US Environmental Protection Agency
Office of Pesticide Programs

Office of Pesticide Programs
Microbiology Laboratory
Environmental Science Center, Ft. Meade, MD

Interim Method for the Evaluation of Bactericidal Activity of Hard, Non-porous Copper-Containing Surface Products

Date Revised: 01-23-2020

Interim Method for the Evaluation of Bactericidal Activity of Hard, Non-porous Copper-Containing Surface Products (01/23/20)

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 hard, non-porous copper-containing surface products with non-food contact surface bactericidal claims. The claim is intended for indoor use only including claims for "continuous reduction of bacteria". The interim method applies to solid copper products, impregnated copper products, and copper coated products (sold as coated pre-market). The interim method includes an efficacy assessment of copper coupons following mechanical abrasion and exposure to certain chemical solutions. Efficacy test results are quantitative and are used to determine the bactericidal activity of the hard, non-porous copper-containing surface products.

Interim Method Overview

In brief, the test method is comprised of two parts; 1) abrasion and chemical treatment, and 2) product efficacy. The method specifies the use of 1" x 1" copper and stainless-steel control carriers. Carriers are exposed to abrasion and chemical treatment five times a day, five days a week for six weeks. The abrasion and chemical exposure process is intended to represent a degree of normal and relevant physical wear, as well as reproduce potential effects resulting from repeated exposure of copper-containing surfaces to three different biocidal materials (chemical solutions). The impact of the abrasion and chemical exposure on the integrity of product carriers is documented – these carriers are considered "exposed" carriers. Under controlled environmental conditions, each product and control carrier receives 20 µL mixture of the test organism and soil load. Following a 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 abrasion and chemical exposure on product efficacy is also determined by a comparison to carriers not exposed to abrasion and chemical treatment.

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 matters experts within the U.S. Department of Health and Human Services (HHS), including experts from the Centers for Disease Control (CDC) and National Institutes for Health (NIH).

Table of Contents

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) *95 – 98% ethanol*. Used to decontaminate carriers prior to efficacy testing.

ii) *De-ionized (DI) Water.* For preparing reagents, media, and rinsing chemical off carriers.

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

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

v) *Neutralizer.* Various 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 (bovine) to 10 mL of PBS, mix thoroughly until dissolved, and autoclave (15 minutes at 121°C), 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) A 3000 ± 150 ppm sodium hypochlorite (NaOCl) solution (e.g., Sigma-Aldrich reagent grade sodium hypochlorite) prepared in deionized water. Verify the final concentration of the solution using a suitable titration method (e.g., Hach digital titrator).

(2) *Solution B*

(a) Use an EPA-registered antimicrobial pesticide product containing hydrogen peroxide (between 3.0% and 6.0%) and peracetic acid as active ingredients that allows spray application to hard, non-porous surfaces. The solution concentration for the peracetic acid component is not limited to a defined range.

(3) *Solution C*

(a) Use an EPA-registered hospital disinfectant product with quaternary ammonium compound as the active ingredient labeled as a one-step cleaner/disinfectant that allows spray 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 (controls):* Die/machine cut 1" x 1" square made from sheet stainless steel (AISI #304). The carriers should physically match the product carriers as closely as possible with respect to thickness, degree of polish and/or brushed surface machining, etc. Carriers are single use.

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

iv) *Copper (test) carriers*: Die/machine cut 1" x 1" square from copper test product, single use.

v) *Cryovials*. For storage of frozen stock culture.

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

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

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

ix) *Kimwipes*. Lint free cloth; used for drying and cleaning carriers.

x) *Microcentrifuge tubes*. For storage of soil single use aliquots.

xi) *3M Scotch Brite Non-scratch scour pads.* UPC 0 5114137319 5

xii) *Petri dishes.* Glass/plastic used as a flat surface for inoculating and incubating carriers. Also used with filter paper for carrier drying, storage, and chemical treatment.

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

xiv) *Spray Bottle*. Used to apply chemical treatments (solutions A, B, and C) to 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 inoculation of test substance on carriers and 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) *Hach Digital Titrator Kit.* For measuring total chlorine.

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

xi) *Microscope* (100x optics and 10x ocular). For observation of Gram stains.

xii) *Refrigerator* (2 – 8°C). Storage of media and post incubated plates.

xiii) *Sonicator capable of producing 45 Hz.* For removal of organism from carriers.

xiv) *Vortex*. For vortex mixing of various solutions including carriers.

2) Carriers

The following section provides guidelines for preparation of both stainless-steel control and test product carriers. Two production lots of the test product should be used to evaluate efficacy. Lot 1 is used for both abrasion/chemical treatments and efficacy. Lot 2 is used for efficacy testing only. See Table 1 for a summary of carrier distribution.

"Exposed" refers to carriers subjected to the physical abrasion and chemical treatment, while "unexposed" refers to those carriers not subjected to the physical abrasion and chemical treatment.

Table 1. Carrier Distribution for Testing Copper-Containing Surface Products

	Carrier Type	# of carriers for *S. aureus*	# of carriers for *P. aeruginosa*
Lot 1	Exposed Product	15	15
	Exposed Stainless Steel	9	9
	Unexposed Product	3	3
	Unexposed Stainless Steel	3	3
Lot 2	Product	5	5
	Stainless Steel	3	3

a) Conduct screening and cleaning of carriers prior to abrasion/chemical exposure and maintain production lot identity throughout the testing process.

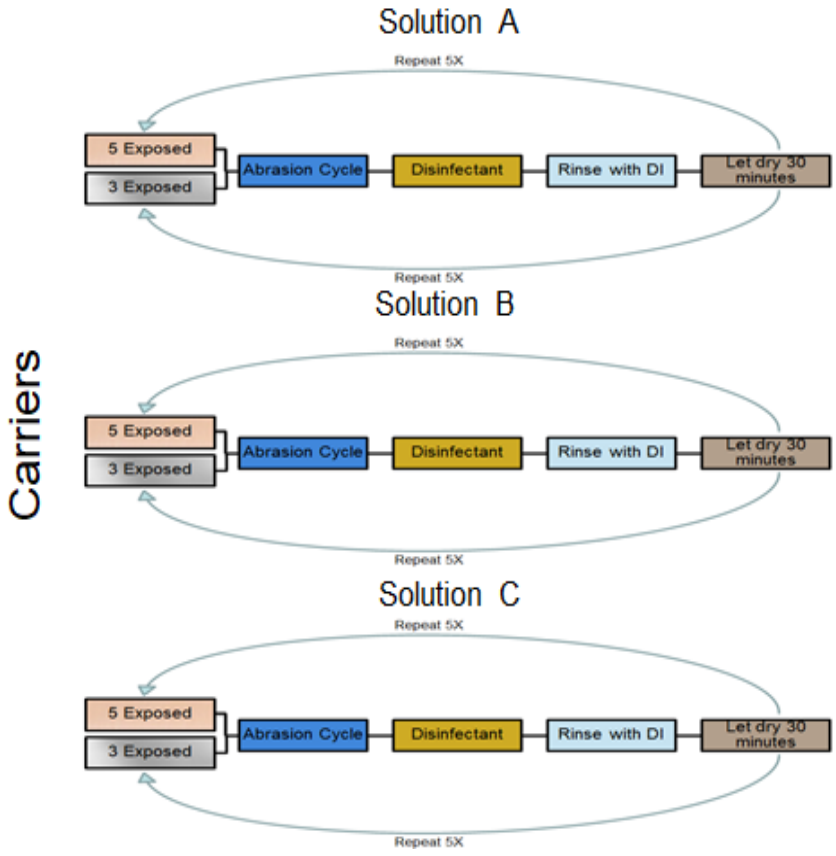
b) Inspect each carrier to ensure uniformity. Discard carriers with visible surface or edge abnormalities (e.g., corrosion/rust, chipping, gouges or deep striations, etc.) (Refer to examples in Appendix B).

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) Prepare at least one additional test and stainless-steel carrier for sterility assessment.

3) Physical Abrasion and Chemical Exposure Treatment Process

Figure 1. Abrasion and Chemical Treatment Diagram – for exposed carriers of a single production lot for 1 day



a) **Preparation for physical abrasion.**

i) Select and prepare carriers as described in Section 2 of this document prior to the abrasion and chemical exposure process.

ii) Orient individual carriers with the copper exposed surface side-up (i.e., the bactericidal copper-containing surface). Maintain this orientation throughout the exposure treatment. For the control stainless steel carriers, either side of the carrier may be selected but maintain the orientation throughout the exposure treatment.

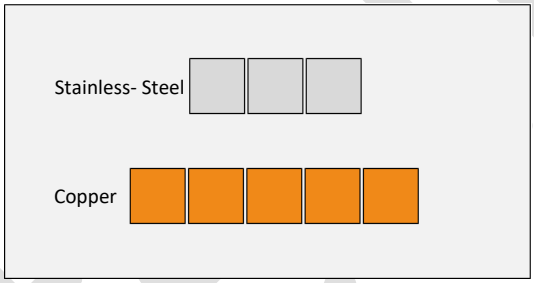
iii) A single abrasion and chemical exposure treatment is twelve passes of an abrasive material (i.e., a non-scratch scour pad) against the product surface, followed by a 20-minute exposure of the product surface to a specific solution.

iv) Perform exposure treatments on Lot 1 five times per day and five days a week for six consecutive weeks, resulting in a total of 150 abrasion and chemical exposures.

v) Maintain all test and control carriers under comparable conditions during each abrasion and chemical solution exposure treatment. Perform the exposure treatment of the test carriers at room temperature.

b) **Conducting the Abrasion Treatment**

Figure 2. Recommended Abrasion Process – Carrier Configuration.



i) Perform the abrasion exposure with the Gardco, Model D10V abrasion tester or comparable equipment. Consult the owner's manual to ensure proper set up and maintenance and calibration.

ii) Calibrate instrument to achieve 2 – 2.5 seconds for one pass and 24 – 30 seconds per abrasion cycle.

iii) The weight of the abrasion boat plus the abrasion material must be 415 to 435 g.

iv) Attach the pad to the abrasion tester as specified in the manual or using double sided tape. Each exposed treatment must have its own abrasion pad. Do not use the same abrasion pad for treated and control carriers. Abrasion pads are to be replaced daily.

v) Load carriers onto abrasion instrument and conduct abrasion cycle.

vi) Copper and stainless-steel carriers should be situated in parallel with one another or back to back for abrasion see Figure 2.

vii) Carriers are not to be adhered to the abrasion instrument; e,g., plastic templates with cut-outs may be used to hold the carriers in place during abrasion process.

viii) One abrasion cycle consists of twelve (12) passes of the abrasive pad against the carrier test surface (the pad to contact the carrier surface twelve times).

ix) Following the abrasion cycle, wipe the carriers with a clean, dry cloth (Kimwipe), and subject the carriers to the chemical solution.

c) **Chemical Solution Treatment**

Table 2. Carrier Exposure to Chemicals per Microbe

Solution A	**Solution B**	**Solution C**
Sodium Hypochlorite (NaOCl)	Hydrogen peroxide	EDTA/phosphoric acid
5 exposed copper product carriers	5 exposed copper product carriers	5 exposed copper product carriers
3 exposed stainless-steel carriers	3 exposed stainless- steel carriers	3 exposed stainless-steel carriers

i) Place the test carriers, copper and stainless steel, abraded surface up on a flat surface (e.g., inside a Petri dish) with absorbant material (e.g., filter paper) so that the carrier does not sit in standing solution. Apply each chemical solution to exposed carriers by spraying (using a mist) two to three pumps of the appropriate solution at 6-8 inches from the carrier surface.

ii) Allow each test carrier to be in contact with the chemical solution for 20 ± 1 minutes at room temperature.

iii) After the contact period, rinse throughly with deionized water (DI) water, air dry, and store uncovered at room temperature until the next exposure cycle.

(1) Wait a minimum of 30 minutes between each exposure cycle.

d) Following the 150 abrasion-chemical exposures, rinse all carriers thoroughly with DI water, air dry, and store at room temperature in covered individual petri dishes lined with filter papers. Visually inspect carrier surfaces (for the designated production lot). Describe and document any physical disruption or degradation of the exposed product surface (e.g., decolorization, cracking, peeling, and chipping).

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

f) Initiate product performance testing within 7 days of completion of the final abrasion-chemical exposure 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 vortexing 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.

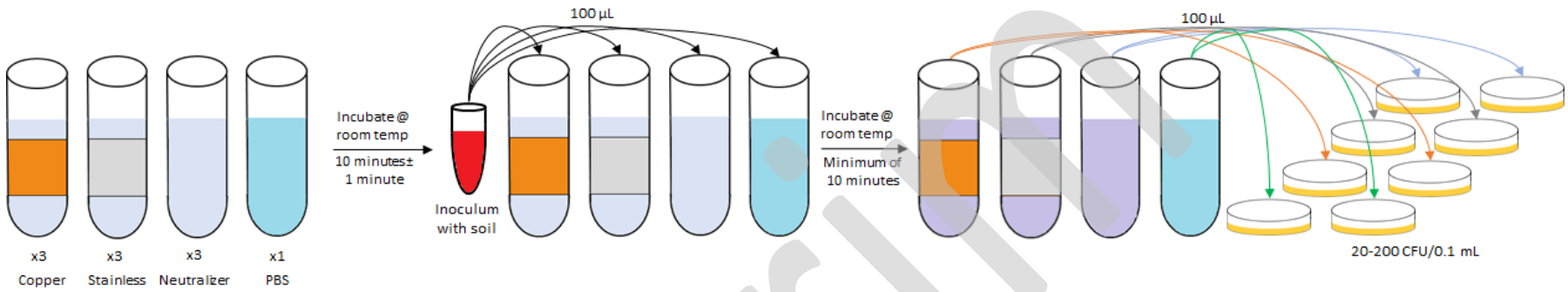
5) Neutralization Assay

The purpose of this section is to assess the effectiveness of the neutralization process associated with this method in neutralizing copper and copper ions. Perform the neutralization assay with both microbes prior to testing to demonstrate the neutralizer's ability to inactivate the test carrier (i.e., residual copper ions in solution). The neutralization of the test carriers is confirmed by using both unexposed copper and unexposed stainless-steel control carriers, the neutralizer (without carriers) as in the test procedure, and a single tube of PBS (used to compare counts from the neutralizer). See Figure 3 for diagram of procedure.

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 test carriers, the control carriers, and the counts for the neutralizer only.

a) Prepare test culture per Section 4: Preparation of Test Culture.
b) In triplicate (x3), add a product carrier (one per production lot) to a tube of neutralizer solution (20 mL). Allow the carrier to remain in the neutralizing solution for 10 ± 1 minute.
c) For controls, add in triplicate (x3), a stainless-steel carrier to a tube of neutralizer solution (20 mL). Allow the carrier to remain in the neutralizer solution for 10 ± 1 minute.
d) Add a 0.1 mL aliquot of a diluted suspension yielding 20 – 200 CFU/0.1 mL per plate. Vortex to mix.
e) Hold the inoculated mixtures for 10 to 20 minutes at room temperature, then vortex-mix. In duplicate, plate 0.1 mL aliquots of this mixed solution using spread plating technique on TSA plates (or TSA with 5% sheep blood).
f) Incubate plates at 36 ± 1°C for 48 ± 4 h. Monitor plates after 24 h of incubation to facilitate appropriate timing for counting the colonies.
g) Following incubation, count the number of colonies and record.

Figure 3. Neutralization Assay



6) Performance Assessment – Efficacy

a) Prior to efficacy testing of test carriers, evaluate three (3) stainless steel carriers from either production lot to determine starting concentration of the inoculum on carriers (T_0).
   i) Neutralize T_0 carriers within 10 minutes of inoculating; do not put into the environmental chamber.

b) After completing the abrasion/chemical exposure (for Lot 1 only) immerse all product and control carriers in 95-98% ethanol for approximately 10 minutes. Using sterile forceps, remove individual carriers and place face up in pre-sterilized Petri dishes (one carrier per dish) lined with filter paper. Allow carriers to dry with lid open. Orient individual carriers with the treated exposed side (i.e., copper surface) up; maintain this orientation. Handle carriers aseptically. Use carriers within 7 days.

c) Efficacy Test Procedure
   i) Prepare test culture per Section 4: Preparation of Test Culture to achieve a final target control count on stainless steel carriers of 4 – 5 logs CFU/carrier after the 2-hour contact time.
   ii) Record the lab and chamber temperature and relative humidity (22 ± 2°C and 30% - 40% relative humidity) during the two-hour contact period.
   iii) Record the time for all timed events.
   iv) Inoculate each carrier with 20 µL of final test culture using a calibrated pipette. 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 with the lid on the Petri dish in the environmental chamber for 2-hours ± 5 minutes. Refer to Appendix C 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 of the appropriate neutralizer solution. Remove and neutralize all carriers within 10 minutes
      (1) The neutralized tube 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 into the neutralizer.
   viii) Prepare serial dilutions of the neutralizer tubes in PBS within 30 minutes.
   ix) Initiate filtration within 30 minutes of preparing dilutions.
   x) 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.
   xi) Use separate 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. For eluates from product carriers remaining in the conical (10^0 dilution), vortex the conical for ~5 s and pour the eluate into the filter unit.

xii) Rinse the treated conical with ~20 mL PBS, vortex for ~5 s and pour the wash into the same filter unit. For dilution tubes, rinse tube once with ~10 mL PBS, briefly vortex, and pour into filter unit.

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

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

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

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

xvii) Incubate control plates at 36±1°C for 48±4 h and unexposed and exposed product plates 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.

xviii) If isolated colonies are present, perform a Gram stain to assess one representative colony per carrier set (exposed, unexposed, and controls).

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

xx) 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) Purity Control:

i) Refer to Section 4.c. The acceptance criteria is culture characteristics consistent with Table 3.

b) Final Test Suspension Purity Control:

i) Refer to Section 4.j. The acceptance criteria is culture characteristics consistent with Table 3.

c) Carrier Sterility Control:

i) Add one un-inoculated test and stainless-steel carrier to individual tubes containing 10 ml of TSB. Incubate at 36 ± 1°C for 48 ± 4 hours and examine for growth. The acceptance criterion for this study control is lack of turbidity in each tube.

d) 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) Calculate the mean log density in viable cells for each microbe for the following treatments: for Lot 1 designated in Table 1.
   i) Exposed product carriers (per chemical)
   ii) Exposed stainless steel carriers (per chemical)
   iii) Unexposed product carriers.
   iv) Unexposed stainless-steel carriers.

b) Calculate the mean log density in viable cells for each microbe for the following treatments: for Lot 2: designated in Table 2.
   i) Product carriers
   ii) Stainless steel carriers

c) Calculate the log reduction values based on the difference in mean log densities associated with the product carriers compared to the stainless-steel control carriers.
   i) Use the exposed stainless-steel control carriers to calculate the log reduction for the exposed product carriers per chemical.
   ii) Use the unexposed stainless-steel control carriers to calculate the log reduction for the unexposed product carriers per chemical.

d) Calculate the difference in log reduction by subtracting the exposed product carriers from the unexposed product carriers.

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

f) 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).

g) 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.

h) Determine the Log Density (LD) of organisms surviving on five exposed test carriers and/or five unexposed test carriers as follows:

$$Mean\ LD = \frac{\text{Log}_{10}(Carrier\ 1) + \text{Log}_{10}(Carrier\ 2) + \text{Log}_{10}(Carrier\ 3) + \text{Log}_{10}(Carrier\ 4) + \text{Log}_{10}(Carrier\ 5)}{5}$$

i) Determine the Log Density (LD) of organisms surviving on three exposed control carriers and/or three nonexposed control carriers as follows:

$$Mean\ LD = \frac{\text{Log}_{10}(Carrier\ 1) + \text{Log}_{10}(Carrier\ 2) + \text{Log}_{10}(Carrier\ 3)}{3}$$

j) Calculate the Log Reduction (LR) of the exposed product carriers compared to exposed control test carriers as follows:

$$LR = Mean\ LD\ (Control\ Carriers) - Mean\ LD\ (Product\ Test\ Carriers)$$

k) Calculate the Log Reduction (LR) of the unexposed product carriers compared to unexposed control test carriers as follows:

$$LR = Mean\ LD\ (Control\ Carriers) - Mean\ LD\ (Product\ Test\ Carriers)$$

l) Calculate the difference between unexposed and exposed log reduction of product carriers.

$$Difference\ in\ LR = LR\ (Unexposed\ Test\ Carriers) - LR\ (Exposed\ Test\ Carriers)$$

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 3. Incubate all plates for 24 ± 2 h at 36 ± 1°C.
    a. Record results at the end of the incubation timeframe. Refer to Table 3 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 3).
    b. Incubate all plates for 24 ± 2 h at 36 ± 1°C. Record results. Refer to Table 3 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 3).
    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 3 at any step in the process, or the Vitek profile is inconsistent with the organism, discard the cultures and re-initiate the process.

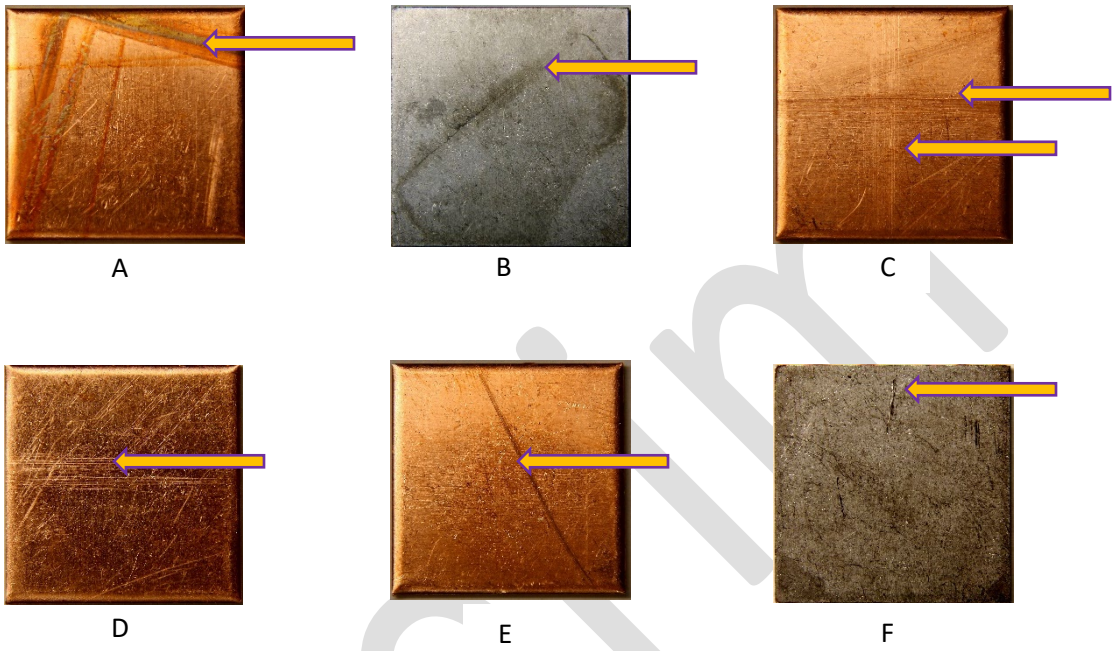
Table 3. Selective media and diagnostic characteristics for *P. aeruginosa* and *S. aureus*.

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

Examples of Failed Physically Screened Carriers



A and B fail due to discoloration of surface.
C and D fail due to surface scratches.
E and F fail due to deep gouge in surface.

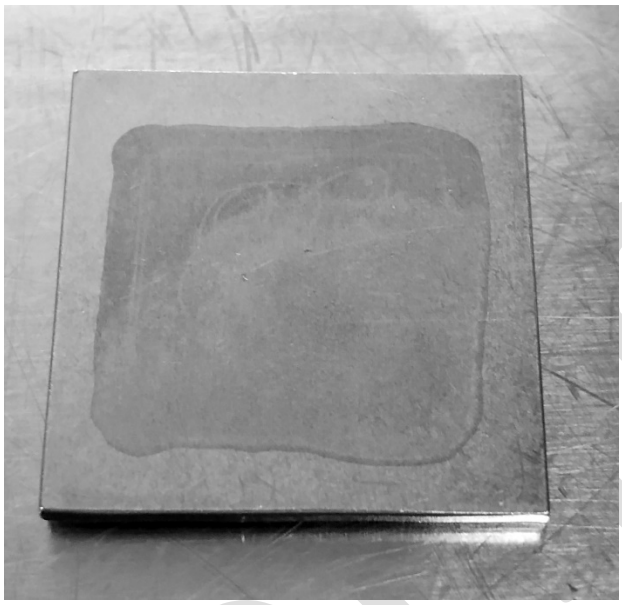
554 **Appendix C**

555

556 **Example of a Dry Inoculated Stainless-Steel Carrier**

557

558



559