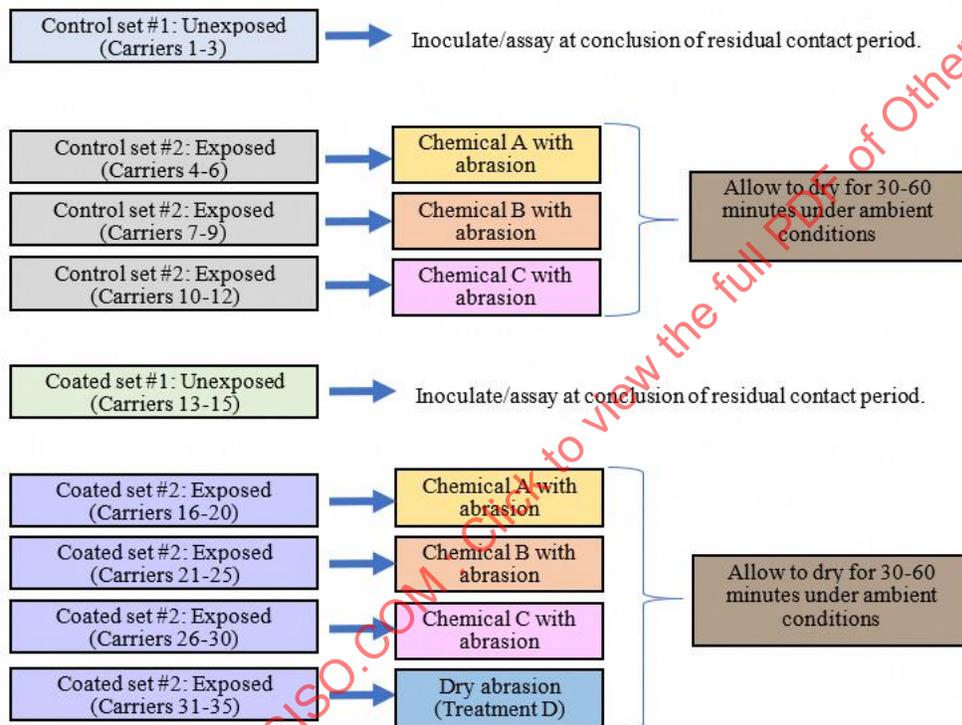
165 \*3 chemical exposures with abrasion

166 \*\*4 exposures (3 chemical exposures with abrasion, 1 dry abrasion exposure)

- 167 a. Screen and clean carriers prior to chemical exposure/abrasion.
- 168 b. Inspect each carrier to ensure uniformity. Discard carriers with visible surface or edge
- 169 abnormalities (e.g., corrosion/rust, chipping, gouges or deep striations, etc.)
- 170 c. Soak screened carriers in a non-ionic detergent solution (e.g., Liquinox) for 2-4 hours to
- 171 degrease and then rinse thoroughly in deionized water. Gently wipe with a clean lint free
- 172 cloth and allow to completely dry.
- 173 d. Steam sterilize carriers in glass petri dishes lined with filter paper prior to use.
- 174 e. Prepare at least one additional carrier for sterility assessment.

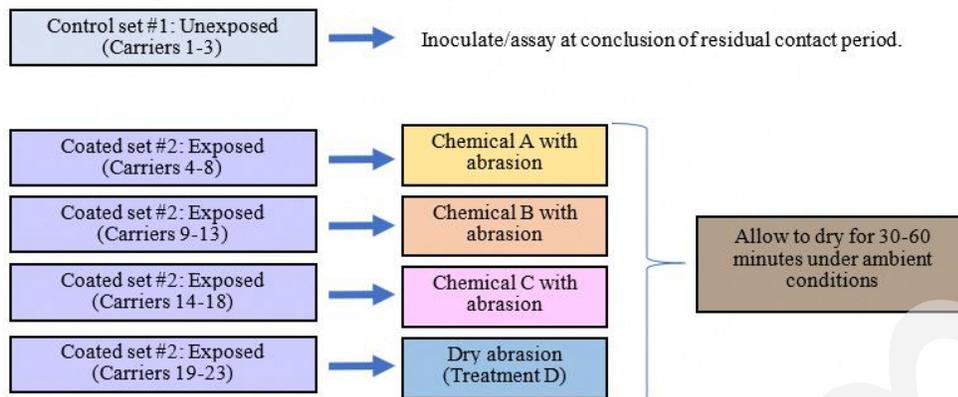
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176 **3) Chemical Exposure and Abrasion Treatment Process**

177  
178 **Figure 1.** Chemical treatment/abrasion and controls for carriers of lot #1 (representing the  
179 chemical exposure/abrasion for 1 cycle)



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182 **Figure 2.** Chemical treatment/abrasion and controls for carriers of lot #2 (representing the  
 183 chemical exposure/abrasion for 1 cycle)



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 186 a. Product (coating) application.
- 187 i. Apply coating to sterile screened carriers (except control sets) with brushed side-  
 188 up the day before the assay using a process consistent with label directions for use  
 189 (according to an approved EPA label or proposed label) and allow carriers to  
 190 completely dry overnight under ambient conditions; document ambient  
 191 temperature and relative humidity.
    - 192 1. Maintain lot identity throughout the testing process.
  - 193 ii. Following treatment and drying, transfer carriers into sterile petri plates. Do not  
 194 use carriers on which the coating coverage is not complete.
  - 195 iii. Define parameters for coating application (spray distance, application coverage,  
 196 application time, etc.) in the study report.
  - 197 iv. Provide digital evidence of what the coated surface looks like after application  
 198 and drying for both lots.
- 199 b. Sponge preparation.
- 200 i. Sterilize sufficient number of sponges for each abrasion treatment (A, B, C and  
 201 D); one sponge per 150 mm glass petri dish for sterilization.
  - 202 ii. Steam sterilize on gravity cycle for 20 minutes. Allow to dry uncovered in the  
 203 BSC overnight before proceeding.
  - 204 iii. With the sponge side down, dispense 20 mL of one test solution (A, B or C) into  
 205 the sterile petri dish with the sponge; allow wetted sponge to stand covered in the  
 206 sterile petri dish for a minimum of 10 minutes. Initiate testing and use wetted  
 207 sponge within 1 hour of application.
    - 208 1. Repeat for each of the three test solutions (A, B or C).
    - 209 2. Apply test solution once per five abrasion cycles; replace treated sponge  
 210 following the fifth abrasion cycle.
    - 211 3. At least one sterile sponge is not wetted and is used for dry abrasion.
- 212 c. Chemical Exposure and Abrasion
- 213 i. Perform the chemical exposure/abrasion with the Gardco, Model D10V abrasion  
 214 tester or comparable equipment. Consult the owner's manual to ensure proper set  
 215 up, operation, maintenance, and calibration.
  - 216 ii. Calibrate instrument to achieve 2-2.5 seconds for one single pass (horizontal  
 217 movement of the abrasion boat.).

- 218 1. 16-20 seconds per abrasion cycle for Treatments A, B and C (8 single  
 219 passes).  
 220 2. 32-40 seconds per abrasion cycle for Treatment D (16 single passes).  
 221 iii. The Gardco sponge abrasion boat with weight weighs approximately 454 g; use  
 222 comparable devices with comparable weight.  
 223 1. Use weight on top of the sponge boat for Treatments A, B, and C.  
 224 2. Use the sponge abrasion boat without the weight for Treatment D.  
 225 iv. Aseptically apply sponge to sponge holder of the abrasion unit (e.g., use ethanol-  
 226 treated or sterile gloves) as specified in the manual so that it fits snugly; cut  
 227 sponge as necessary to fit into the sponge boat.  
 228 1. The sponge must extend a minimum of 5 mm beyond the rim of the  
 229 sponge boat; spacer material such as a foam pad, additional sterile dry  
 230 sponge, or other material may be used to achieve the correct set-up (see  
 231 Figure 3).  
 232

233 **Figure 3.** Proper sponge placement



- 234  
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 236 v. Treat abrasion platform with ethanol (e.g., 70% ethanol) and allow to air dry prior  
 237 to use and in between cycles.  
 238 vi. Load carriers onto abrasion instrument. Orient individual carriers with the coated  
 239 brushed surface side-up. Maintain this orientation throughout the exposure  
 240 treatment. For the control carriers, orient the carrier with the brushed surface side-  
 241 up. Situate carriers from Control Set #2 (3 carriers) and Coated Set #2 (5 carriers)  
 242 in parallel with one another for abrasion, see Figure 4.  
 243 1. Use one sponge boat per carrier set.  
 244 2. Do not adhere carriers to the abrasion instrument; plastic templates with  
 245 cut-outs, magnets, or other means may be used to hold the carriers in place  
 246 during abrasion process.  
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258 **Figure 4.** Recommended abrasion process – carrier configuration.



One sponge boat

Two sponge boats

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- vii. Conduct chemical exposure/abrasion cycle for a single chemical exposure/abrasion treatment (Treatments A, B, and C) using a wetted sponge in the sponge boat of an abrasion unit to perform eight single passes across the carriers.
  - 1. 8 single passes across the surface of the carrier = 1 abrasion cycle for Treatments A, B, and C.
- viii. Conduct the dry abrasion treatment (Treatment D) using a dry sponge in the sponge boat of an abrasion unit without additional weight to perform sixteen single passes across the carriers.
  - 1. 16 single passes across the surface of the carrier = 1 abrasion cycle for Treatment D.
- f. Following the abrasion cycles, aseptically transfer the carrier to its own sterile petri dish ; store carriers under ambient conditions.
- g. Wait 30-60 minutes between each chemical treatment/abrasion exposure cycle.
- h. Perform 10 abrasion cycles (80 single passes across the surface of the carrier for Treatments A, B, and C; 160 single passes across the surface of the carrier for Treatment D) with appropriate dry times between abrasion cycles (see Tables 2 and 3) on Lot 1 to support a 1-week residual claim. The number of abrasion cycles performed can be adjusted for residual claims of up to a maximum of 4 weeks.

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- O o et of carriers for Control Set #1: Unexposed and Coated Set #1:
- n n Unexposed are necessary over the 10 abrasion cycles.
- l e Replace treated sponge following the fifth abrasion cycle; discard sponge daily.
- y s Replace the non-wetted sterile sponge following the fifth abrasion cycle.
- 285 iv. All 10 abrasion cycles must be started and completed within 5 consecutive days.

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295 **Table 2.** Durability regimen for one exposure/abrasion treatment\*

<b>Chemical Exposure/Abrasion**</b>				
Cycle 1	8 single passes	→	Dry	→ Proceed with Cycle 2
Cycle 2	8 single passes	→	Dry	→ Proceed with Cycle 3
Cycle 3	8 single passes	→	Dry	→ Proceed with Cycle 4
Cycle 4	8 single passes	→	Dry	→ Proceed with Cycle 5
Cycle 5	8 single passes	→	Dry	→ Proceed with Cycle 6
Cycle 6	8 single passes	→	Dry	→ Proceed with Cycle 7
Cycle 7	8 single passes	→	Dry	→ Proceed with Cycle 8
Cycle 8	8 single passes	→	Dry	→ Proceed with Cycle 9
Cycle 9	8 single passes	→	Dry	→ Proceed with Cycle 10
Cycle 10	8 single passes	→	Dry/rinse	<b>Efficacy evaluation</b>

296  
297 \*Repeat for each exposure/abrasion treatment (test solutions A, B, and C)

298 \*\*Each cycle (8 single passes) takes 16-20 seconds.

299

300 **Table 3.** Durability regimen for one dry abrasion treatment (Treatment D)

<b>Dry Abrasion*</b>				
Cycle 1	16 single passes	→	Dry	→ Proceed with Cycle 2
Cycle 2	16 single passes	→	Dry	→ Proceed with Cycle 3
Cycle 3	16 single passes	→	Dry	→ Proceed with Cycle 4
Cycle 4	16 single passes	→	Dry	→ Proceed with Cycle 5
Cycle 5	16 single passes	→	Dry	→ Proceed with Cycle 6
Cycle 6	16 single passes	→	Dry	→ Proceed with Cycle 7
Cycle 7	16 single passes	→	Dry	→ Proceed with Cycle 8
Cycle 8	16 single passes	→	Dry	→ Proceed with Cycle 9
Cycle 9	16 single passes	→	Dry	→ Proceed with Cycle 10
Cycle 10	16 single passes	→	Dry	<b>Efficacy evaluation</b>

301  
302 \*Each cycle (16 single passes) takes 32-40 seconds.

303

- 304 i. Following the number of chemical exposure/abrasions corresponding to the duration of  
305 residual activity requested on the label, individually and gently rinse all carriers exposed  
306 to Treatments A, B, and C for 3-5 seconds with sterile DI water three times using a sterile  
307 squirt bottle. Do not rinse carriers for Treatment D.
- 308 j. Transfer each carrier to its own individual petri dish, air dry in the BSC (lids ajar for  
309 drying), and store at room temperature in covered individual petri dishes lined with filter  
310 paper.
- 311 k. After drying, note any changes to the surface characteristics of the carrier (e.g., flaking,  
312 removal, discoloration of the coating).
- 313 l. Include all carrier storage conditions (temperature and humidity range) in the study  
314 report.
- 315 m. Initiate product performance testing within 7 days of completion of the final chemical  
316 exposure/abrasion process.

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321 **4) Preparation of Test Culture**

- 322 a. Refer to Appendix A for preparation of the frozen stock cultures.
- 323 b. Defrost a cryovial rapidly to avoid loss in the viability of the preserved cells. Each
- 324 cryovial is single use only.
- 325 c. Add 100  $\mu$ L of defrosted stock culture to 10 mL TSB, briefly vortex mix and incubate for
- 326 18-24 h at  $36\pm 1^\circ\text{C}$ . In addition, inoculate an agar plate (e.g., TSA or TSA with 5% sheep
- 327 blood) with a loopful from the inoculated tube and streak for isolation. Incubate plate
- 328 with the test culture and examine for purity.
- 329 d. Following incubation, use the broth cultures to prepare a test suspension for each
- 330 organism.
- 331 e. For *P. aeruginosa*, inspect culture prior to harvest; discard if pellicle has been disrupted
- 332 (fragments in culture). Remove visible pellicle on surface of medium and around
- 333 associated interior edges of the tube by pipetting or with vacuum suction. Using a
- 334 serological pipette, withdraw the remaining broth culture (approx. 7-8 mL) avoiding any
- 335 sediment on the bottom of the tube and transfer it into a 15 mL centrifuge tube.
- 336 Alternatively, the culture may be removed by gently aspirating the broth away from the
- 337 pellicle material.
- 338 f. For *S. aureus*, briefly vortex the 18-24 h culture and transfer to a 15 mL centrifuge tube.
- 339 g. Centrifuge the 18-24 h broth cultures at 5,000 g for  $20\pm 5$  min.
- 340 h. Remove the supernatant without disrupting the pellet. Re-suspend the pellet in a
- 341 maximum of 10 mL PBS. Resuspension of the pellet in a smaller volume (e.g., 5 mL) is
- 342 permissible to concentrate culture.
- 343 i. For *S. aureus*, disrupt the pellet using vortex mixing or repetitive tapping/striking
- 344 against a hard surface to disaggregate the pellet completely prior to re-suspending
- 345 it in a maximum of 10 mL PBS. If necessary, add 1 mL of PBS to the pellet to aid
- 346 in the disaggregation.
- 347 ii. For efficacy testing, further dilute the resuspended culture as necessary in PBS to
- 348 achieve a mean control carrier count level of 4.0-5.0 logs CFU/carrier for *S.*
- 349 *aureus* and *P. aeruginosa*.
- 350 i. Use the diluted culture to prepare the final test suspension with the addition of the soil
- 351 load.
- 352 i. Vortex-mix the test suspension for 10-30 seconds.
- 353 ii. To obtain 500  $\mu$ L of the final test suspension with soil load, vortex each
- 354 component and combine the following:
- 355 1. 25  $\mu$ L BSA stock
- 356 2. 35  $\mu$ L yeast extract stock
- 357 3. 100  $\mu$ L mucin stock
- 358 4. 340  $\mu$ L microbial test suspension.
- 359 iii. Use final test suspension with soil load to inoculate carriers within 30 minutes of
- 360 preparing.
- 361 iv. Vortex-mix the final test suspension for 10 seconds following the addition of the
- 362 soil load and also immediately prior to use; use the final test suspension within 30
- 363 min for carrier inoculation.
- 364 j. Streak inoculate an agar plate (TSA or TSA with 5% sheep blood) with a loopful of the
- 365 final test suspension. Incubate at  $36\pm 1^\circ\text{C}$  for  $48\pm 4$  hours and visually examine for purity.
- 366 The purity plate should be free of contamination.

367 k. Optical density/absorbance (at 650 nm) may be used as a tool to monitor/adjust the  
368 diluted test suspension.

369369

**370 5) Neutralization Assay**

- 371 a. Perform the neutralization assay with both microbes prior to testing to demonstrate the  
372 neutralizer's ability to inactivate the residual antimicrobial coating. The neutralization of  
373 the coated carriers is confirmed in triplicate by using coated carriers, the neutralizer  
374 (without carriers) as in the test procedure, and PBS (used to compare counts from the  
375 neutralizer and coated carriers).
- 376 b. Select a neutralizing medium that is not inhibitory to the test microbe. The acceptance  
377 criteria for acceptable neutralization is  $\leq 50\%$  difference in colony counts between the  
378 coated carriers, the neutralizer-only treatment, and the PBS treatment.
- 379 c. Prepare test culture per Section 4b-h.i: Preparation of Test Culture.
- 380 i. Dilute test suspension in PBS so that the average challenge will be 20-200 CFU  
381 per 0.1 mL.
- 382 ii. Prepare the diluted test suspension with the soil load per Section 4i.ii.
- 383 d. In triplicate (x3), add a coated carrier (one per market relevant lot) to a tube of neutralizer  
384 solution (20 mL or other appropriate volume in a 50 mL conical tube); vortex-mix for 30  
385 seconds on highest vortex setting.
- 386 e. Immediately add 0.1 mL of a diluted suspension with the soil load yielding 20-200 CFU.  
387 Vortex to mix.
- 388 i. More than one dilution of the suspension with the soil load may be utilized to  
389 meet the 20-200 CFU/0.1 mL target concentration.
- 390 f. Hold the inoculated mixtures for 10 minutes at room temperature, then vortex-mix.
- 391 g. Filter entire contents of each 50 mL conical tube onto a PES filter membrane and plate on  
392 TSA plates (or TSA with 5% sheep blood).
- 393 h. Incubate plates at  $36\pm 1^\circ\text{C}$  for 48-72 h. Monitor plates after 24 h of incubation to facilitate  
394 appropriate timing for counting the colonies.
- 395 i. Following incubation, count the number of colonies and record.

396396

**397 6) Performance Assessment – Efficacy**

- 398 a. In preparation for efficacy testing, it is advisable to determine the appropriate dilution of  
399 the test suspension that will ensure control counts in the appropriate range after drying by  
400 inoculating uncoated control carriers, placing them in the environmental chamber for 1-2  
401 hours (relative to the target contact time), and determining the counts per carrier. Adjust  
402 the inoculum as necessary to achieve the target control counts for efficacy testing (4.0 to  
403 5.0 logs/carrier).
- 404 b. Within 7 days of completing the chemical exposure/abrasion cycles, conduct efficacy  
405 testing on all coated and control carriers.
- 406 c. Efficacy Test Procedure
- 407 i. Prepare test culture per Section 4: Preparation of Test Culture to achieve a final  
408 target control count on Control Set #1 and Control Set #2 (Lot #1) and Control  
409 Set #1 (Lot #2) of 4.0-5.0 logs CFU/carrier after 1-2 hours (relative to the target  
410 contact time).

- 411 ii. Set environmental chamber to achieve 22±2°C and 30-40% relative humidity  
412 during the 1-2 hour contact period; record temperature and humidity over the  
413 contact period.
- 414 iii. Record the time for all timed events.
- 415 iv. Inoculate each carrier with 20 µL of final test culture using a calibrated  
416 micropipette suitable to deliver 20 µL. Spread the inoculum to within 1/8 inch of  
417 the edge of each carrier, using a sterile transfer loop or the pipette tip. Place in  
418 environmental chamber within 10 minutes of inoculation.
- 419 v. Allow carriers to remain in a flat, horizontal position in individual petri dishes  
420 with the lid on in the environmental chamber for 1-2 hours. Refer to Appendix B  
421 for picture of dried inoculum on carrier.
- 422 vi. Following the contact time, sequentially and aseptically transfer each carrier to a  
423 50 mL conical tube containing 20 mL (or other appropriate volume) of the  
424 appropriate neutralizer solution. Remove and neutralize all carriers within 5  
425 minutes of the contact time.
- 426 1. The tube with the 20 mL neutralizer and the carrier represents the 10<sup>0</sup>  
427 dilution.
- 428 vii. After all the carriers have been transferred into the neutralizer, vortex-mix for 30  
429 seconds then sonicate for 5 minutes±30 seconds at 45 Hz to suspend any  
430 surviving organism in the neutralizer.
- 431 viii. Process coated carriers first and control carriers last.
- 432 ix. Initiate serial dilutions of the neutralizer tubes in PBS within 30 minutes.
- 433 x. Initiate filtration within 30 minutes of preparing dilutions.
- 434 xi. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS.
- 435 xii. Apply vacuum to filter contents; leave the vacuum on for the duration of the  
436 filtration process.
- 437 xiii. Use separate PES membrane filters for each eluate; however, the same filtration  
438 unit may be used for processing eluates from a given carrier set starting with the  
439 most dilute sample first.
- 440 xiv. Pour the eluate into the filter unit. Rinse tubes (conical tube and/or dilution blank)  
441 once with ~10 mL PBS, briefly vortex-mix, and pour into filter unit.
- 4424 7 xv.xvi. xvii.

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S h ents of the filter unit and quickly filter with limited pooling of liquid in the filter  
w e apparatus.  
i c Rinse the inside of the surface of the funnel unit with ~20 mL PBS and filter  
r o contents.  
l n Aseptically remove the membrane filter and place onto TSA. Avoid trapping any  
t t air bubbles between the filter and agar surface.  
448 xviii. Filter appropriate dilutions which yield countable numbers (up to 200 CFU per  
449 plate).  
450 xix. Incubate plates from Control Set #1 at  $36\pm 1^{\circ}\text{C}$  for  $48\pm 4$  h and plates from Control  
451 Set #2, Coated Set #1, and Coated Set #2 for  $72\pm 4$  h; incubate plates with no  
452 growth an additional  $48\pm 4$  h and count the number of colonies.  
453 1. Monitor filters after 24 h of incubation to facilitate appropriate timing for  
454 counting the colonies.  
455 2. Plates with  $>200$  CFU result in TNTC.

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- 456 xx. If isolated colonies are present, perform a Gram stain to assess one representative  
 457 colony per carrier set (Control Set #1, Control Set #2, Coated Set #1, Coated Set  
 458 #2).  
 459 xxi. If confluent growth is present, perform a streak isolation on the appropriate agar  
 460 on growth taken from at least 1 carrier.  
 461 xxii. If additional verification of the test organism is required, perform further  
 462 confirmatory analyses (e.g. VITEK or biochemical analyses) and isolation streaks  
 463 on selective media.  
 464

465 **7) Study Controls**

- 466 a. The results of the purity controls (section 4.c. and 4.j.) must be consistent with  
 467 characteristics in Table 6.  
 468 b. Carrier Sterility Control:  
 469 i. Add one sterile uncoated carrier to a tube containing 10 mL of TSB. Incubate at  
 470  $36\pm 1^\circ\text{C}$  for  $48\pm 4$  h and examine for growth. The acceptance criterion for this  
 471 study control is lack of turbidity in each tube.  
 472 c. Neutralizer Assay Control:  
 473 i. Add 1 mL of neutralizer into 9 mL of TSB and visually examine for growth after  
 474 incubation at  $36\pm 1^\circ\text{C}$  after  $48\pm 4$  hours. The acceptance criterion is lack of growth.  
 475

476 **8) Calculations/Data Analysis**

- 477 a. Use values with at least three significant figures when performing calculations (e.g., log  
 478 density, mean log density). Report the final log reduction and differences in log densities  
 479 with two significant figures.  
 480 b. Calculate the Colony Forming Units (CFU)/carrier using the following equation:

481 
$$\text{Log}_{10} \frac{\sum_{i=1}^n (r_{ii})}{\sum_{i=1}^n (C_{ii} \times D_{ii})} \times V \times W$$

482 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).

- 483  
 484 c. Calculate the mean log density (LD) of viable cells for each microbe for the carrier sets in  
 485 Lot 1 [Control Set #1, Control Set #2 (per chemical exposure/abrasion treatment, 3 total),  
 486 Coated Set #1, Coated Set #2 (per chemical exposure/abrasion treatment, 4 total)] as  
 487 follows:

488 
$$\text{Mean LD} = \frac{\sum \text{Log}_{10}(\text{CarrCCer } 1 + \text{CarrCCer } 2 + \dots + \text{CarrCCer } XX)}{XX}$$
, where "X" refers to the total  
 489 number of carriers assayed:

- 490 d. Calculate the mean LD of viable cells for each microbe for the carrier sets in Lot 2  
 491 [(Control Set #1 and Coated Set #2 (per chemical exposure/abrasion treatment, 4 total)  
 492 using the above equation.  
 493 e. When TNTC (Too Numerous to Count) values are observed for each dilution filtered,  
 494 substitute 200 for the TNTC at the highest (most dilute) dilution and account for the  
 495 dilution factor in the calculation.  
 496 f. See Table 4 for additional calculations for Lot #1 and Table 5 for additional calculations  
 497 for Lot #2.  
 498  
 499

**Table 4.** Additional calculations for Lot #1

<b>LOG DIFFERENCE BETWEEN CONTROL SETS</b>		<b>Outcome (Difference)</b>
Difference between Control Set #1 and Control Set #2	Mean LD Control Set #1 – Mean LD Control Set #2: Solution A	≤ 0.5
	Mean LD Control Set #1 – Mean LD Control Set #2: Solution B	≤ 0.5
	Mean LD Control Set #1 – Mean LD Control Set #2: Solution C	≤ 0.5
<b>LOG REDUCTION CALCULATIONS</b>		<b>Outcome (LR)</b>
LR Coated Set #1	Mean LD Control Set #1 – Mean LD Coated Set #1	≥ 3.0
LR Coated Set #2	Mean LD Control Set #1 – Mean LD Coated Set #2: Solution A	≥ 3.0
	Mean LD Control Set #1 – Mean LD Coated Set #2: Solution B	≥ 3.0
	Mean LD Control Set #1 – Mean LD Coated Set #2: Solution C	≥ 3.0
	Mean LD Control Set #1 – Mean LD Coated Set #2: Dry abrasion	≥ 3.0
<b>LOG DIFFERENCE BETWEEN COATED SETS</b>		<b>Outcome (Difference)</b>
Difference between Coated Set #1 vs. Coated Set #2	Mean LR Coated Set #1 – Mean LR Coated Set #2: Solution A	≤ 1.0
	Mean LR Coated Set #1 – Mean LR Coated Set #2: Solution B	≤ 1.0
	Mean LR Coated Set #1 – Mean LR Coated Set #2: Solution C	≤ 1.0
	Mean LR Coated Set #1 – Mean LR Coated Set #2: Dry abrasion	≤ 1.0

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