US Environmental Protection Agency
Office of Pesticide Programs

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Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Test Method for Evaluating the Efficacy of Antimicrobial Surface Coatings

SOP Number: MB-40-00

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Title	Test Method for Evaluating the Efficacy of Antimicrobial Surface Coatings
Scope	Describes the methodology used for evaluating the durability and efficacy of coated surfaces against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and viruses.
Application	The methodology described in this SOP is limited to antimicrobial surface coating used on hard, non-porous surfaces against the prescribed test microbes.

	Approval	Date
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Test Method for Evaluating the Efficacy of Antimicrobial Surface Coatings

Scope

The Environmental Protection Agency (EPA) Office of Pesticide Programs (OPP) recommends that applicants utilize this test 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 test method includes an efficacy assessment of the coated coupons following exposure to certain chemical solutions/mechanical abrasion. The test method provides guidance for the evaluation of durability and the baseline efficacy of these coated surfaces against *Staphylococcus aureus, Pseudomonas aeruginosa*, and viruses; the test method can be adapted for additional organisms. A minimum 3 log reduction of test organisms within 1-2 hours is the required level of performance. This test method is a revised version of the interim method posted on 10/26/2020 (EPA-HQ-OPP-2020-0529).

Method Overview

In brief, the test method is comprised of two parts: 1) chemical treatment and abrasion, and 2) product efficacy. The test method uses 1" × 1" brushed stainless-steel carriers coated with the residual antimicrobial chemical and uncoated control carriers. To support a 1-week residual claim, sets of carriers are abraded using 5 cycles of alternating wet (with liquid treatment) and dry (no liquid treatment) abrasion for each of three prescribed liquid treatments; in addition, carriers are abraded using an independent 10 cycle dry abrasion treatment. 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 residual surface coatings to three different biocidal materials (chemical solutions) and the impact of dry abrasion. Following completion of chemical exposure and abrasion, 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 abraded carriers is calculated in relation to the viable test organisms on the unabraded control carriers. The impact of the chemical exposure and abrasion on product efficacy is also determined by comparing unabraded coated and uncoated carriers.

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).

Table of Contents

1. Terminology

a. *Abraded carriers, n*—stainless steel carriers that are exposed to chemical exposure and abrasion cycles via a cleaning sponge on the surface of the abrasion tester tray.

b. *Coated carriers, n*—stainless steel carriers with residual antimicrobial product present on the surface.

c. *Control, n*—uncoated carriers with no residual product present

d. *Cycle (abrasion cycle), n*—a series of 4 sequential, single passes across the surface of carriers.

e. *Dry Abrasion, n*—abrasion cycles performed using a dry sponge in a sponge box (with weight) followed by a minimum 5 min hold time before proceeding to the next cycle.

f. *Min, n*—minutes.

g. *Pass, n*—within an abrasion cycle, one movement across the surface of the carriers.

h. *S, n*—seconds.

i. *Unabraded carriers, n*—stainless steel carriers that are not exposed to chemical exposure and abrasion cycles via a cleaning sponge on the surface of the abrasion tester tray.

j. *Uncoated carriers, n*—stainless steel carriers with no residual antimicrobial product present on the surface.

k. *Wet abrasion, n*—abrasion cycles performed using a wetted sponge (treatments A, B, or C) in a sponge box (with weight) followed by a minimum 10 min dry time before proceeding to the subsequent dry component of the two-component cycle.

2. Apparatus

a. *-20°C Freezer.* Used for storage of soil aliquots.

b. *-80°C Freezer.* Used for storage of frozen stock cultures.

c. *0.2 µm Pore Diameter Polyethersulfone (PES) Membrane Filters, 47 mm diameter*. Used for recovery of the test microbe (bacteria). Filtration units (reusable or disposable) may be used.

d. *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 A for carrier specifications and passivation instructions.

e. *Centrifuge* (with rotor capable of achieving 5,000 g). Used for bacterial test culture preparation.

f. *Certified timer*. Readable in minutes and seconds, used for tracking of timed events and intervals.

g. *Cleaning Sponge.* Scotch-Brite™ General Purpose Scouring Sponge #74 or equivalent highly conformable web laminate with a cellulose sponge backing. Minimum dimensions: 10 cm x 8 cm x 1.5cm. Used for the abrasion process.

i. Prior to sterilization, cut sponge to fit snugly into sponge box (10 cm x 8 cm).

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

i. *Cryovials*. Used for storing frozen stock cultures.

j. *Dilution tubes* (glass/plastic tubes). Used for preparing serial dilutions.

k. *Environmental chamber*. Used for efficacy testing to hold carriers during microbe contact time at 21±3°C and 30-40% relative humidity.

l. *Filter paper* (Whatman No. 2). Used to line Petri dishes associated with carriers.

m. *Forceps, sterile.* Used to handle carriers and membrane filters.

n. *Gardco Model D10V or comparable abrasion instrument*. Used for simulating wear on carriers.

o. *Identification system (optional)*. Used for appropriate identification of test microbes (e.g., VITEK identification system).

p. *Incubator.* Used to incubate test cultures and growth medium plates at 36±1°C.

q. *Incubator with 5% CO_2*. Used for growing cell lines and viruses (when applicable).

r. *Liquid nitrogen dewar or comparable freezer capable of long-term storage of cell lines.* Used for storing viral cell lines.

s. *Microcentrifuge tubes*. Used for storage of single-use soil aliquots.

t. *Micropipettes, calibrated*. Used with corresponding tips for preparing dilutions.

u. *Microscope (e.g., 100X optics and 10X ocular)*. Used for microbial observation.

v. *Neodymium magnets (optional).* Used for securing carriers in place on abrasion tester tray during chemical exposure and abrasion treatments.

w. *Petri dishes.* Multiple sizes:

i. 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.

ii. 150 mm glass/plastic dishes. Glass dishes used to sterilize sponges; plastic or glass dishes used to hold hydrated sponges during the test day.

x. *Positive displacement pipette, capable of delivering 20 µL, calibrated.* Used with corresponding tips for carrier inoculation.

y. *Refrigerator (2-8°C)*. Used for storage of media and post-incubated plates.

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

aa. *Sonicator* (capable of producing 45 Hz*)*. Used for removal of organism from carriers. If necessary, verify the sonicator to determine the impact of sonication on the culture by placing the standardized broth culture into sonicator for 5 min, serially dilute, and

recover. Compare sonicated counts to a non-sonicated control. The sonicated and non-sonicated counts should be comparable.

bb. *Spacer Material.* Used in sponge box to ensure that cleaning sponge extends a minimum of 5 mm beyond the outer edge of the sponge box (e.g., foam pad, additional sponge, etc.) to prevent the sponge box from contacting the carriers or abrasion unit plate.

cc. *Sponge box with weight*. To achieve total weight of approximately 454 g (sponge box, 224 g + weight, 230 g = approx. 454 g (without sponge or spacer material)).

i. Use weight (approximately 230 g) for Treatments A, B, C, and D.

dd. *Sterile Squirt Bottle*. Used for rinsing carriers after chemical exposure and abrasion treatments.

ee. *Template for use with Abrasion Tester Tray (optional).* Used for securing carriers in place on Abrasion tester tray during chemical exposure and abrasion treatments. See Appendix B for specifications. Brushed carrier surface should be at or slightly above template recesses.

ff. *Titration kit (e.g., Hach digital titrator)*. Used for measuring total chlorine.

gg. *Vacuum source* (in-house line or suitable vacuum pump). Used to facilitate rapid membrane filtration.

hh. *Vortex*. Used for vortex mixing of various solutions including carriers.

3. Bacterial Reagents

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

b. Culture media for *P. aeruginosa* and *S. aureus*.

i. *10% (w/v) dextrose solution.* Used as a supplement to synthetic broth. Add 5.0 g dextrose to 50 mL de-ionized water (to bring to volume in a volumetric flask) and mix by stirring. Filter sterilize the solution using a 0.2 µm filter. Store the sterile solution at 2-8°C for up to 30 days.

ii. *Phosphate buffered saline stock solution (e.g., 10X).* Used for preparing 1X phosphate buffered saline. The stock solution has a pH of approximately 7.2±0.2.

iii. *Phosphate buffered saline (PBS), 1X.* Used for dilution blanks and filtration. PBS has a pH of approximately 7.0±0.5.

iv. *Selective media (optional)*. Cetrimide agar (*P. aeruginosa*) and Mannitol salt agar (*S. aureus*). See Table 6 in Appendix C for use. Purchase from a reputable source or prepare according to manufacturer's instructions.

v. *Synthetic broth (SB).* Used as the growth medium for test cultures. Commercial media (HIMEDIA, Synthetic Broth, AOAC, #M334-500G). Store prepared SB at 2-8°C.

1. Alternatively, SB made in-house per the recipe provided in Appendix D and AOAC Methods 955.15, 964.02, and 955.14 may be substituted.

vi. *Trypticase Soy Agar (TSA)*. Used as a recovery medium for bacterial enumeration and purity checks. Prepare TSA according to manufacturer's instructions.

1. Equivalent commercially prepared agar culture medium may be purchased.

vii. *Trypticase Soy Agar with 5% sheep blood (BAP).* Used for performing streak isolation of microbial cultures as a purity check (quality control purposes).

viii. *Trypticase Soy Broth (TSB), (30g/L).* Used for rehydrating lyophilized/frozen vegetative culture of test microorganism. Prepare TSB according to manufacturer's instructions.

ix. *TSB with 15% (v/v) glycerol.* Used as a cryoprotectant solution. Suspend 7.5 g TSB in 212.5 mL de-ionized water. Using a positive displacement pipette, dispense 37.5 mL glycerol and stir, warm slightly to dissolve. Dispense into bottles and steam sterilize for 15 min at 121°C.

1. Alternatively, purchase broth from a reputable source or prepare according to manufacturer's instructions.

c. *Gram stain kit.* Used for diagnostic staining.

4. Viral Reagents

a. Test virus: use appropriate virus for efficacy testing required for intended label claim.

b. Cell line: Use an appropriate cell line for virus selected for efficacy testing.

c. Viral media

i. *Complete Growth Media (CGM).* Consisting of Minimum Essential Media and FBS or other medium specified for the test virus. Used for cell line propagation, viral propagation, and serial dilution. Antibiotics and/or antifungals may be added to reduce potential contamination.

1. *Minimum Essential Media (MEM).* Liquid or powder form (e.g., Eagle's or Dulbecco's). Used to prepare complete growth media. Prepare per manufacturer's guidelines.

2. *Heat Inactivated Fetal Bovine Serum (FBS).* Compatible for use with cell lines. Often used to prepare CGM.

ii. *Dulbecco's Phosphate buffered saline (DPBS).* Or other equivalent buffer (e.g., PBS, Earle's Balanced Salt Solution). Prepare per manufacturer's guidelines.

1. *Antibiotic/antifungal.* 100x Amphotericin B/Penicillin/Streptomycin solution or other equivalent antibiotic/antimycotic solution. May be used to prevent contamination of cell culture.

5. Common Reagents

a. *70% (v/v) ethanol (diluted in deionized water).* Used for treating the abrasion platform prior to the chemical exposure/abrasion processes.

b. *De-ionized (DI) water, non-sterile and sterile.* Used for preparation of reagents and media; used for rinsing test solutions off carriers prior to efficacy testing.

c. *Liquinox (or equivalent non-ionic detergent solution).* Used to clean and degrease carriers.

d. *Neutralizer medium.* 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.

    i. The recommended neutralizer for the viral test system is the same medium used to grow the virus (e.g., CGM). If the neutralization confirmation assay demonstrates that CGM is ineffective, other neutralizers may be used.

e. *Soil Load,3-part.* The standard soil load to be incorporated in the final test suspension

    i. *Bovine Serum Albumin (BSA).* Add 0.5 g BSA (radio immunoassay (RIA) grade or equivalent, CAS# 9048-46-8) to 10 mL of PBS, mix and pass through a 0.2 µm pore diameter polyethersulfone membrane filter, aliquot, and store frozen at -20±2°C for up to one year. Aliquots are single use only; do not refreeze once thawed.

    ii. *Yeast Extract.* Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter polyethersulfone membrane filter, aliquot, and store frozen at -20±2°C for up to one year. Aliquots are single use only; do not refreeze once thawed.

    iii. *Mucin.* Add 0.04 g mucin (from bovine submaxillary gland, CAS# 84195-52-8) to 10 mL of PBS, stir or vortex-mix until thoroughly dissolved, and pass through a 0.2 µm pore diameter polyethersulfone membrane filter, aliquot, and store frozen at -20±2 °C for up to one year. Note: mucin may require vigorous stirring or vortex-mixing to fully dissolve. Aliquots are single use only; do not refreeze once thawed.

    iv. See sections 13j and 14e for addition of soil load to bacterial and viral inoculum respectively.

f. Treatments used in chemical exposure of carriers

    i. *Treatment A*. 5 cycles of wet/dry abrasion (10 total cycle components). For wet abrasion cycle components, use 2000±100 ppm reagent grade (e.g., Sigma-Aldrich) sodium hypochlorite (NaOCl). Prepare a 2000±100 ppm NaOCl solution in sterile deionized water. Verify the final concentration of the solution using a suitable titration method (e.g., Hach digital titrator). Identify concentration in the study report.

    ii. *Treatment B*. 5 cycles of wet/dry abrasion (10 total cycle components). For wet abrasion cycle components, use an EPA-registered hospital disinfectant 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. Identify product in the study report.

iii. *Treatment C.* 5 cycles of wet/dry abrasion (10 total cycle components). For wet abrasion cycle components, 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 for wet abrasion cycles. Identify the product in the study report.

iv. *Treatment D.* 10 cycles of dry abrasion only. No wet abrasion cycles/components are included in Treatment D.

6. Carriers

a. See Appendix A for carrier specifications and passivation instructions. The test product is the formulation used to coat the stainless steel carriers. Two market relevant lots of the test product should be used to evaluate efficacy. "Abraded" refers to carriers subjected to the chemical treatment/physical abrasion, while "unabraded" refers to those carriers not subjected to the chemical treatment/physical abrasion. "Coated" refers to coated carriers with residual product present and "Control" refers to uncoated carriers with no residual product present. Lot 1 of test product is used for chemical treatments/abrasion and efficacy testing on abraded and unabraded control and coated carriers; two additional controls are included. Lot 2 is used to compare the unabraded control carriers to the abraded coated carriers only. Note: Control Set #2 serves as a control for Treatment D. See Table 1 for a summary of carrier distribution and Appendix E for a visual representation of carrier distribution.

Table 1. Carrier sets used in residual product testing

	Carrier Set	**# of carriers for *S. aureus***	**# of carriers for *P. aeruginosa***
Lot 1	Control Set #1: No residual product, Unabraded	3	3
	Control Set #2: No residual product, Abraded	3 per exposure* (9 total)	3 per exposure* (9 total)
	Coated Set #1: Residual product, Unabraded	3	3
	Coated Set #2: Residual product, Abraded	5 per exposure** (20 total)	5 per exposure** (20 total)
	Total Carriers for Lot #1	**35**	**35**
Lot 2	Control Set #1: (No residual product, Unabraded)	3	3
	Coated Set #2: (Residual product, Abraded)	5 per exposure** (20 total)	5 per exposure** (20 total)
	Total Carriers for Lot #2	**23**	**23**

*3 separate treatments (A, B, and C) with abrasion
**4 separate treatments (A, B, C, and D) with abrasion

b. Screen and clean carriers prior to chemical exposure/abrasion according to Appendix A.

c. Inspect each carrier to ensure uniformity. Discard carriers with visible surface or edge abnormalities (e.g., corrosion/rust, chipping, gouges, or deep striations, etc.); refer to examples of acceptable carriers in Appendix A.

d. 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 dry completely.

e. Steam-sterilize carriers in glass Petri dishes lined with filter paper prior to use; place ≤ 5 carriers per dish (carriers should not overlap in the dish).

f. Prepare at least one additional carrier for sterility assessment.

7. Chemical Exposure and Abrasion Treatment Process: Overview

a. To support a 1-week residual claim (see Tables 2 and 3) on Lot 1 (see Table 1 and Appendix E), perform each of the following on an independent set of carriers:

i. Treatment A; 5 cycles of wet/dry abrasion (10 total cycle components)

ii. Treatment B; 5 cycles of wet/dry abrasion (10 total cycle components)

iii. Treatment C; 5 cycles of wet/dry abrasion (10 total cycle components)

iv. Treatment D; 10 dry abrasion cycles

b. Dry time and hold time intervals:

i. Allow a 10 min minimum dry time interval following all wet abrasion cycles.

ii. Allow a 5 min minimum hold time interval following all dry abrasion cycles.

c. Complete all 5 chemical exposure/abrasion cycles for treatments A, B, and C, and all 10 independent dry abrasion cycles for treatment D within 5 consecutive days.

d. See Tables 2 and 3 for durability regimen flow for chemical exposure/abrasion treatments for Treatments A, B, C (Table 2), and D (Table 3).

Table 2. Durability regimen for one WET/DRY abrasion treatment (Treatments A, B, and C)*

Chemical Exposure/Abrasion**				Time Interval	
Cycle 1	Wet Component	4 single passes	→	Dry ≥ 10 min →	Dry Component
	Dry Component	4 single passes	→	Hold ≥ 5 min →	Proceed to Cycle 2
Cycle 2	Wet Component	4 single passes	→	Dry ≥ 10 min →	Dry Component
	Dry Component	4 single passes	→	Hold ≥ 5 min →	Proceed to Cycle 3
Cycle 3	Wet Component	4 single passes	→	Dry ≥ 10 min →	Dry Component
	Dry Component	4 single passes	→	Hold ≥ 5 min →	Proceed to Cycle 4

Cycle 4	Wet Component	4 single passes	→	Dry ≥ 10 min →	Dry Component
	Dry Component	4 single passes	→	Hold ≥ 5 min →	Proceed to Cycle 5
Cycle 5	Wet Component	4 single passes	→	Dry ≥ 10 min →	Dry Component
	Dry Component	4 single passes	→	Hold ≥ 5 min →	**Efficacy Evaluation**

*Repeat for each abrasion treatment (A, B, and C)
**Each cycle (4 single passes) requires 8-10 s with proper instrument calibration.

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

Dry Abrasion*			Hold Time	
Dry Cycle 1	4 single passes	→	≥ 5 min →	Proceed to Dry Cycle 2
Dry Cycle 2	4 single passes	→	≥ 5 min →	Proceed to Dry Cycle 3
Dry Cycle 3	4 single passes	→	≥ 5 min →	Proceed to Dry Cycle 4
Dry Cycle 4	4 single passes	→	≥ 5 min →	Proceed to Dry Cycle 5
Dry Cycle 5	4 single passes	→	≥ 5 min →	Proceed to Dry Cycle 6
Dry Cycle 6	4 single passes	→	≥ 5 min →	Proceed to Dry Cycle 7
Dry Cycle 7	4 single passes	→	≥ 5 min →	Proceed to Dry Cycle 8
Dry Cycle 8	4 single passes	→	≥ 5 min →	Proceed to Dry Cycle 9
Dry Cycle 9	4 single passes	→	≥ 5 min →	Proceed to Dry Cycle 10
Dry Cycle 10	4 single passes	→	≥ 5 min →	**Efficacy Evaluation**

*Each cycle (4 single passes) requires 8-10 s with proper instrument calibration.

Figure 1. Chemical exposure/abrasion and controls for carriers of lot #1

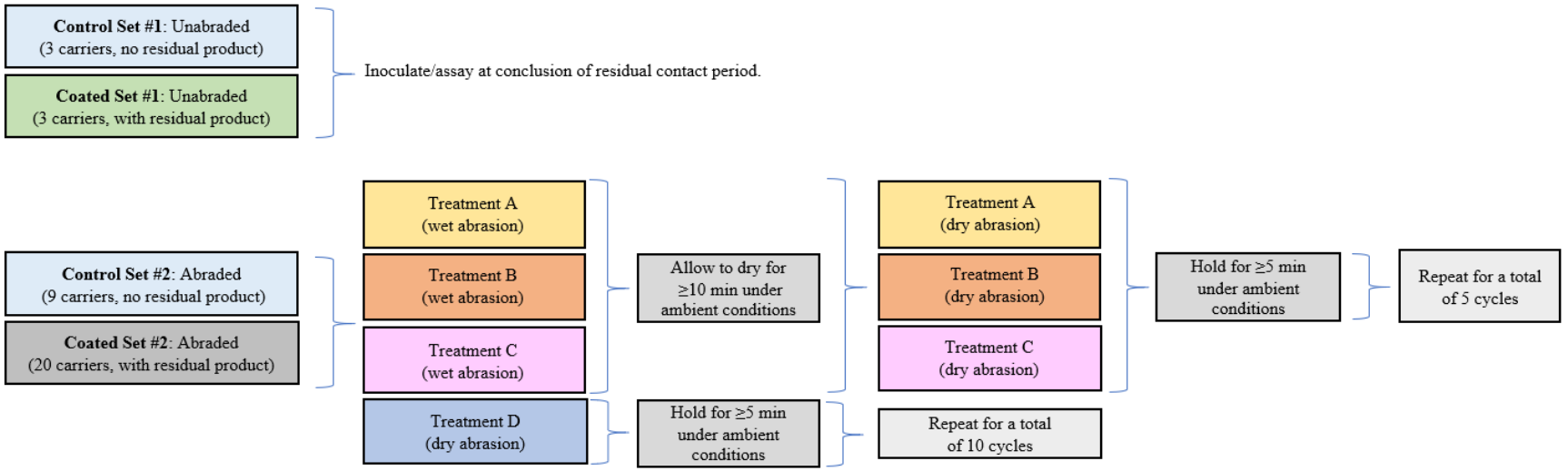
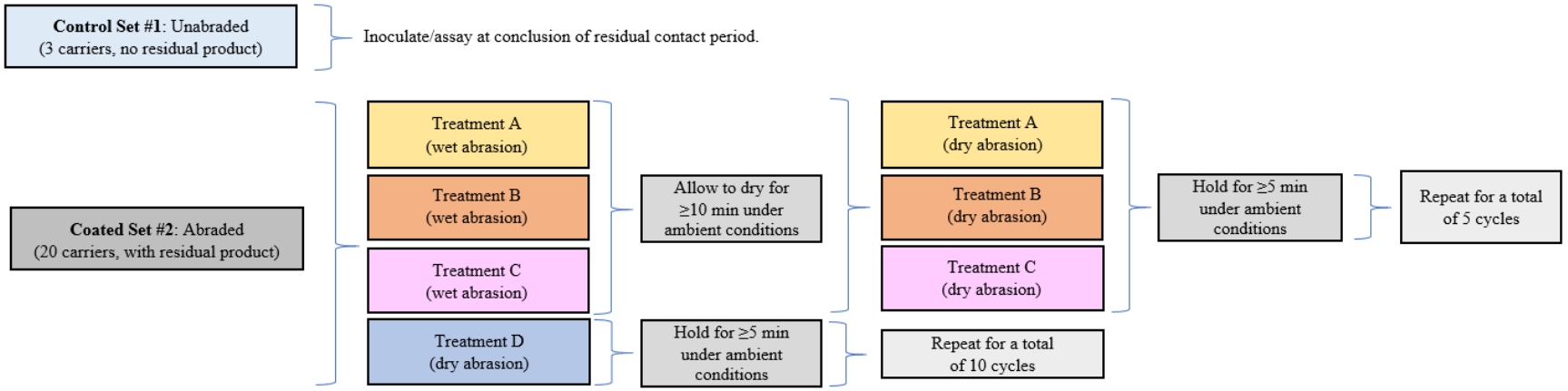


Figure 2. Chemical exposure/abrasion and controls for carriers of lot #2



8. Product (Coating) Application.

a. Apply coating to the brushed side of sterile screened carriers only according to an approved EPA label or proposed label use directions, document ambient temperature and relative humidity.

   i. Maintain lot identity throughout the testing process.

b. Following coating, transfer carriers into individual sterile Petri dishes lined with filter paper, one carrier per dish. Do not use carriers on which the coating coverage is not complete.

c. Define parameters for coating application (spray distance, application coverage, application time, etc.) in the study report.

d. Provide digital evidence of what the coated surface looks like after application and drying for both lots.

9. Sponge Preparation.

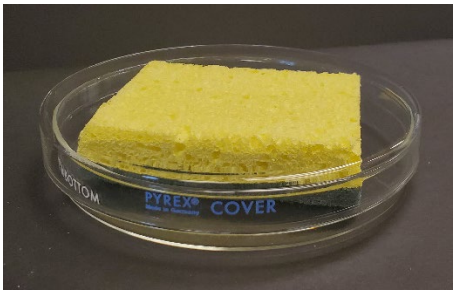
a. Prepare a sufficient number of sponges for each abrasion treatment; allow one sponge per 150 mm glass Petri dish for sterilization. See Figure 3.

   i. Prepare one sponge for each wet and each dry component for Treatments A, B, C, and one sponge for Treatment D. For example, for abrasion required for lot #1 (Control Set #2 and Coated Set #2), a minimum of 13 sterile sponges will be required. For abrasion required for lot #2 (Coated Set #2), a minimum of 7 sterile sponges will be required.

b. Cut sponge to fit within abrasion tester sponge box (10 cm x 8 cm).

c. Preclean cut sponges by submerging or saturating sponges completely with/in deionized water, rinse with deionized water, and squeeze dry before steam sterilization.

Figure 3. One sponge, pre-cleaned and cut to fit snugly in abrasion tester sponge box, shown in 150 mm glass Petri dish prepared for sterilization.



d. Steam-sterilize covered Petri dishes containing sponges on gravity cycle for 20 min.

e. After sterilization, allow sponges to dry uncovered in the biosafety cabinet overnight before proceeding.

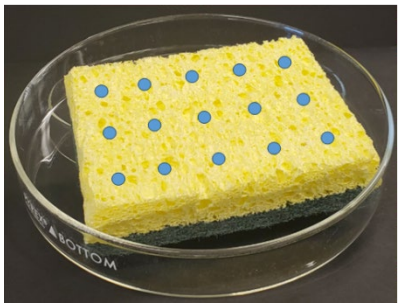
i. Sponges used for dry abrasion cycles may be aseptically gently manipulated to ensure the sponge is not convex; the surface of the sponge must contact the surface of the carriers during abrasion cycles.

f. With the sponge side of the sponge up (scrub pad down), dispense 15 mL in 15, 1 mL aliquots of one test solution (A, B, or C) in an evenly spaced 5 × 3 grid (see Figure 4) across the sponge side/surface of the sponge. Allow wetted sponge to stand covered in the sterile Petri dish until uniformly hydrated (up to 60 min); testing may begin upon complete saturation. Initiate testing and use wetted sponge within 1 hour of hydration end time.

i. Repeat for treatments A, B, and C.

ii. Apply treatments A, B, or C once per five wet abrasion cycles; discard treated sponge either following the fifth wet abrasion cycle or three hours post saturation end time, whichever occurs first.

Figure 4. 15-1mL aliquots distributed in a 5 × 3 grid pattern on the sponge side of the sponge.



g. Use one dry sterile sponge for each dry component of Treatments A, B, C, and one dry sterile sponge for Treatment D.

i. For example, for lot #1 testing, a total of 3 dry sterile sponges are required for Control Set #2 and a total of 4 dry sterile sponges are required for Coated Set #2.

h. Be sure to use the same sponge for each treatment/carrier set throughout the entire test. For example, for lot #1 testing, label Petri dishes containing the sterile sponges as follows:

i. Sponge 1: Control Set #2/Treatment A/Wet Component

ii. Sponge 2: Control Set #2/Treatment A/Dry Component

iii. Sponge 3: Control Set #2/Treatment B/Wet Component

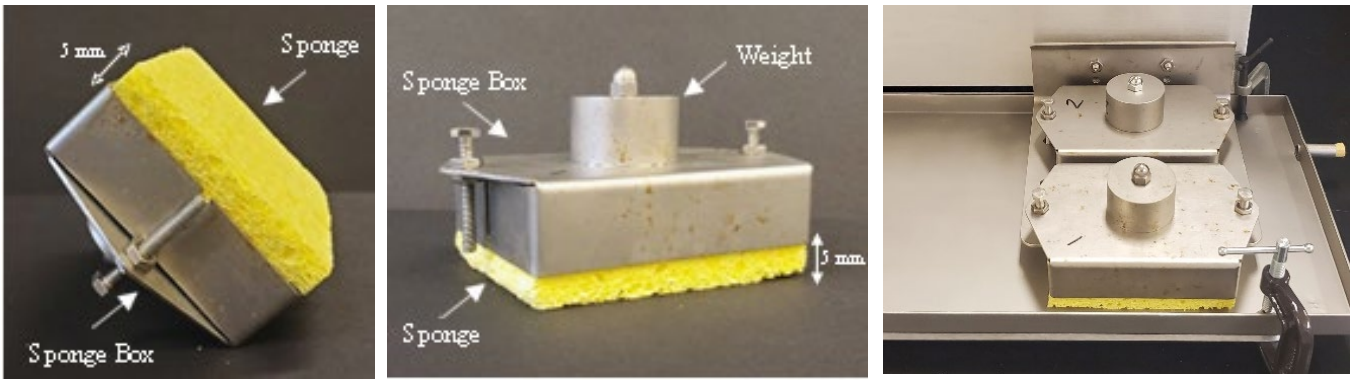
iv. Sponge 4: Control Set #2/Treatment B/Dry Component, etc.

10. Abrasion Tester Set-Up

a. Perform the chemical exposure/abrasion with a variable speed abrasion tester. Consult the owner's manual to ensure proper set up, operation, maintenance, and calibration.

b. Calibrate instrument to achieve 2-2.5 s for one single pass (horizontal movement of the abrasion boat).
    i. 8-10 s per each wet and dry abrasion component for Treatments A, B, and C (4 single passes).
    ii. 8-10 s per dry abrasion cycle for Treatment D (4 single passes).
c. The variable speed abrasion tester sponge box with weight weighs approximately 454 g; use comparable devices with comparable weight.
    i. Use weight on top of the sponge box for Treatments A, B, C, and D.
d. Aseptically (e.g., use ethanol-treated or sterile gloves) apply sponge to sponge holder/sponge box of the abrasion unit as specified in the manual so that it fits snugly.
    i. The sponge must extend a minimum of 5 mm beyond the rim of the sponge box to prevent the contact of sponge box with the surface of the coated carriers; 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 5).

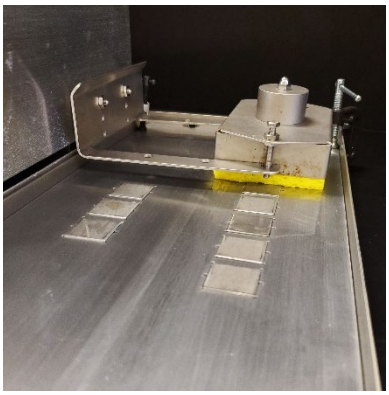
Figure 5. Proper sponge placement



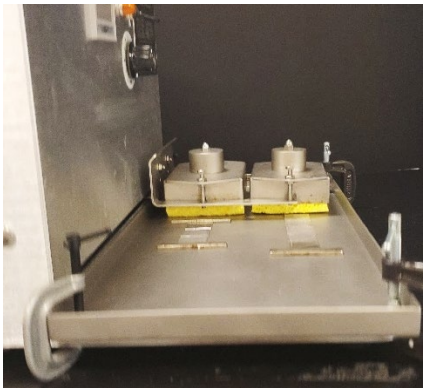
e. Treat abrasion platform with 70% (v/v) ethanol and allow to air dry prior to use and in between cycles.
f. Load carriers onto abrasion instrument. For coated and uncoated control carriers, orient individual carriers with the coated or uncoated brushed surface side-up. Maintain brushed side-up orientation throughout the exposure treatment. Situate carriers from Control Set #2 (3 carriers) and Coated Set #2 (5 carriers) in parallel with one another for abrasion, see Figure 6.
    i. Use one sponge box per carrier set.
    ii. Do not adhere carriers to the abrasion instrument; templates, magnets, or other means may be used to hold the carriers in place during abrasion process (see Figure 6 for example use of template and magnets).
        1. If using abrasion template, brushed carrier surface should be at or slightly above template recesses.

2. If using magnets, place one magnet on bottom and one magnet on top of abrasion platform to hold the carriers in place during abrasion process; magnets should sit at the beginning and end of carriers in place for abrasion.

Figure 6. Recommended abrasion process – carrier configuration.



One sponge box
(Machine-cut aluminum template to hold carriers in place)



Two sponge boxes
(Neodymium magnets to hold carriers in place; magnets situated under platform not shown here)

11. Wet/Dry Chemical Exposure and Abrasion: Treatments A, B, and C

a. Conduct chemical exposure/abrasion cycle for a single chemical exposure/abrasion treatment (Treatments A, B, and C). Perform four single passes across the carriers for both the wet component (using the appropriately labeled wetted sponge per treatment/carrier set) and the dry component (using the appropriately labeled dry sponge per treatment/carrier set).

   i. 4 single passes across the surface of the carrier with wet abrasion (chemical wetted sponge) followed by 4 single passes across the surface of the carrier dry abrasion (dry sponge) = 1 abrasion cycle for Treatments A, B, and C.

   ii. Allow a minimum 10 min dry time interval following all wet abrasion cycles.

   iii. Allow a minimum 5 min hold time interval following all dry abrasion cycles.

   iv. Carriers may stay on the abrasion tester platform or be transferred into the appropriate sterile Petri dish for the duration of the dry time and hold time intervals.

   v. Discard sponges after completing 5 wet or dry cycles for one chemical exposure/abrasion treatment or after three hours post saturation end time, whichever occurs first. Do not re-use sponges.

b. Following the completion of all wet/dry abrasion cycles, individually and gently rinse both sides of carriers abraded with Treatments A, B, and C for 3-5 s with sterile DI water using a sterile squirt bottle. Place each carrier abraded, brushed side up into a new,

individual sterile Petri dish lined with filter paper and air dry in the BSC (lids ajar for drying).

c. Once dry, store covered at room temperature (i.e., 21±3°C).

   i. Note any changes to the surface characteristics of the carrier (e.g., flaking, removal, discoloration of the coating).

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

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

12. Dry Abrasion: Treatment D

a. Conduct the independent dry abrasion treatment (Treatment D) using a dry sponge in the sponge box of an abrasion unit to perform four single passes across the carriers.

   i. 4 single passes across the surface of the carrier = 1 abrasion cycle for Treatment D.

   ii. Allow a minimum 5 min hold time interval following all dry abrasion cycles.

b. For Treatment D, use one dry sterile sponge for all 10 independent dry abrasion cycles.

   i. Sponges used for dry abrasion cycles may be aseptically gently manipulated to ensure the sponge is not convex; the surface of the sponge must contact the surface of the carriers during abrasion cycles.

c. Following 10 independent dry abrasion cycles for Treatment D, aseptically place each carrier abraded, brushed side up into its own individual sterile Petri dish lined with filter paper: store carriers under ambient conditions (i.e., room temperature 21±3°C). Do not rinse carriers for Treatment D.

   i. After transfer to sterile Petri dishes lined with filter paper, note any changes to the surface characteristics of the carrier (e.g., flaking, removal, discoloration of the coating).

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

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

13. Preparation of Test Culture: *P. aeruginosa* and *S. aureus*

a. Refer to Appendix C 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. No more than 15 min prior to inoculation, use a calibrated micropipette to aseptically add 0.1 mL of 10% sterile dextrose (w/v) solution to each 10 mL tube of SB in a 20×150 mm glass culture tube with a Morton closure; mix well.

d. Using a calibrated micropipette, add 100 µL of defrosted stock culture to the same tube containing 10 mL SB + 0.1 mL dextrose, briefly vortex-mix and incubate for 24±2 h at 36±1°C.
    i. In addition, inoculate TSA or TSA with 5% sheep blood plate from the inoculated tube and streak for isolation. Incubate plate with the test culture.
    ii. Incubate without disrupting the culture (i.e., do not move culture while incubating).
e. Following incubation, use the SB cultures to prepare a test suspension for each organism. Record results from the streak isolation plate.
f. The 24±2 h culture should exhibit a titer of at least 10^8 CFU/mL. Record time of culture harvest.
    i. For *P. aeruginosa*, inspect culture prior to harvest; visible pellicle on the surface of the culture is expected to form during incubation (record its presence). Discard the culture if pellicle has been disrupted (fragments in culture).
        1. Remove visible pellicle on surface of medium and around associated interior edges of the tube with vacuum suction.
        2. 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 conical centrifuge tube.
        3. Record approximate volume harvested and transferred to 15 mL conical tube.
    ii. For *S. aureus*, briefly vortex the 24±2 h culture and transfer to a 15 mL centrifuge tube.
        1. Record approximate volume harvested and transferred to 15 mL conical tube
g. Within 15 minutes, centrifuge the 24±2 h harvested broth cultures at 5,000×g for 20 min. Record time of centrifugation.
h. Remove the supernatant without disrupting the pellet. Once supernatant is removed, re-suspend the pellet in 5-10 mL PBS. Record resuspension volume and time of resuspension.
    i. If necessary, disrupt the pellet using vortex-mixing or repetitive tapping/striking against a hard surface to disaggregate the pellet completely. If necessary, add 1 mL of PBS to the pellet first to aid in disaggregation.
i. For efficacy testing, further dilute the 5-10 mL of resuspended culture in PBS as necessary to achieve a mean control carrier count level of 4.0-5.0 logs CFU/carrier for *P. aeruginosa* and *S. aureus*. Each inoculated carrier must be within this range following the 1 to 2-hour exposure time for a valid test. Record time of test suspension dilution.
    i. Optical density/absorbance (e.g., 650 nm) may be used as a tool to monitor/adjust the diluted test suspension; record if measured.

j. Use the diluted culture within 30 min to prepare the final test suspension with the addition of the soil load.
    i. Vortex-mix the test suspension for 10-30 s.
    ii. To obtain 500 µL of the final test suspension with the 3-part soil load, vortex-mix each component and combine in the following order using a calibrated micropipette:
        1. 25 µL BSA stock
        2. 35 µL yeast extract stock
        3. 100 µL mucin stock
        4. Vortex soil suspension for 10 s prior to adding microbial test suspension.
        5. 340 µL microbial test suspension.

k. Briefly vortex the final test suspension with 3-part soil load (at room temperature, 21±3°C) and use to inoculate carriers within 90 min of preparation. Record time of final test suspension preparation.
    i. Within the 35 carriers, there are a total of 9 independent "sets" of carriers (5 sets of 3 carriers, 4 sets of 5 carriers). A 90-minute use period for the culture with the 3-part soil load provides the opportunity to stagger inoculations, 10 min per carrier set.
    ii. Inoculate 1 carrier from Control Set #1 first. Continue inoculations with Treated Set #1, Treated Set #2, Control Set #2; then inoculate the remaining 2 carriers from Control Set #2.

l. 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 h and visually examine for purity.

14. Preparation of the Test Culture – Viruses

a. Propagate the test virus on the appropriate cell line.
    i. Note: Concentration of the test virus stock (~100,000×g for 4 h at 4ºC) may be necessary to achieve adequate control counts.

b. Defrost a cryovial rapidly to avoid loss in the viability of the preserved virus (e.g., place in a 37ºC water bath and use within 15 min after thawing). Record time placed in water bath.

c. Dilute the virus stock with CGM to achieve control counts in the range of 4.0 to 5.0 logs virus particles/carrier. Record time of virus stock dilution.

d. Use the diluted virus within 30 min to prepare the final test suspension with the addition of the soil load.

e. To obtain 500 µL of the final test suspension with the 3-part soil load, vortex-mix each component and combine in the following order using a calibrated micropipette (smaller volumes may be used proportionally):

i. 25 µL BSA stock

ii. 35 µL yeast extract stock

iii. 100 µL mucin stock

iv. Vortex soil suspension for 10 s prior to adding viral test suspension.

v. 340 µL virus test suspension

f. Briefly vortex the final test suspension with 3-part soil load (at room temperature, 21±3°C) and use to inoculate carriers within 90 min of preparation. Record time of final test suspension preparation.

i. Within the 35 carriers, there are a total of 9 independent "sets" of carriers (5 sets of 3 carriers, 4 sets of 5 carriers). A 90-minute use period for the culture with the 3-part soil load provides the opportunity to stagger inoculations, 10 min per carrier set.

ii. Inoculate 1 carrier from Control Set #1 first. Continue inoculations with Treated Set #1, Treated Set #2, Control Set #2; then inoculate the remaining 2 carriers from Control Set #2.

15. Efficacy Assessment

a. Conduct efficacy testing on all coated and control carriers within 7 days of completing the chemical exposure/abrasion cycles.

b. Perform the neutralization assay prior to testing to demonstrate the neutralizer's ability to inactivate the residual antimicrobial coating; see Appendix F (bacteria) or Appendix H (viruses).

c. 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 h (relative to the target contact time), and determining the counts per carrier.

d. Prepare test cultures per section 13 (bacteria) or 14 (viruses) 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 or viral particles per carrier after 1-2 h (relative to the target contact time).

e. Set environmental chamber to achieve 21±2°C and 30-40% relative humidity during the 1-2 h contact period; record temperature and humidity over the contact period.

f. Record the time for all timed events.

g. Process coated carriers first and control carriers last.

h. Inoculate each carrier with 20 µL of final test suspension using a calibrated positive displacement 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. Contact time begins immediately after inoculation; place in environmental chamber within 10 min of inoculation.

i. Allow carriers to remain in a flat, horizontal position in individual Petri dishes with the lid on in the environmental chamber for 1-2 h. Refer to Appendix A for picture of dried inoculum on carrier.

16. Bacterial Recovery

a. Following the contact time, sequentially and aseptically transfer each carrier to a 50 mL conical tube containing 20 mL of the verified neutralizer solution. Remove carriers from the environmental chamber and neutralize all carriers within 10 min of the contact time.
    i. The tube with the neutralizer and the carrier represents the 10^0 dilution.

b. After all the carriers have been transferred into the neutralizer, vortex-mix for 30 s then sonicate for 5 min±30 s at 45 Hz to suspend any surviving organism in the neutralizer. If necessary, refer to section 2aa for sonicator verification.

c. Initiate serial dilutions of the neutralizer tubes in PBS within 30 min.

d. Initiate filtration within 30 min of preparing dilutions.

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

f. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS. Apply vacuum to filter contents; leave the vacuum on for the duration of the filtration process regardless of filtration apparatus used (e.g., filter manifold, single filter unit).

g. 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.

h. 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.

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

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

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

l. Carrier Sterility Control: add one sterile uncoated carrier to a tube containing 20 mL of TSB. Incubate at 36±1°C for 48±4 h and examine for growth. The acceptance criterion is lack of turbidity/growth in the tube.

m. Neutralizer Sterility Control: add 1 mL of neutralizer into 9 mL of TSB. Incubate at 36±1°C for 48±4 h and examine for growth. The acceptance criterion is lack of turbidity/growth in the tube.

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

i. Monitor all plates (filters) after 24 h of incubation to facilitate appropriate timing for counting colonies.

ii. For colony counts on filters in excess of 200 CFU, record as Too Numerous to Count (TNTC).

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

p. If confluent growth is present, perform a streak isolation on the appropriate agar on growth taken from at least 1 representative filter per carrier set.

q. The results of the streak isolation plates (sections 13d, 13l, and 16o) should be consistent with characteristics in Table 6.

r. 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.

17. Virus Recovery

a. Following the contact time, sequentially and aseptically transfer each carrier to a 50 mL conical tube containing 20 mL of the verified neutralizer solution. Remove carriers from the environmental chamber and neutralize all carriers within 10 min of the contact time.

i. The tube with the neutralizer and the carrier represents the 10^0 dilution.

b. After all the carriers have been transferred into the neutralizer, vortex-mix for 30 s to suspend any surviving organism in the neutralizer. If necessary to increase viral recovery, sonicate for 5 min±30 s at 45 Hz; refer to section 2aa for sonicator verification.

c. Initiate dilutions within 30 min after neutralization and vortex-mixing.

d. Initiate inoculation of cell line within 30 min of preparing the dilutions.

e. Titrate the samples for virus infectivity.

f. Plate a minimum of 80% of the volume (8 mL for 10 mL volumes, 16 mL for 20 mL volumes) of the 10^0 vessel and of each dilution tube.

g. Remove the growth medium from each well of the plate with a confluent monolayer of cells and replace with the maximum volume of the well (i.e., add 1 mL per well for a 24 well plate) working from most dilute to least dilute.

h. The elution steps for control carriers are the same as for the test carriers; use 10-fold dilutions to achieve 4.0-5.0 logs virus particles/carrier.

i. For each test, use at least one well as a negative control (CGM alone) and one well as a positive growth control (e.g., one of the dilutions from a control carrier).

j. If cytotoxicity was observed in the neutralization testing and/or on the cytotoxicity control, CGM may be removed from all wells in the affected dilutions at the appropriate time (one hour minimum). Wash the wells with pre-warmed PBS, then replace the PBS with fresh CGM.

k. Incubate test and control plates as appropriate for the test system.

l. Record all observations (presence/absence of viable virus particles) and use in calculations to estimate the log reduction based on the $TCID_{50}$ or MPN (most probable number) technique.

18. 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. For bacteria, 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. For viruses, calculate the $TCID_{50}$/carrier or MPN/carrier. Calculate the log density of each carrier by taking the $\log_{10}$ of the density (per carrier).

d. Calculate the mean log density (LD) of viable cells or virus particles 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.

e. Calculate the mean LD of viable cells or virus particles 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.

f. For bacteria, 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.

g. Conduct additional calculations for Lot #1 and Lot #2; include in study report. See Tables 4 and 5 for calculations and outcome requirements.

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

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

19. References

a. Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD. *P. aeruginosa* p. 164.

b. Sneath, P., Mair, N., Sharpe, M.E., and Holt, J. eds. 1986. Bergey's Manual of Systematic Bacteriology Volume 2. Williams & Wilkins, Baltimore, MD. *S. aureus* p. 1015.

c. ASTM Method E1482-12. Standard Practice for Use of Gel Filtration Columns for Cytotoxicity Reduction and Neutralization (Reapproved 2017)

Appendix A

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 with uniform thickness (example: 18-20 gauge); carrier manufacturer will provide thickness of the original stainless steel sheet.
- Flatness: Carrier height not to exceed 110% of the thickness of the uncut sheet of stainless steel from which the carriers are manufactured.

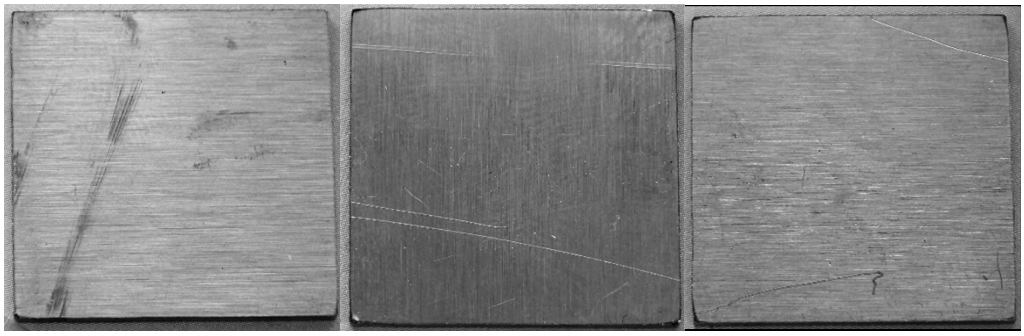
Finish: A ground unidirectional finish obtained with 150 grit abrasive (AISI) on the top side of the stainless steel sheet.

Burr Removal: If necessary, remove burrs from the edges on the bottom side of the carrier using a manual process.

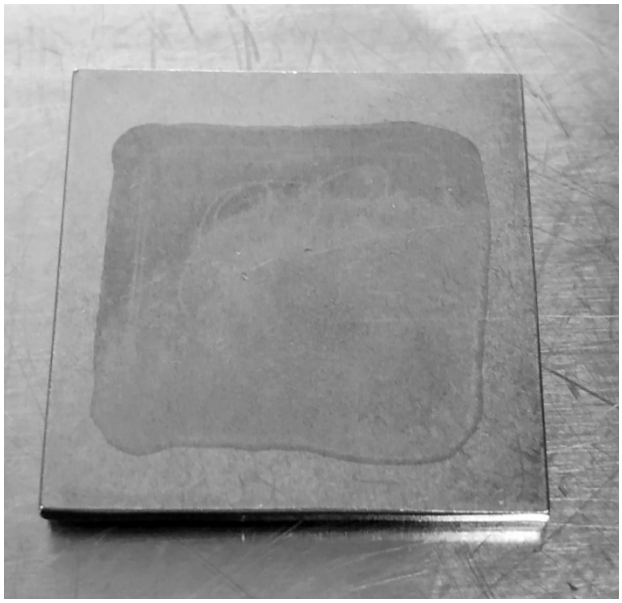
Passivation: Parts are passivated by the carrier manufacturer according to ASTM A967 in a citric acid solution and prepared as follows:

- 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

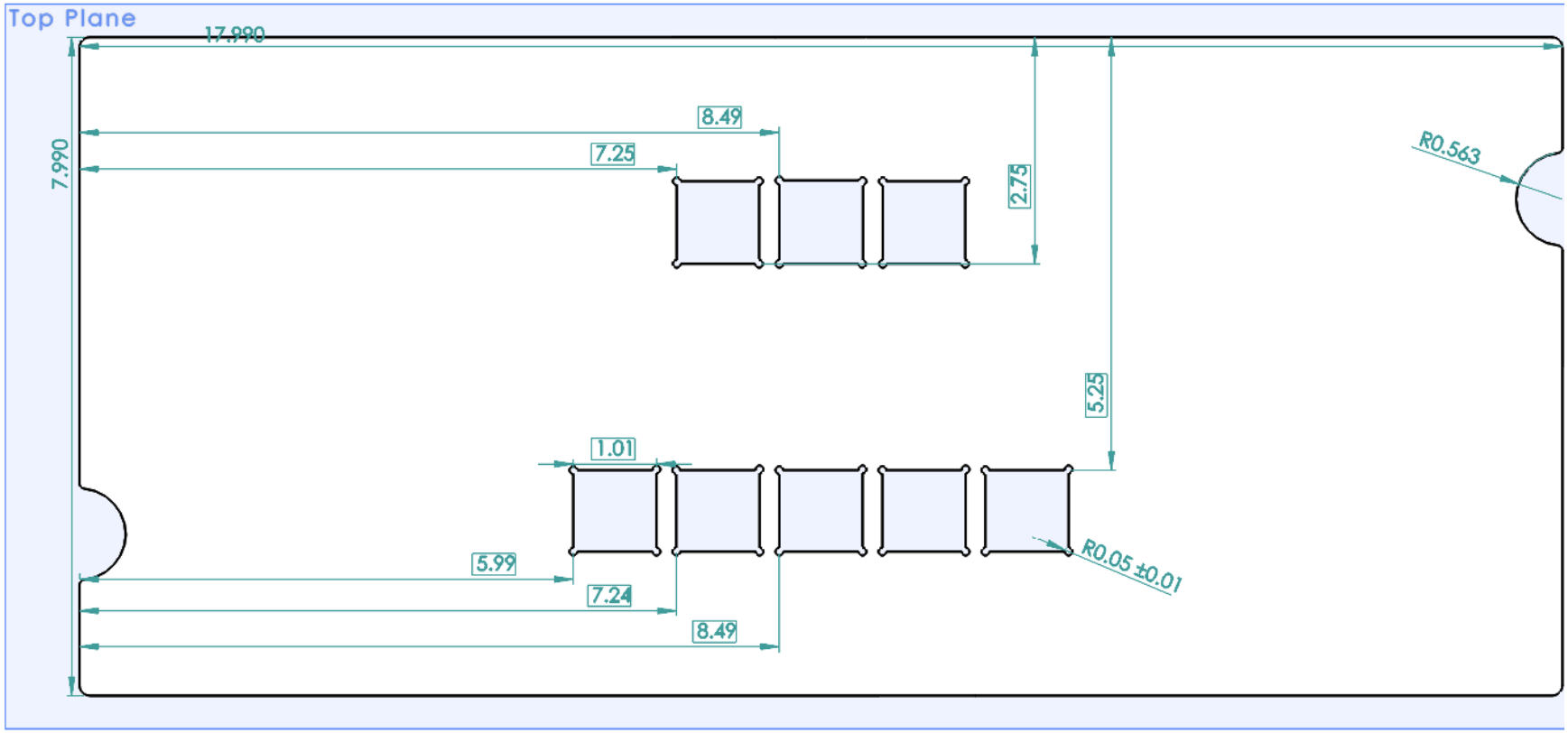


Example of a Dry Inoculated Carrier



Appendix B

Gardco Template Specifications*



*All dimensions are given in inches.

Appendix C

Preparation of Bacterial Frozen Stock Cultures

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 *P. aeruginosa*, select colonies from each of the two possible phenotypes present. For *S. aureus*, select only golden yellow colonies. 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* (see sections 19a-b)

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 D

Synthetic Broth Recipe

Solution A: Dissolve the following in 500 mL H_2O containing 18 mL 1 N NaOH:

- 0.05 g L-cystine
- 0.37 g DL-methionine
- 0.4 g L-arginine×HCl
- 0.3 g DL-histidine
- 0.85 g L-lysine×HCl
- 0.21 g L-tyrosine
- 0.5 g DL-threonine
- 1.0 g DL-valine
- 0.8 g L-leucine
- 0.44 g DL-isoleucine
- 0.06 g glycine
- 0.61 g DL-serine
- 0.43 g DL-alanine
- 1.3 g L-glutamic acid×HCl
- 0.45 g L-aspartic acid
- 0.26 g DL-phenylalanine
- 0.05 g DL-tryptophan
- 0.05 g L-proline

Solution B: Dissolve the following in 500 mL H_2O:

- 3.0 g NaCl
- 0.2 g KCl
- 0.1 g $MgSO_4 \times 7\ H_2O$
- 1.5 g KH_2PO_4
- 4.0 g Na_2HPO_4
- 0.01 g thiamine×HCl
- 0.01 niacinamide

Mix Solutions A and B, and steam sterilize 20 min at 121°C.

- Final pH should be 7.1±0.1.

Appendix E

Visual Representation of the Evaluation of Lot 1 Carriers

Carriers Required for Lot 1 Testing

Control Set 1
- No Residual Coating
- Unabraded
- In Petri dish until efficacy testing

1 2 3

Coated Set 1
- Residual Coating present
- Unabraded
- In Petri dish until efficacy testing

Key: Carriers

No Residual Coating

Residual Coating present

	Control Set 2 • No Residual Coating • Abraded • On Gardco tray, second row	**Coated Set 2** • Residual Coating present • Abraded • On Gardco tray, front row
Treatment A	4 5 6	
Treatment B	7 8 9	
Treatment C	10 11 12	
D **Dry Abrasion**	N/A (Note: Control Set #2 serves as a control for Treatment D)	

Appendix F

Bacterial Neutralization Assay

1. The neutralization of the coated carriers is confirmed in triplicate by using coated carriers in neutralizer, neutralizer only (without carriers), and PBS only (used to compare counts from the neutralizer and coated carriers).

2. **Preparation of the Test Organism**

    a. Refer to the preparation of test cultures section (13a-h) of for preparation of the test cultures. Conduct preliminary tests as necessary to determine appropriate dilution(s) of *Test Suspension A* (used to prepare *Test Suspension B*) to achieve the target challenge of 20-200 CFU per 20 µL.

    b. **Prepare Test Suspension A (without soil load)**. Serially dilute the microbial test suspension with PBS (e.g., through 10^{-4} or 10^{-5}). Select appropriate dilutions of *Test Suspension A* so that after the addition of the soil load, *Test Suspension B* will achieve an average challenge of 20-200 CFU per 20 µL. Use *Test Suspension A* within 30 min of preparation.

        i. Two separate serial dilutions of *Test Suspension A* may be used to prepare two different concentrations of *Test Suspension B* to ensure at least one dilution with an average challenge of 20-200 CFU per 20 µL.

        ii. A calibration curve (OD @ 650nm) may be used to estimate the number of viable organisms in *Test Suspension A*.

    c. **Prepare Test Suspension B (with soil load)**. Prepare the 3-part soil load: using a vortex, mix each component and combine 25 µL bovine serum albumin (BSA), 35 µL yeast extract, and 100 µL of mucin; then vortex-mix the solution. Add 340 µL of diluted *Test Suspension A* to the 160 µL of the soil load (SL) and vortex-mix for 10 s.

        i. Ensure at least one preparation of *Test Suspension B* provides an average challenge of 20-200 CFU per 20 µL.

3. **Neutralization Assay Components**

    a. ***Treatment 1: Neutralizer Effectiveness.*** Add a coated carrier (one per market relevant lot) to each of three 50 mL conical tubes. At timed intervals, add 20 mL of neutralizer to each 50 mL conical tube and vortex-mix for 30 s on highest vortex setting. Immediately add 20 µL *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix F, section 4.

    b. ***Treatment 2: Neutralizer Toxicity Control.*** Add 20 mL of neutralizer to each of three 50 mL conical tubes. At timed intervals, add 20 µL of *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix F, section 4.

    c. ***Treatment 3: Titer Control.*** Add 20 mL of PBS to each of three 50 mL conical tubes. At timed intervals, add 20 µL of *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix F, section 4.

4. **Processing and Recovery**

a. Hold the mixtures from Appendix F, section 3 for 10±1 min at room temperature (21±3°C).

b. At the conclusion of the holding period, vortex-mix each tube.

c. Initiate filtration as soon as possible (e.g., within 30 min).

d. Prior to filtration, pre-wet each membrane filter with ~10 mL PBS. Filter each mixture through a separate, pre-wetted 0.2 µm PES membrane filter. Apply vacuum to filter contents; leave the vacuum on for the duration of the filtration process regardless of filtration apparatus used (e.g., filter manifold, single filter unit).

e. Wash each tube with ~20 mL PBS and vortex-mix; filter the wash through the same filter membrane. Finish the filtering process by rinsing the inside of the funnel unit with ~20 mL PBS and filter the rinsing liquid through the same filter membrane.

f. Remove the membrane aseptically with sterile forceps and place it carefully over the surface of the TSA. Avoid trapping air bubbles between the filter and the agar surface.

g. Incubate plates at 36±1°C for 48±4 h and count the colonies.

h. Incubate plates with no growth or few colonies an additional 24±4 h and count the number of colonies.

5. **Data Analysis/Calculations**

a. Compare the average CFU of the **Titer Control** with the average CFU of the **Neutralizer Toxicity Control** and **Neutralizer Effectiveness** treatment. Determine the percent difference in CFU.

b. For determining the suitability of the neutralizer, ensure that the average CFU in the **Neutralizer Toxicity Control** is at least 50% of the **Titer Control**. A count lower than 50% indicates that the neutralizer is harmful to the test organism.

   i. Average CFU for the **Neutralizer Toxicity Control** that are higher than the **Titer Control** (e.g., 120% of the **Titer Control**) are also deemed valid.

c. To verify effectiveness of the neutralizer, the average number of CFU in the **Neutralizer Effectiveness** treatment is at least 50% of the **Titer Control**.

   i. Average CFU for the **Neutralizer Effectiveness** treatment that are higher than the **Titer Control** (e.g., 120% of the **Titer Control**) are also deemed valid.

d. If the criteria are not met, verify another neutralizer or mixture of neutralizers.

Appendix G

Cytotoxicity Determination

Prior to performing the neutralization assay, ensure the proposed neutralizer, neutralizer and test chemical, and the soil used do not impact the quality of the cell line by performing the following:

1. **Neutralizer Effect on Cell Line (for neutralizers other than CGM with 2% FBS).**
   a. Add 0.5 mL of the proposed neutralizer to 4.5 mL CGM with 2% (v/v) FBS, equilibrated to 37±1°C (this is the 10^{-1} dilution). It is suggested to do further dilutions out to 10^{-2} or 10^{-3} depending on the expected cytotoxicity of the neutralizer.
   b. Remove the CGM from the wells of a 24 well plate with a confluent monolayer of cells and add 1 mL per well of the neutralizer plus CGM solution. Plate at least 4 wells per dilution. Have at least one well as a negative control (e.g., CGM with 2% FBS alone).
   c. Incubate plate as appropriate and observe closely for cytotoxicity.
   d. If cytotoxicity is observed after one hour, remove the media in a single well of the affected dilution, rinse once with pre-warmed DPBS (the DPBS wash step may be omitted if the cytotoxicity is mild), and replace media.
   e. If cell death occurs in under one hour, the neutralizer cannot be tested.
   f. The effect of the media change in the single well can be compared to the other wells in the dilution and the negative control. If cytotoxicity cannot be overcome with washing and replacing of media, column filtration (e.g., Sephadex) may be used in future testing. See section 19c for further information on column filtration.
2. **Neutralizer Plus Residual Coating Effect on Cell Line.**
   a. Add the carrier to one coated test carrier (representative of Lot 1 testing) 20 mL of neutralizer, equilibrated to 21±3°C, and vortex 2-3 seconds. Let this solution sit at room temperature for 10 minutes.
   b. Add 1.0 mL of this solution to 9 mL CGM with 2% (v/v) FBS, equilibrated to 37±1°C (this is the 10^{-1} dilution). It is suggested to do further dilutions out to 10^{-2} depending on the expected cytotoxicity.
   c. Remove the CGM from the wells of a 24 well plate with a confluent monolayer of cells and add 1 mL per well of the neutralizer plus coated test carrier solution and dilutions. Plate at least 8 wells for the 10^{0} dilution, 6 wells for the 10^{-1} dilution, and 4 wells is for the 10^{-2} dilution. Extra wells will be needed to observe the effect of no media changes or for further media changes as needed.
   d. For highly toxic test chemicals, washing the cells with pre-warmed DPBS before the addition of CGM with 2% FBS will help remove cytotoxicity.
   e. Have at least one well on each plate as a negative control (e.g., CGM with 2% (v/v) FBS alone).
   f. At a minimum, change the media in the wells as outlined below. Change the media at the lower time interval if they look more toxic. Other media changes can be made at

other times if necessary.

i. For the 10^0 dilution:
    i. On the day of the test, change two 1-2 hours (1-hour minimum) after the neutralized test chemical mixture was added to the cells.
    ii. Change two more additional wells 3-5 hours after the neutralized test chemical mixture was added to the cells
    iii. The following day change one 1-2 hour well, one 3-5 hour well, and one previously unchanged well, plus one of the two previously changed well.

ii. For the 10^{-1} dilution:
    i. On the day of the test, change tow wells 3-5 hours after the neutralized test chemical mixture was added to the cells.
    ii. The following day, change one 3-5 hour well and one previous unchanged well

iii. For the 10-2 dilution:
    i. On the following day after the test, change one well.

g. Incubate the plate as appropriate and observe the cells for cytotoxicity. The test cells should be compared to the negative control cells to determine toxicity.

h. Score the cells as toxic or non-toxic in each in each test conditions.

i. Identify the test condition that removed the cytotoxicity and use that condition for further neutralization and efficacy testing. Use the test condition that allows the media to stay on the cells for as long as possible.
    i. **Example:** In the 10^0 dilution, if the unchanged wells are toxic, but both the 1 hour and 4 hour media changes are non-toxic, change the media in the 10^0 dilutions after 4 hours in all future testing.

j. If cell death occurs in under one hour, that test condition cannot be used.

k. Cytotoxicity past the 10^{-1} dilution is unacceptable for testing. Alternative neutralizers or column filtration (e.g., Sephadex) may be used to mitigate cytotoxicity. See section 19c for further information on column filtration.

3. **3-Part Soil Effect on Cell Line.**

a. Make the 3-part soil (see section 14e but withhold the virus).

b. Add 10 µL of the soil to 20 mL of CGM, equilibrated to 37±1°C.

c. Remove the CGM from the cells and add 1 mL of this solution to 4 wells on a 24 well plate with a confluent monolayer of cells. Have at least one well as a negative control (e.g., CGM alone).

d. Incubate plate as appropriate and observe daily for cytotoxicity. No cytotoxicity should be observed.

Appendix H
Viral Neutralization Assay

1. The purpose of this section is to assess the effectiveness of the neutralization processes associated with this method. Perform the neutralization assay prior to testing to demonstrate the neutralizer's ability to inactivate the chemical.
2. Select a neutralizing medium that is not inhibitory to the virus and is not cytotoxic to the cells. The acceptance criteria for acceptable neutralization are 0.5 log differences between the neutralization effectiveness, neutralization toxicity control, and titer control. Interaction between the neutralizer and product and its effect on the cell line must be determined prior to testing.
3. **Prepare *Test Suspension A***. Dilute the virus stock suspension in CGM to achieve an average recovered concentration of approximately 2-3 logs (i.e., 100-1000 virus particles) per vessel for the Titer Control sample. To achieve this, dilute the virus stock suspension through 10^{-4} (or as necessary).
4. **Prepare *Test Suspension B***. Prepare the soil load: vortex each component and combine 25 µL bovine serum albumin (BSA), 35 µL yeast extract, 100 µL of mucin, and add 340 µL of *Test Suspension A* (0.5 mL total volume) and mix well. Use *Test Suspension B* within 30 minutes of preparation.
5. **Neutralization Treatments**
    a. ***Treatment 1: Neutralizer Effectiveness.*** Add a coated carrier (one per market relevant lot) to each of three 50 mL conical tubes. At timed intervals, add 20 mL of neutralizer to each 50 mL conical tube and vortex-mix for 30 s on highest vortex setting. Immediately add 20 µL *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix H, section 6.
    b. ***Treatment 2: Neutralizer Toxicity Control.*** Add 20 mL of neutralizer to each of three 50 mL conical tubes. At timed intervals, add 20 µL of *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix H, section 6.
    c. ***Treatment 3: Titer Control.*** Add 20 mL CGM to each of three 50 mL conical tubes. At timed intervals, add 20 µL of *Test Suspension B* to each vessel using a micropipette and briefly vortex-mix. Proceed with Appendix H, section 6.
        i. Note: Steps should be conducted at timed intervals (e.g., 30 s) to ensure consistent time of contact.
6. **Processing and Recovery**
    a. Hold the mixtures from Appendix H, section 5 for 10±1 min at room temperature (21±3°C).
    b. At the conclusion of the holding period, vortex each tube for 3-5 s. Serially dilute the sample as needed (e.g., remove 1 mL of sample and dilute in 9 mL of CGM).

i. Initiate dilution and plating as soon as possible (e.g., within 5 minutes). Two analysts are recommended to perform vortexing and dilution steps to reduce holding time after vortexing.

ii. Titrate the samples for virus infectivity using the appropriate cell line – plate a minimum of 80% of the 10^0 vessel and all dilutions.

iii. For each well plated, add the maximum volume of the well (i.e., add 1 mL per well for a 24 well plate).

iv. Note: If any 10^0 (vessel) dilution is used that does not contain CGM (e.g., Treatment 2 with proposed neutralizer), allow it to adsorb on the cells for 1 hr, then remove and replace with fresh CGM.

7. If cytotoxicity was observed in pre-neutralization testing, CGM may be removed from all wells in the affected dilutions at the appropriate time (one hour minimum), the wells washed with pre-warmed PBS, and then the PBS replaced with fresh CGM. Follow the same procedure for dilutions from the control carriers.
8. Incubate test and control plates as appropriate for the test system.
9. For the neutralizer to be considered effective:
    a. Ensure that the recovered virus in the **Titer Control** using *Test Suspension B* is between approximately 2-3 logs per vessel.
    b. The recovered virus in the **Neutralizer Effectiveness** treatment is within 0.5 logs of the **Titer Control**; this verifies effective neutralization. A log reduction greater than 0.5 logs indicates that the neutralizer was not effective. Note: a value higher than the **Titer Control** is also deemed valid.
    c. The recovered virus in the **Neutralizer Toxicity Control** is within 0.5 logs of the **Titer Control**. A log reduction greater than 0.5 logs indicates that the neutralizer is harmful to the test system. Note: a value higher than the **Titer Control** is also deemed valid.
10. All criteria in Appendix H, section 9 must be met. If the criteria are not met, another neutralizer or mixture of neutralizers must be identified and verified.