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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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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1) Special Apparatus and Materials

a. Test microbes: *Pseudomonas aeruginosa* (ATCC #15442) and *Staphylococcus aureus* (ATCC #6538)

b. Culture media

i. *Tryptic Soy Agar (TSA) and TSA with 5% sheep blood (BAP).* Used for culturing, isolation, and characterization of the test microbes. Purchase plates from a reputable source or prepare according to manufacturer's instructions.

ii. *Tryptic Soy Broth (TSB).* Used to rehydrate lyophilized cultures and grow overnight cultures. Purchase broth from a reputable source or prepare according to manufacturer's instructions.

iii. *TSB with 15% (v/v) glycerol.* Used as a cryoprotectant solution. Purchase broth from a reputable source or prepare according to manufacturer's instructions.

c. Reagents

i. *Ethanol (e.g., 70%)*. Used to treat abrasion platform.

ii. *De-ionized (DI) Water.* For preparing reagents and media; use sterile DI water for rinsing test solutions off carriers prior to efficacy testing.

iii. *Gram stain kit.* Used for diagnostic staining of *P. aeruginosa* and *S. aureus*.

iv. *Liquinox or equivalent non-ionic detergent solution.* To clean carriers.

v. *Neutralizer.* Various confirmed neutralizers may be used, including letheen broth. If necessary, other ingredients may be added to letheen broth. Purchase letheen broth from a reputable source or prepare according to manufacturer's instructions.

vi. *Phosphate buffered saline stock solution (e.g., 10X).* Use to prepare 1X phosphate buffered saline. The stock solution has a pH of approximately 7.2±0.2.

vii. *Phosphate buffered saline (PBS), 1X.* Use for dilution blanks and filtration. PBS with a pH of approximately 7.0±0.5 is desirable.

viii. *Soil Load.* The soil load to be incorporated in the test suspension is a mixture of the following stock solutions in PBS:

1. BSA: Add 0.5 g bovine serum albumin (BSA) to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter membrane filter, aliquot and store at approximately -20°C.
2. Yeast extract: Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter membrane filter, aliquot and store at approximately -20°C.
3. Mucin: Add 0.04 g mucin (from bovine submaxillary gland or equivalent) to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter PES membrane filter, aliquot and store at approximately -20°C.
4. The stock solutions of the soil load solutions are single use only. Do not refreeze once thawed; store up to one year at -20±2°C.

ix. Solutions used in chemical exposure of carriers:

1. *Solution A*. A 2000±100 ppm sodium hypochlorite (NaOCl) solution (e.g., Sigma-Aldrich reagent grade sodium hypochlorite) prepared in sterile deionized water. Verify the final concentration of the solution using a suitable titration method (e.g., Hach digital titrator).
2. *Solution B*. Use an EPA-registered antimicrobial pesticide product containing hydrogen peroxide (between 3.0% and 6.0%) and peracetic acid as active ingredients that allows liquid application to hard, non-

porous surfaces. The solution concentration for the peracetic acid component is not limited to a defined range.

3. *Solution C*. Use an EPA-registered hospital disinfectant product with quaternary ammonium compound as the active ingredient labeled as a one-step cleaner/disinfectant that allows liquid application to hard, non-porous surfaces.

d. Materials

i. *0.2 µm Polyethersulfone (PES) membrane filters*. For recovery of the test microbe. Filtration units (reusable or disposable) may be used.

ii. *Carriers:* Die/machine cut 1 × 1 inch square made of AISI Type 304 stainless steel with 150 grit unidirectional brushed finish on one side. Carriers are single use. See Appendix B for carrier specifications.

iii. *Conical tubes*. (e.g., 15 mL, 50 mL) Capable of being centrifuged at 5,000 g. Used for neutralization.

iv. *Cryovials*. For storage of frozen stock culture.

v. *Dilution tubes*. Glass/plastic tubes that are used for preparing dilutions.

vi. *Filter paper*. Whatman No. 2, used to line Petri plates.

vii. *Forceps.* Use appropriate tips (smooth or curved) to pick up carriers for placement in conical tubes and for membrane filtration.

viii. *Microcentrifuge tubes*. For storage of soil single use aliquots.

ix. *Cleaning Sponge.* Example: Scotch Brite Non-Scratch Scrub Sponge, item number C05068 or equivalent.

1. Prior to sterilization, cut sponge to fit snugly into sponge boat, sponge-side down.

x. *Spacer Material.* Use to ensure that the sponge extends a minimum of 5 mm beyond the sponge boat (example: foam pad, additional sponge, etc.) so that the sponge boat does not contact the carriers or abrasion unit plate.

xi. *Petri dishes.* Multiple sizes

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

xii. *Serological Pipettes.* (e.g., 10 mL, 25 mL) Used for removing/adding larger volumes of liquid.

xiii. *Sterile Squirt Bottle*. Used to rinse carriers after chemical treatments.

xiv. *Neodymium Magnets (optional).* Place magnets on bottom and top of abrasion platform to hold the carriers in place during abrasion process. Placed at the beginning and end of the abraded carriers.

e. Equipment

i. *-20°C Freezer.* For storage of soil aliquots.

ii. *-80°C Freezer.* For storage of frozen stock cultures.

iii. *Calibrated 20 µL positive displacement pipette.* With corresponding tips for carrier inoculation.

iv. *Calibrated micropipettes*. (e.g., 200 µL, 1 mL) With 20-200 and 100-1000 µL tips. For preparing dilutions.

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

2) Carriers

Two market relevant lots of the test product should be used to evaluate efficacy. The test product is the formulation used to coat the stainless-steel carriers. Lot 1 of test product is used for chemical treatments/abrasion and efficacy testing on exposed and unexposed control and coated carriers; two additional controls are included. Lot 2 is used to compare the unexposed control carriers to the exposed coated carriers only. See Table 1 for a summary of carrier distribution. "Exposed" refers to carriers subjected to the chemical treatment/physical abrasion, while "unexposed" refers to those carriers not subjected to the chemical treatment/physical abrasion. Test carriers are coated with the residual product while controls are uncoated carriers.

Table 1. Carrier distribution

	Carrier Type	# of carriers for *S. aureus*	# of carriers for *P. aeruginosa*
Lot 1	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)
	Total Carriers for Lot #1	**35**	**35**
Lot 2	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)
	Total Carriers for Lot #2	**23**	**23**

*3 chemical exposures with abrasion

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

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

3) Chemical Exposure and Abrasion Treatment Process

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

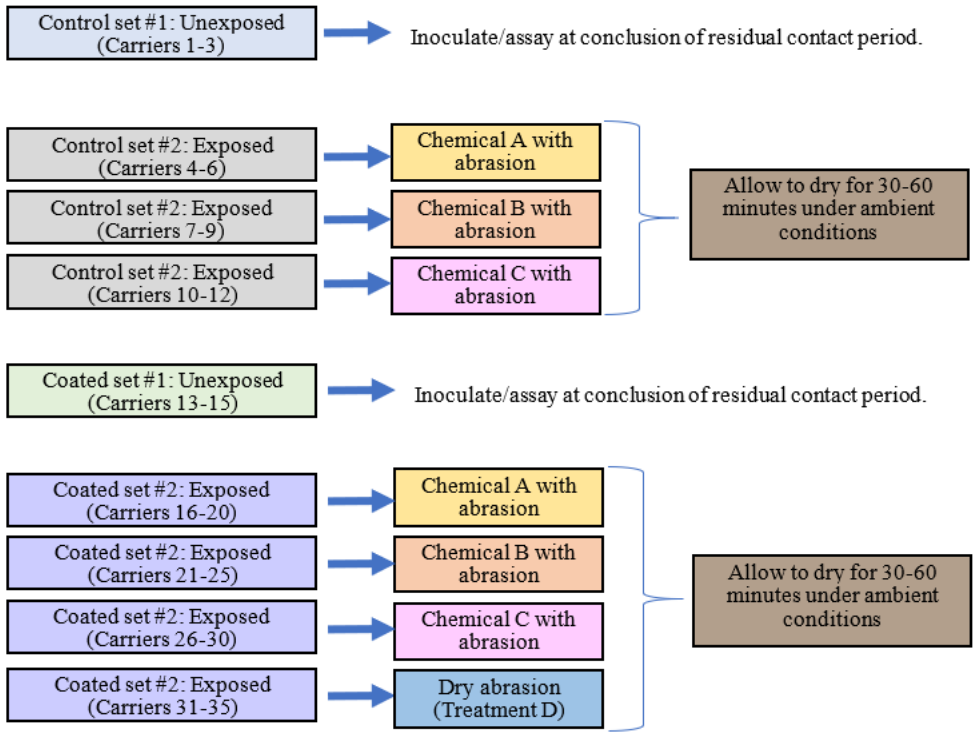
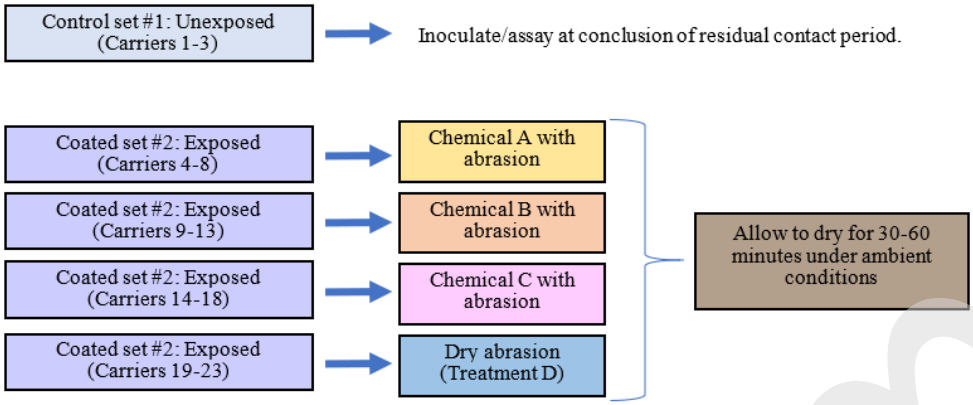


Figure 2. Chemical treatment/abrasion and controls for carriers of lot #2 (representing the chemical exposure/abrasion for 1 cycle)



a. Product (coating) application.
    i. Apply coating to sterile screened carriers (except control sets) with brushed side-up the day before the assay using a process consistent with label directions for use (according to an approved EPA label or proposed label) and allow carriers to completely dry overnight under ambient conditions; document ambient temperature and relative humidity.
        1. Maintain lot identity throughout the testing process.
    ii. Following treatment and drying, transfer carriers into sterile petri plates. Do not use carriers on which the coating coverage is not complete.
    iii. Define parameters for coating application (spray distance, application coverage, application time, etc.) in the study report.
    iv. Provide digital evidence of what the coated surface looks like after application and drying for both lots.

b. Sponge preparation.
    i. Sterilize sufficient number of sponges for each abrasion treatment (A, B, C and D); one sponge per 150 mm glass petri dish for sterilization.
    ii. Steam sterilize on gravity cycle for 20 minutes. Allow to dry uncovered in the BSC overnight before proceeding.
    iii. With the sponge side down, dispense 20 mL of one test solution (A, B or C) into the sterile petri dish with the sponge; allow wetted sponge to stand covered in the sterile petri dish for a minimum of 10 minutes. Initiate testing and use wetted sponge within 1 hour of application.
        1. Repeat for each of the three test solutions (A, B or C).
        2. Apply test solution once per five abrasion cycles; replace treated sponge following the fifth abrasion cycle.
        3. At least one sterile sponge is not wetted and is used for dry abrasion.

c. Chemical Exposure and Abrasion
    i. Perform the chemical exposure/abrasion with the Gardco, Model D10V abrasion tester or comparable equipment. Consult the owner's manual to ensure proper set up, operation, maintenance, and calibration.
    ii. Calibrate instrument to achieve 2-2.5 seconds for one single pass (horizontal movement of the abrasion boat.).

1. 16-20 seconds per abrasion cycle for Treatments A, B and C (8 single passes).
2. 32-40 seconds per abrasion cycle for Treatment D (16 single passes).

iii. The Gardco sponge abrasion boat with weight weighs approximately 454 g; use comparable devices with comparable weight.
1. Use weight on top of the sponge boat for Treatments A, B, and C.
2. Use the sponge abrasion boat without the weight for Treatment D.

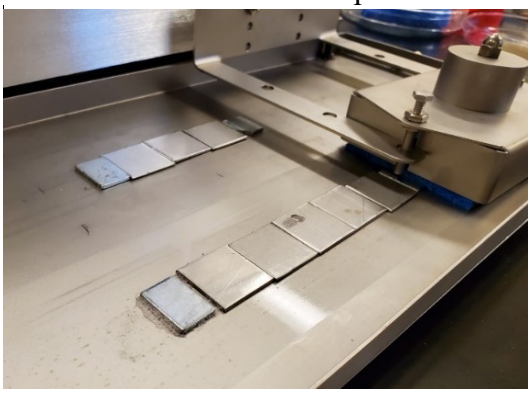
iv. Aseptically apply sponge to sponge holder of the abrasion unit (e.g., use ethanol-treated or sterile gloves) as specified in the manual so that it fits snugly; cut sponge as necessary to fit into the sponge boat.
1. The sponge must extend a minimum of 5 mm beyond the rim of the sponge boat; spacer material such as a foam pad, additional sterile dry sponge, or other material may be used to achieve the correct set-up (see Figure 3).

Figure 3. Proper sponge placement

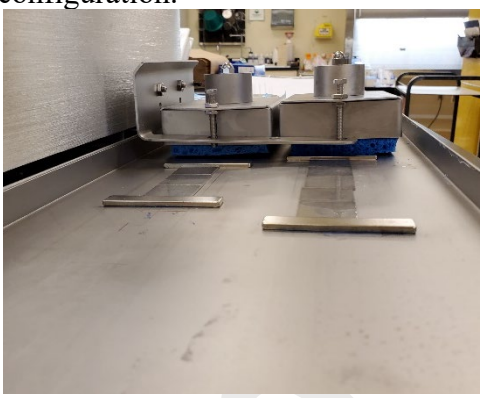
v. Treat abrasion platform with ethanol (e.g., 70% ethanol) and allow to air dry prior to use and in between cycles.

vi. Load carriers onto abrasion instrument. Orient individual carriers with the coated brushed surface side-up. Maintain this orientation throughout the exposure treatment. For the control carriers, orient the carrier with the brushed surface side-up. Situate carriers from Control Set #2 (3 carriers) and Coated Set #2 (5 carriers) in parallel with one another for abrasion, see Figure 4.
1. Use one sponge boat per carrier set.
2. Do not adhere carriers to the abrasion instrument; plastic templates with cut-outs, magnets, or other means may be used to hold the carriers in place during abrasion process.

Figure 4. Recommended abrasion process – carrier configuration.



One sponge boat



Two sponge boats

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.
    i. Only one set of carriers for Control Set #1: Unexposed and Coated Set #1: Unexposed are necessary over the 10 abrasion cycles.
    ii. Replace treated sponge following the fifth abrasion cycle; discard sponge daily.
    iii. Replace the non-wetted sterile sponge following the fifth abrasion cycle.
    iv. All 10 abrasion cycles must be started and completed within 5 consecutive days.

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

Chemical Exposure/Abrasion**				
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 →	**Efficacy evaluation**

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

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

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

Dry Abrasion*				
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 →	**Efficacy evaluation**

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

i. Following the number of chemical exposure/abrasions corresponding to the duration of residual activity requested on the label, individually and gently rinse all carriers exposed to Treatments A, B, and C for 3-5 seconds with sterile DI water three times using a sterile squirt bottle. Do not rinse carriers for Treatment D.

j. Transfer each carrier to its own individual petri dish, air dry in the BSC (lids ajar for drying), and store at room temperature in covered individual petri dishes lined with filter paper.

k. After drying, note any changes to the surface characteristics of the carrier (e.g., flaking, removal, discoloration of the coating).

l. Include all carrier storage conditions (temperature and humidity range) in the study report.

m. Initiate product performance testing within 7 days of completion of the final chemical exposure/abrasion process.

4) Preparation of Test Culture

a. Refer to Appendix A for preparation of the frozen stock cultures.
b. Defrost a cryovial rapidly to avoid loss in the viability of the preserved cells. Each cryovial is single use only.
c. Add 100 µL of defrosted stock culture to 10 mL TSB, briefly vortex mix and incubate for 18-24 h at 36±1°C. In addition, inoculate an agar plate (e.g., TSA or TSA with 5% sheep blood) with a loopful from the inoculated tube and streak for isolation. Incubate plate with the test culture and examine for purity.
d. Following incubation, use the broth cultures to prepare a test suspension for each organism.
e. For *P. aeruginosa*, inspect culture prior to harvest; discard if pellicle has been disrupted (fragments in culture). Remove visible pellicle on surface of medium and around associated interior edges of the tube by pipetting or with vacuum suction. Using a serological pipette, withdraw the remaining broth culture (approx. 7-8 mL) avoiding any sediment on the bottom of the tube and transfer it into a 15 mL centrifuge tube. Alternatively, the culture may be removed by gently aspirating the broth away from the pellicle material.
f. For *S. aureus*, briefly vortex the 18-24 h culture and transfer to a 15 mL centrifuge tube.
g. Centrifuge the 18-24 h broth cultures at 5,000 g for 20±5 min.
h. Remove the supernatant without disrupting the pellet. Re-suspend the pellet in a maximum of 10 mL PBS. Resuspension of the pellet in a smaller volume (e.g., 5 mL) is permissible to concentrate culture.
    i. For *S. aureus*, 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 a maximum of 10 mL PBS. If necessary, add 1 mL of PBS to the pellet to aid in the disaggregation.
    ii. For efficacy testing, further dilute the resuspended culture as necessary in PBS to achieve a mean control carrier count level of 4.0-5.0 logs CFU/carrier for *S. aureus* and *P. aeruginosa*.
i. Use the diluted culture to prepare the final test suspension with the addition of the soil load.
    i. Vortex-mix the test suspension for 10-30 seconds.
    ii. To obtain 500 µL of the final test suspension with soil load, vortex each component and combine the following:
        1. 25 µL BSA stock
        2. 35 µL yeast extract stock
        3. 100 µL mucin stock
        4. 340 µL microbial test suspension.
    iii. Use final test suspension with soil load to inoculate carriers within 30 minutes of preparing.
    iv. Vortex-mix the final test suspension for 10 seconds following the addition of the soil load and also immediately prior to use; use the final test suspension within 30 min for carrier inoculation.
j. Streak inoculate an agar plate (TSA or TSA with 5% sheep blood) with a loopful of the final test suspension. Incubate at 36±1°C for 48±4 hours and visually examine for purity. The purity plate should be free of contamination.

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

5) <u>Neutralization Assay</u>

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

6) <u>Performance Assessment – Efficacy</u>

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

ii. Set environmental chamber to achieve 22±2°C and 30-40% relative humidity during the 1-2 hour contact period; record temperature and humidity over the contact period.

iii. Record the time for all timed events.

iv. Inoculate each carrier with 20 µL of final test culture using a calibrated micropipette suitable to deliver 20 µL. Spread the inoculum to within 1/8 inch of the edge of each carrier, using a sterile transfer loop or the pipette tip. Place in environmental chamber within 10 minutes of inoculation.

v. Allow carriers to remain in a flat, horizontal position in individual petri dishes with the lid on in the environmental chamber for 1-2 hours. Refer to Appendix B for picture of dried inoculum on carrier.

vi. Following the contact time, sequentially and aseptically transfer each carrier to a 50 mL conical tube containing 20 mL (or other appropriate volume) of the appropriate neutralizer solution. Remove and neutralize all carriers within 5 minutes of the contact time.

1. The tube with the 20 mL neutralizer and the carrier represents the 10^0 dilution.

vii. After all the carriers have been transferred into the neutralizer, vortex-mix for 30 seconds then sonicate for 5 minutes±30 seconds at 45 Hz to suspend any surviving organism in the neutralizer.

viii. Process coated carriers first and control carriers last.

ix. Initiate serial dilutions of the neutralizer tubes in PBS within 30 minutes.

x. Initiate filtration within 30 minutes of preparing dilutions.

xi. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS.

xii. Apply vacuum to filter contents; leave the vacuum on for the duration of the filtration process.

xiii. Use separate PES membrane filters for each eluate; however, the same filtration unit may be used for processing eluates from a given carrier set starting with the most dilute sample first.

xiv. Pour the eluate into the filter unit. Rinse tubes (conical tube and/or dilution blank) once with ~10 mL PBS, briefly vortex-mix, and pour into filter unit.

xv. Swirl the contents of the filter unit and quickly filter with limited pooling of liquid in the filter apparatus.

xvi. Rinse the inside of the surface of the funnel unit with ~20 mL PBS and filter contents.

xvii. Aseptically remove the membrane filter and place onto TSA. Avoid trapping any air bubbles between the filter and agar surface.

xviii. Filter appropriate dilutions which yield countable numbers (up to 200 CFU per plate).

xix. Incubate plates from Control Set #1 at 36±1°C for 48±4 h and plates from Control Set #2, Coated Set #1, and Coated Set #2 for 72±4 h; incubate plates with no growth an additional 48±4 h and count the number of colonies.

1. Monitor filters after 24 h of incubation to facilitate appropriate timing for counting the colonies.
2. Plates with >200 CFU result in TNTC.

xx. If isolated colonies are present, perform a Gram stain to assess one representative colony per carrier set (Control Set #1, Control Set #2, Coated Set #1, Coated Set #2).

xxi. If confluent growth is present, perform a streak isolation on the appropriate agar on growth taken from at least 1 carrier.

xxii. If additional verification of the test organism is required, perform further confirmatory analyses (e.g. VITEK or biochemical analyses) and isolation streaks on selective media.

7) Study Controls

a. The results of the purity controls (section 4.c. and 4.j.) must be consistent with characteristics in Table 6.

b. Carrier Sterility Control:

i. Add one sterile uncoated carrier to a tube containing 10 mL of TSB. Incubate at 36±1°C for 48±4 h and examine for growth. The acceptance criterion for this study control is lack of turbidity in each tube.

c. Neutralizer Assay Control:

i. Add 1 mL of neutralizer into 9 mL of TSB and visually examine for growth after incubation at 36±1°C after 48±4 hours. The acceptance criterion is lack of growth.

8) Calculations/Data Analysis

a. Use values with at least three significant figures when performing calculations (e.g., log density, mean log density). Report the final log reduction and differences in log densities with two significant figures.

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

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

where:

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).

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

$$Mean\ LD = \sum \frac{\mathrm{Log}_{10}(Carrier\ 1 + Carrier\ 2 + \ldots + Carrier\ X)}{X}$$, where "X" refers to the total number of carriers assayed:

d. Calculate the mean LD of viable cells for each microbe for the carrier sets in Lot 2 [(Control Set #1 and Coated Set #2 (per chemical exposure/abrasion treatment, 4 total) using the above equation.
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 calculation.
f. See Table 4 for additional calculations for Lot #1 and Table 5 for additional calculations for Lot #2.

Table 4. Additional calculations for Lot #1

LOG DIFFERENCE BETWEEN CONTROL SETS		**Outcome (Difference)**
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
LOG REDUCTION CALCULATIONS		**Outcome (LR)**
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
LOG DIFFERENCE BETWEEN COATED SETS		**Outcome (Difference)**
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

502 **Table 5.** Additional calculations for Lot #2

LOG REDUCTION CALCULATIONS		Outcome (LR)
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

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Appendix A

Preparation of Frozen Stock Culture

1. Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from ATCC (or other 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. 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±1°C for 24±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 6. Incubate all plates for 24±2 h at 36±1°C.
    a. Record results at the end of the incubation timeframe. Refer to Table 6 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±2 h at 36±1°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 6).
    b. Incubate all plates for 24±2 h at 36±1°C. Record results. Refer to Table 6 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 6).
    b. Incubate all plates for 24±2 h at 36±1°C.
    c. Record results.

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 Vitek confirmation from growth taken from the BAP. Conduct VITEK according to the manufacturer's instructions.
f. Record all confirmation results.

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 6 at any step in the process, or the Vitek profile is inconsistent with the organism, discard the cultures and re-initiate the process.

Table 6. Selective media and diagnostic characteristics for *P. aeruginosa* and *S. aureus*

Aspect	***P. aeruginosa****	***S. aureus***
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
	Typical Microscopic Characteristics	
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 B

Carrier Specifications

General Description: 1 × 1 inch square made of AISI Type 304 Stainless Steel (SS) with 150 grit unidirectional brushed finish on one side.

Material: AISI Type 304 Austensic stainless steel consisting of 18% to 20% Chromium, 8% to 10.5% Nickel, and a maximum of 0.8% Carbon.

- European Specification X5CrNi18-10 Number 1.4301
- Japanese Specification: JIS 4303 SUS 304

Carrier Dimensions:

- 1 inch by 1 inch square
- Stainless Steel Sheet Thickness: 22 gauge; carrier manufacturer will provide thickness of the original stainless steel sheet (in mm).
- Flatness: Carrier height not to exceed 110% of the thickness of the uncut sheet of stainless steel from which the carriers are manufactured.

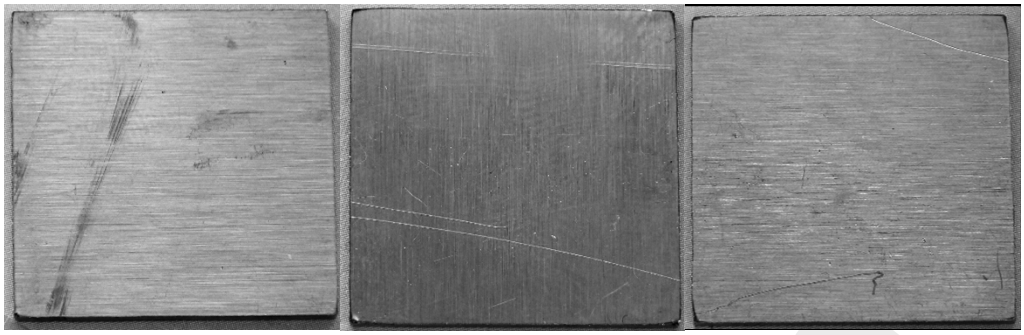
Finish: A ground unidirectional finish obtained with 150 grit abrasive (AISI) on the top side of the stainless steel sheet.

Burr Removal: Remove burrs from the edges of the discs on the bottom side of the carrier using a manual process.

Passivation: Parts are passivated by the carrier manufacturer according to ASTM A967 in a citric acid solution and prepared as follows:

- Degrease with citrus-based degreaser by soaking in the degrease solution for 1 hour
- Rinse with de-ionized water
- Passivate by soaking carriers:
    - 7% citric acid solution
    - 20-30 min at 35±5°C.
- Rinse with de-ionized water
- Air dry

Examples of Failed Physically Screened Carriers



Example of a Dry Inoculated Carrier