MB-41-02	MB-41-02
Title	Method for Evaluation of Antimicrobial Activity of Hard, Non-porous Copper-Containing Surface Products
Revisions Made	• New SOP format ➢ Subsection numbers may have changed from previous versions. • Minor changes to definitions. • Added consistency throughout • Minor editorial changes

MB-41-01	MB-41-01
Title	Method for Evaluation of Antimicrobial Activity of Hard, Non-porous Copper-Containing Surface Products
Revisions Made	• Updated SOP to MLB SOP format which include: • Dextrose addition for test cultures was changed to on the day of inoculated. • Added information on how soil can be removed from viral culture. • Viral control counts increased to 4.0-5.8 logs virus particles/carrier. • The neutralization assay can also be conducted concurrently with testing. • Control counts for viral neutralization assay increased to 3-4 logs per vessel. • Examples of $TCID_{50}$ calculations added as an Appendix. • An example timing scheme was added as an Appendix. • Added test sheets (stored separately from the SOP).

SOP Number:	MB-41-02
Title:	Method for the Evaluation of Antimicrobial Activity of Hard, Non-porous Copper-Containing Surface Products
Scope:	Describes the methodology used for evaluating the durability and efficacy of solid copper products, impregnated copper products, and copper-coated products (sold as coated pre-market) against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and viruses.
Application:	This method provides $\log_{10}$ reduction (LR) as the quantitative measure of efficacy for copper-containing surfaces against the prescribed test microbes.
	Approval
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1.0 Definitions

1.1 Additional abbreviations/definitions are provided in the text.

1.2 Control carriers = stainless steel carriers

1.3 Product carriers = copper or copper-coated carriers

1.4 Cycle (abrasion cycle) = a series of 12 sequential, single passes across the surface of carriers.

1.5 Pass = within an abrasion cycle, one movement across the surface of the carriers.

1.6 Exposed carriers = carriers that are exposed to chemical exposure and abrasion cycles via a non-scratch scour pad on the surface of the abrasion tester tray.

1.7 Unexposed carriers = carriers that are not exposed to chemical exposure and abrasion cycles via a non-scratch scour pad on the surface of the abrasion tester tray.

1.8 g = gram(s)

1.9 h = hour(s).

1.10 min = minute(s).

1.11 s = second(s).

2.0 Health and Safety

2.1 Follow appropriate biosafety practices specified in SOP MB-01, Laboratory Biosafety.

2.2 Consult the Safety Data Sheet for specific hazards associated with antimicrobial products.

3.0 Personnel Qualification and Training

3.1 Refer to SOP ADM-04, OPP Microbiology Laboratory Training.

4.0 Instrument Calibration

4.1 Refer to SOPs EQ-01 (pH meters), EQ-02 (thermometers), EQ-03 (weigh balances), EQ-04 (spectrophotometers), EQ-05 (timers), QC-05 (environmental monitoring system), and QC-19 (pipettes) for details on method and frequency of calibration.

5.0 Sample Handling and Storage

5.1 Refer to SOP MB-22, Preparation and Sampling Procedures for Antimicrobial Test Substances, and SOP COC-01, Chain of Custody Procedures.

6.0 Quality Control

6.1 For quality control purposes, the required information is documented on the appropriate form(s) (see Section 14).

6.2 For viral testing, assess all new reagents and media in parallel with existing reagents and media to ensure that they perform as expected and are sterile. Perform quality control and sterility testing on the cell line to ensure there is no unexpected cytotoxicity.

6.3 Refer to SOP MB-10, Media and Reagents Used in Microbiological Assays, for QC of media and reagents.

7.0 Interferences

7.1 Inadequate neutralization may lead to errors in the measurement of test substance efficacy. Prior to or concurrently with efficacy testing, verify neutralizer effectiveness using the procedure outlined in Appendix 4 for bacteria and/or Appendix 6 for viruses.

7.2 For viral testing:

7.2a The test substance or the neutralizer may interfere with or be cytotoxic to the cell line. Determine this experimentally prior to testing using Appendix 5.

7.2b Cytotoxicity of the cell line may compromise the test system.

7.2c If cells are not viable due to cytotoxicity past the 10^{-1} dilution, utilize an alternative neutralizer or column filtration to reduce cytotoxicity.

7.2d Avoid excess alkalinity of the base mediums and complete growth medium (CGM). Place medium in a CO_2 incubator with a loose cap to allow it to reach normal pH if needed. pH is indicated by the color of the media.

7.2e Presence of atypical cytopathic effect (CPE) may be indicative of a viral or *Mycoplasma* contaminate.

7.2f Extended exposure of the cell line to the air during media removal can result in cell death.

8.0 Non-conforming Data

8.1 For a valid bacterial test, the mean $\log_{10}$ density (LD) for control carriers is 4.0-5.0 log CFU/carrier, with each control carrier exhibiting a LD of 4.0 to 5.0.

8.2 For a valid viral test, the mean $\log_{10}$ density (LD) for control carriers is 4.0-5.8 log viral particles/carrier, with each control carrier exhibiting a LD of 4.0 to 5.8.

8.3 Any level of contamination which interferes with the recording and interpretation of results will result in invalid data.

9.0 Data Management

9.1 Archive data consistent with SOP ADM-03, Records and Archives.

9.2 See Section 13 for appropriate calculations and default values.

9.3 Use approved spreadsheets for calculations.

10.0 Cautions

10.1 Avoid extended soaking of the carriers in water or detergent and prolonged rinsing to reduce risk of corrosion or rusting.

10.2 For storage, ensure carriers are completely dry.

10.3 Ensure quality of media (sterility and performance) is adequate.

10.4 For viral testing:

10.4a Use of different cell culture media, incubation conditions, or animal serum concentrations other than those specified for the viral system can produce inaccurate results.

10.4b Ensure that the cell monolayer in 24 well plates is at the appropriate confluency before testing.

10.4c Ensure that the cell line is free from *Mycoplasma* contamination. Test cells regularly with a *Mycoplasma* detection kit (e.g., MycoAlert Plus).

10.4d Freezing virus stock in aliquots of less than 1 mL will negatively affect the long-term viability of the virus.

11.0 Special Apparatus and Materials

11.1 Apparatus

11.1a -20°C Freezer. Used for storage of soil aliquots.

11.1b -80°C Freezer. Used for storage of frozen stock cultures.

11.1c 0.2 µm pore diameter polyethersulfone (PES) membrane filters, 47 mm diameter. Used for recovery of bacteria. Filtration units (reusable or disposable) may be used.

11.1d 3M Scotch Brite Non-scratch scour pads. UPC 0 5114137319 5

11.1e Abrasion Boat. Used to abrade carriers with non-scratch scour pad attached.

11.1f Copper Carriers, Product carriers (copper or copper-coated). Die/machine cut 1” × 1” square from copper test product, single use.

11.1g Stainless-steel Carriers, Control carriers. Die/machine cut 1” × 1” square made from sheet stainless steel (AISI #304), single use. 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.

11.1h Centrifuge (with rotor capable of achieving 5,000g). Used for bacterial test culture preparation.

11.1i Certified timer. Readable in minutes and seconds, used for tracking of timed events and intervals.

11.1j Conical tubes (e.g., 15 mL, 50 mL). Capable of being centrifuged at 5,000g. Used for culture preparation and neutralization.

11.1k Cryovials. Used for storage of frozen stock culture.

11.1l Dilution tubes (glass/plastic tubes). Used for preparing serial dilutions.

11.1m Environmental chamber. Used for efficacy testing, to hold carriers during microbe contact time at 21±3°C and 30-40% relative humidity.

11.1n Filter paper (Whatman No. 2). Used to line Petri dishes associated with carriers.

11.1o Forceps, sterile. Used to handle carriers and membrane filters.

11.1p Gardco Model D10V or comparable abrasion instrument. Used for simulating wear on carriers.

11.1q Identification system (optional). Used for appropriate identification of test microbes (e.g., VITEK identification system).

11.1r Incubator. Used to incubate test cultures and growth medium plates at 36±1°C.

11.1s Incubator with 5% CO_2. Used for growing cell lines and viruses (when applicable).

11.1t Lint-free cloth (e.g., Kimwipes). Used for drying and cleaning carriers.

11.1u Microcentrifuge tubes. Used for storage of single-use soil aliquots.

11.1v Microscope (e.g., 100X optics and 10X ocular). Used for microbial observation.

11.1w Micropipettes, calibrated. Used with corresponding tips for preparing dilutions.

11.1x 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.

11.1y Positive displacement pipette, capable of delivering 20 µL, calibrated. Used with corresponding tips for carrier inoculation.

11.1z Refrigerator (2-8°C). Used to store media and post-incubated plates.

11.1aa Serological Pipettes (e.g., 10 mL, 25 mL). Used for removing/adding larger volumes of liquid.

11.1bb 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.

11.1cc Spray Bottle. Used to apply chemical treatments (solutions A, B, and C) to carriers.

11.1dd Titration kit (e.g., Hach digital titrator). Used for measuring total chlorine.

11.1ee Vacuum source (in-house line or suitable vacuum pump). Used to facilitate rapid membrane filtration.

11.1ff Vortex. Used for vortex mixing of various solutions including carriers.

11.2 Bacterial Media and Reagents

11.2a Test microbes: *Pseudomonas aeruginosa* (ATCC #15442) and *Staphylococcus aureus* (ATCC #6538)

11.2b 10% (w/v) dextrose solution. Used as a supplement to synthetic broth. Add 5.0 g dextrose to 50 mL deionized 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.

11.2c 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.

11.2d Phosphate buffered saline (PBS), 1X. Used for dilution blanks and filtration. PBS has a pH of approximately 7.0±0.5.

11.2e Selective media (optional). Cetrimide agar (*P. aeruginosa*) and Mannitol salt agar (*S. aureus*). See Table 1 in Appendix 1 for use. Purchase from a reputable source or prepare according to manufacturer's instructions.

11.2f 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.

11.2f.i Alternatively, SB made in-house per the recipe provided in Appendix 7 and AOAC Methods 955.15, 964.02, and 955.14 may be substituted.

11.2g Trypticase Soy Agar (TSA). Used as a recovery medium for bacterial enumeration and purity checks. Prepare TSA according to manufacturer's instructions.

11.2g.i Equivalent commercially prepared agar culture medium may be purchased.

11.2h Trypticase Soy Agar with 5% sheep blood (BAP). Used for performing streak isolation of microbial cultures as a purity check (quality control purposes).

11.2i Trypticase Soy Broth (TSB), (30 g/L). Used for rehydrating lyophilized/frozen vegetative culture of test microorganism. Prepare TSB according to manufacturer's instructions.

11.2j TSB with 15% (v/v) glycerol. Used as a cryoprotectant solution. Suspend 7.5 g TSB in 212.5 mL deionized 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.

11.2j.i Alternatively, purchase broth from a reputable source or prepare according to manufacturer's instructions.

11.2k Gram stain kit. Used for diagnostic staining.

11.3 Viral Media and Reagents

11.3a Test virus: use appropriate virus for efficacy testing required for intended label claim.

11.3b Cell line: use an appropriate cell line for virus selected for efficacy testing.

11.3c Complete Growth Media (CGM). Consisting of Minimum Essential Media and FBS or other medium specified for the test virus. Used for serial dilution. Antibiotics and/or antifungals may be added to reduce potential contamination.

11.3d 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.

11.3e Serum, Heat Inactivated Fetal Bovine Serum (FBS), Horse Serum, etc. that is compatible for use with cell lines. Often used to prepare CGM.

11.3f Dulbecco's Phosphate buffered saline (DPBS) or other equivalent buffer (e.g., PBS, Earle's Balanced Salt Solution). Prepare per manufacturer's guidelines.

11.3g Antibiotic/antifungal. 100x Amphotericin B/Penicillin/Streptomycin solution or other equivalent antibiotic/antimycotic solution. May be used to prevent contamination of cell culture.

11.4 Common Reagents

11.4a 95-98% ethanol. Used to decontaminate carriers prior to efficacy testing.

11.4b Deionized (DI) water. Used for preparation of reagents, media, and rinsing test solutions off carriers.

11.4c Liquinox (or equivalent non-ionic detergent solution). Used to clean and degrease carriers.

11.4d Neutralizer. Various confirmed neutralizers may be used.

11.4d.i The recommended neutralizer for the viral test system is the same medium used to grow the virus (e.g., CGM). If necessary, other ingredients may be added to CGM.

11.4e Soil Load, 3-part. The standard soil load to be incorporated in the final test suspension.

11.4e.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 PES 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.

11.4e.ii Yeast Extract. Add 0.5 g yeast extract to 10 mL of PBS, mix, and pass through a 0.2 µm pore diameter PES 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.

11.4e.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 PES 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.

11.4e.iv See Section 12.3 and 12.4 for addition of soil load to bacterial and viral inoculum, respectively

11.4f Treatments used in chemical exposure of carriers (Appendix 11).

11.4f.i Treatment A. A 3000±150 ppm sodium hypochlorite (NaOCl) solution (e.g., Sigma-Aldrich reagent grade sodium hypochlorite) prepared in sterile deionized water. Verify the final concentration of the treatment using a suitable

titration method (e.g., Hach digital titrator). Identify concentration in the study report.

11.4f.ii Treatment B. Use an EPA-registered hospital disinfectant 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 treatment concentration for the peracetic acid component is not limited to a defined range. Identify product in the study report.

11.4f.iii Treatment C. Use an EPA-registered hospital disinfectant product with quaternary ammonium compound as the active ingredient labeled as a one-step cleaner/disinfectant that allows spray application to hard, non-porous surfaces. Identify product in the study report.

12.0 Procedures and Analysis

12.1 Carriers

12.1a The following section provides guidelines for preparation of both control carriers and product carriers. Two production lots of the 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 Appendix 8 for a summary of carrier distribution. “Exposed” refers to carriers subjected to the physical abrasion/chemical treatment, while “unexposed” refers to those carriers not subjected to the physical abrasion/chemical treatment.

12.1b Screen and clean carriers prior to abrasion/chemical exposure and maintain production lot identity throughout the testing process.

12.1c 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 2.

12.1d Soak screened carriers in a non-ionic detergent solution (e.g., Liquinox) for 2-4 h to degrease and then rinse thoroughly in deionized water. Gently wipe with a clean lint-free cloth and allow to completely dry.

12.1e Prepare at least one additional product carrier and one additional control carrier for sterility assessment.

12.2 Chemical Exposure and Abrasion Treatment Process

12.2a Preparation for physical abrasion

12.2a.i Select and prepare carriers as described in 12.1 of this document prior to the abrasion and chemical exposure process.

12.2a.ii Orient individual carriers with the exposed copper surface side-up (i.e., the antimicrobial copper-containing surface). Maintain this orientation throughout the exposure treatment. For the control carriers, either side of the carrier may be selected but maintain the orientation throughout the exposure treatment.

12.2a.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-min exposure of the product surface to a specific treatment.

12.2a.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.

12.2a.v Maintain all product carriers and control carriers under comparable conditions during each abrasion and chemical treatment exposure treatment. Perform the exposure treatment of the carriers at room temperature (21±3°C).

12.2b Conducting the Abrasion Treatment

12.2b.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.

12.2b.ii Calibrate instrument to achieve 2-2.5 s for one pass and 24-30 s per abrasion cycle.

12.2b.iii 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 product carriers and control carriers. Replace abrasion pads daily.

12.2b.iv The total weight of the abrasion boat plus the abrasion material must be 415 to 435 g.

12.2b.v Load carriers onto abrasion instrument and conduct abrasion cycle.

12.2b.vi Product carriers and control carriers should be situated in parallel with one another (see Appendix 10) or abraded separately.

12.2b.vii Do not adhere carriers to the abrasion instrument; use a template with cut-outs or similar device to hold the carriers in place during abrasion process.

12.2b.viii One abrasion cycle consists of twelve passes of the abrasive pad against the carrier surface (the pad to contact the carrier surface twelve times).

12.2b.ix Following the abrasion cycle, wipe the carriers with a clean, dry, lint-free cloth (Kimwipe), and subject the carriers to the chemical treatment.

12.2c Chemical Treatment

12.2c.i Place the product carriers and control carriers, exposed surface up on a flat surface (e.g., inside a Petri dish) with absorbent material (e.g., filter paper) to absorb excess chemical treatment. Apply each chemical treatment to the exposed carriers by spraying two to three pumps of the appropriate treatment at 6-8 in from the carrier surface.

12.2c.ii Allow each carrier to be in contact with the chemical treatment for 20±1 min at room temperature.

12.2c.iii After the contact period, rinse each carrier thoroughly with deionized water, air dry, and store uncovered at room temperature until the next exposure cycle.

12.2c.iv Wait a minimum of 30 min between each exposure cycle.

12.2d Following the 150 abrasion/chemical exposures, rinse all carriers thoroughly with deionized water, air dry, and store at room temperature in individual covered Petri dishes lined with filter paper. 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).

12.2e After completing the abrasion/chemical exposure (for Lot 1 only) immerse all product carriers and control carriers in 95-98% ethanol for approximately 10 min. 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.

12.2f Include all carrier storage conditions (temperature and humidity range) in the study report.

12.2f.i Initiate efficacy testing within 7 days of completion of the final abrasion/chemical exposure process.

12.3 Preparation of Test Cultures: *P. aeruginosa* and *S. aureus*

12.3a Refer to Appendix 1 for preparation of the frozen stock cultures.

12.3b Defrost a cryovial rapidly to avoid loss in the viability of the preserved cells. Each cryovial is single use only.

12.3c Prior to inoculation, on the day inoculated, 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.

12.3d 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.

12.3d.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.

12.3d.ii Incubate without disrupting the culture (i.e., do not move culture while incubating).

12.3e Following incubation, use the SB cultures to prepare a test suspension for each organism. Record results from the streak isolation plate.

12.3f The 24±2 h culture should exhibit a titer of at least 10^8 CFU/mL. Record time of culture harvest.

12.3g 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).

12.3g.i Remove visible pellicle on surface of medium and around associated interior edges of the tube with vacuum suction.

12.3g.ii 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.

- If necessary, the culture may be harvested from two 10 mL 24 ± 2 h broth cultures to centrifuge a maximum of

10 mL of *P. aeruginosa*, record the associated information.

12.3g.iii Record approximate volume harvested and transferred to 15 mL conical tube.

12.3h For *S. aureus*, briefly vortex the 24±2 h culture and transfer to a 15 mL centrifuge tube.

12.3h.i Record approximate volume harvested and transfer to 15 mL conical tube.

12.3i Within 15 min, centrifuge the 24±2 h harvested broth cultures at 5,000×g for 20 min. Record time of centrifugation.

12.3j 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.

12.3j.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.

12.3k 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 2-h exposure time for a valid test. Record time of test suspension dilution.

12.3k.i Optical density/absorbance (e.g., 650 nm) may be used as a tool to monitor/adjust the diluted test suspension; record if measured.

12.3l Use the diluted culture within 30 min to prepare the final test suspension with the addition of the soil load.

12.3m Vortex-mix the test suspension for 10-30 s.

12.3n 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:

12.3n.i 25 µL BSA stock

12.3n.ii 35 µL yeast extract stock

12.3n.iii 100 µL mucin stock

12.3n.iv Vortex soil suspension for 10 s prior to adding microbial test suspension.

12.3n.v 340 µL microbial test suspension.

12.3o Briefly vortex the final test suspension with 3-part soil load (at room temperature, 21±3°C) and use to inoculate carriers within 30 min of preparation. Record time of final test suspension preparation.

12.3p 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.

12.4 Preparation of Test Culture: Viruses

12.4a Propagate the test virus on the appropriate cell line.

12.4a.i Note: Concentration of the test virus stock (e.g., ultracentrifugation) may be necessary to achieve adequate control counts.

12.4b On the day of testing, defrost a cryovial to avoid loss in the viability of the preserved virus (e.g., place in a 37°C water bath and use within 15 min after thawing). Record time placed in water bath.

12.4b.i Note: Frozen viral stock may be washed in cell culture medium to remove soil prior to testing.

12.4c Dilute the virus stock with cell growth medium to achieve control counts in the range of 4.0 to 5.8 logs virus particles/carrier. Record time of virus stock dilution.

12.4d Use the diluted virus within 30 min to prepare the final test suspension with the addition of the soil load.

12.4e 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):

12.4e.i 25 µL BSA stock

12.4e.ii 35 µL yeast extract stock

12.4e.iii 100 µL mucin stock

12.4e.iv Vortex soil suspension for 10 s prior to adding viral test suspension.

12.4e.v 340 µL virus test suspension

12.4f Briefly vortex the final test suspension with 3-part soil load (at room temperature, 21±3°C) and use to inoculate carriers within 30 min of preparation. Record time of final test suspension preparation.

12.5 Efficacy Assessment

12.5a Conduct efficacy testing on all product carriers and control carriers within 7 days of completing the abrasion/chemical exposure cycles.

12.5b Perform the neutralization assay prior to or concurrently with testing to demonstrate the neutralizer's ability to inactivate the copper and copper ions; see Appendix 4 (bacteria) or Appendix 6 (viruses).

12.5c 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 3 control carriers, placing them in the environmental chamber for 1-2 h (relative to the target contact time), and determining the counts per carrier.

12.5d Prepare test cultures:

12.5d.i For bacteria, see Section 12.3 to achieve a final target control count on control carriers of 4.0-5.0 logs CFU per carrier after 1-2 h (relative to the target contact time).

12.5d.ii For viruses, see Section 12.4 to achieve a final target control count on control carriers of 4.0-5.8 viral particles per carrier after 1-2 h (relative to the target contact time).

12.5e Set environmental chamber to achieve 21±3°C and 30-40% relative humidity during the 1-2 h contact period, record temperature and humidity over the contact period.

12.5f Record the time for all timed events.

12.5g Process product carriers first and stainless carriers last. See Appendix 14 for example timing scheme for Lot #1.

12.5h Inoculate each carrier with 20 µL of final test suspension using a calibrated positive displacement pipette suitable to deliver 20 µL. Spread the inoculum to within 1/8 inch of the edge of each carrier using a sterile transfer loop or the pipette tip. Place in environmental chamber within 10 min of inoculation. Contact time begins once carrier is placed in the environmental chamber.

12.5i Allow carriers to remain in a flat, horizontal position with the lid on the Petri dish in the environmental chamber for 1-2 h. Refer to Appendix 3 for picture of dried inoculum on carrier.

12.6 Bacterial Recovery

12.6a 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.

12.6a.i The tube with the neutralizer and the carrier represents the 10^0 dilution.

12.6b After all the carriers have been transferred into the neutralizer, vortex-mix for 30 s then sonicate for 5±0.5 min at 45 Hz to suspend any surviving organism in the neutralizer. If necessary, refer to Section 11 for sonicator verification.

12.6c Initiate serial dilutions of the neutralizer tubes in PBS within 30 min.

12.6d Initiate filtration within 30 min of preparing dilutions.

12.6e Filter appropriate dilutions which yield countable numbers (up to 200 CFU per plate).

12.6f 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).

12.6g 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.

12.6h 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.

12.6i Swirl the contents of the filter unit and quickly filter with limited pooling of liquid in the filter apparatus.

12.6j Rinse the inside of the surface of the funnel unit with ~20 mL PBS and filter contents.

12.6k Aseptically remove the membrane filter and place onto TSA. Avoid trapping any air bubbles between the filter and agar surface.

12.6l Carrier Sterility Control: add one sterile product carrier and one sterile stainless steel carrier to separate tubes 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.

12.6m Neutralizer Sterility Control: filter approximately 10 mL of neutralizer through PES membrane filter. Incubate at 36±1°C for 48±4 h and examine for growth. The acceptance criterion is lack of growth on filter.

12.6n Incubate plates from Unexposed Stainless Steel carriers at 36±1°C for 48±4 h. Incubate plates from Exposed Product, Exposed Stainless Steel, and Unexposed Product carriers at 36±1°C for 72±4 h; after

72±4 h, incubate plates with no growth an additional 48±4 h and count the number of colonies.

12.6o Monitor all plates (filters) after 24 h of incubation to facilitate appropriate timing for counting colonies.

12.6p For colony counts on filters in excess of 200 CFU, record as Too Numerous to Count (TNTC).

12.6q If isolated colonies are present, perform a Gram stain to assess one representative colony per carrier set (product exposed, product unexposed, stainless steel exposed, stainless steel unexposed).

12.6r 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.

12.6s The results of the streak isolation plates should be consistent with characteristics in Table 1.

12.6t 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.

12.7 Virus Recovery

12.7a 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.

12.7a.i The tube with the neutralizer and the carrier represents the 10^0 dilution.

12.7b 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±0.5 min at 45 Hz; refer to Section 11 for sonicator verification.

12.7c Initiate dilutions within 30 min after neutralization and vortex-mixing.

12.7d Initiate inoculation of cell line within 30 min of preparing the dilutions.

12.7e Plate a minimum of 80% of the volume (8 mL for 10 mL volumes, 16 mL for 20 mL volumes) from the 10^0 vessel and of each dilution tube.

12.7f 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.

12.7g The elution steps for control carriers are the same as for the product carriers; use 10-fold dilutions to achieve 4.0-5.8 logs virus particles/carrier.

12.7h 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).

12.7i 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 (1 h minimum). Wash the wells with pre-warmed PBS, then replace the PBS with fresh CGM.

12.7j Incubate test and control plates as appropriate for the test system.

12.7j.i 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.

13.0 Data Analysis/Calculations

13.1 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.

13.1a 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

i = lower limit of summation (the fewest number of dilutions).

13.1b For viruses, calculate the $TCID_{50}$/carrier (see Appendix 15) or MPN/carrier. Calculate the log density of each carrier by taking the $\log_{10}$ of the density (per carrier).

13.2 Calculate the mean log density (LD) of viable cells or virus particles for each microbe for the carrier sets in Lot 1 (Exposed Product (per chemical

exposure/abrasion treatment, 3 total), Exposed Stainless Steel (per chemical exposure/abrasion treatment, 3 total), Unexposed Product, Unexposed Stainless Steel) 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.

13.3 Calculate the mean LD of viable cells or virus particles for each microbe for the carrier sets in Lot 2 (Unexposed Product, Unexposed Stainless Steel) using the above equation.

13.4 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.

13.5 Conduct additional calculations for Lot #1 and Lot #2; include in study report. See Appendix 12 and 13 for calculations and outcome requirements.

14.0 Forms and Data Sheets

14.1 Test Sheets are stored separately from the SOP under the following file names:

14.1a MB-41-02_F1.docx: Copper Method for Bactericidal Activity: Test Processing Sheet

14.1b MB-41-02_F2.docx: Copper Method for Bactericidal Activity: Test Information Sheet

14.1c MB-41-02_F3.docx: Copper Method for Bactericidal Activity: Serial Dilutions/Plating Tracking Form

14.1d MB-41-02_F4.docx: Copper Method for Bactericidal Activity: Results Sheet

14.1e MB-41-02_F5.docx: Copper Method for Bactericidal Activity: Test Microbe Confirmation Sheet

14.1f MB-41-02_F6.docx: Copper Method for Bactericidal Activity: Neutralization Test Processing Sheet

14.1g MB-41-02_F7.docx: Copper Method for Bactericidal Activity: Neutralization Test Information Sheet

14.1h MB-41-02_F8.docx: Copper Method for Bactericidal Activity: Neutralization Time Recording and Results Sheet

14.1i MB-41-02_F9.docx: Copper Method for Bactericidal Activity: Neutralization Test Suspension Preparation Sheet

14.1j MB-41-02_F10.docx: Copper Method for Virucidal Activity: Test Processing Sheet

14.1k MB-41-02_F11.docx: Copper Method for Virucidal Activity: Test Information Sheet

14.1l MB-41-02_F12.docx: Copper Method for Virucidal Activity: Results Sheet

14.1m MB-41-02_F13.docx: Copper Method for Virucidal Activity: Neutralizer Test Processing Sheet

14.1n MB-41-02_F14.docx: Copper Method for Virucidal Activity: Neutralization Test Information Sheet

14.1o MB-41-02_F15.docx: Copper Method: Test Information Sheet for Abrasion/Chemical Treatment

14.1p MB-41-02_F16.docx: Copper Method: Processing Sheet for Abrasion/Chemical Treatment

14.1q MB-41-02_F17.xlsx: Copper Method for Bactericidal Efficacy: Spreadsheet

14.1r MB-41-02_F18.xlsx: Copper Method for Virucidal Efficacy: Spreadsheet

14.1s MB-41-02_F19.xlsx: Copper Method for Bactericidal Neutralization: Spreadsheet

14.1t MB-41-02_F20.xlsx: Copper Method for Virucidal Neutralization: Spreadsheet

15.0 References

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

15.2 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.

15.3 ASTM Method E1482-23 Standard Practice for Use of Gel Filtration Columns for Cytotoxicity Reduction and Neutralization

Appendix 1

Preparation of Bacterial Frozen Stock Cultures

A1.1 Initiate new stock cultures from lyophilized cultures of *Pseudomonas aeruginosa* and *Staphylococcus aureus* from ATCC (or other reputable vendor) at least every 18 months.

A1.1a New frozen stock culture may be initiated one time using an existing, unexpired frozen stock culture as the source. Begin process at step A1.3 below, by streaking a loopful of the frozen stock culture onto 2 TSA plates.

A1.2 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.

A1.2a For lyophilized cultures received on loops/swabs, as pellets, etc., open and rehydrate the lyophilized organism as indicated by the manufacturer. Transfer the rehydrated culture to a tube of 5-6 mL TSB (e.g., swish inoculated swab/loop in the TSB tube or transfer the rehydrated pellet to the TSB tube). Incubate broth culture at 36±1°C for 24±2 h.

A1.3 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 1. Incubate all plates for 24±2 h at 36±1°C.

A1.3a Record results at the end of the incubation timeframe. Refer to Table 1 for results on selective media and diagnostic characteristics of the test microbes.

A1.4 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).

A1.4a 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 1).

A1.4b Incubate all plates for 24±2 h at 36±1°C. Record results. Refer to Table 1 for results on selective media and diagnostic characteristics of the test microbes.

A1.5 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.

A1.6 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.

A1.7 Immediately after mixing, dispense 0.5-1.0 mL aliquots of the harvested suspension into cryovials; these represent the frozen stock cultures.

A1.7a 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 1).

A1.7b Incubate all plates for 24±2 h at 36±1°C.

A1.7c Record results.

A1.7d After incubation, perform a Gram stain on growth from the BAP; observe the Gram reaction by using brightfield microscopy at 1000X magnification (oil immersion).

A1.7e Conduct confirmation using an automated identification system (i.e., Vitek) or biochemical and antigenic analyses from growth taken from the BAP according to the manufacturer's instructions.

A1.7f Record all confirmation results.

A1.8 Store the cryovials at approximately -80°C for a maximum of 18 months. These cultures are single use only.

A1.9 If the characteristics of the organism are not consistent with the information in Table 1 at any step in the process, or the Vitek profile is inconsistent with the organism, discard the cultures and re-initiate the process.

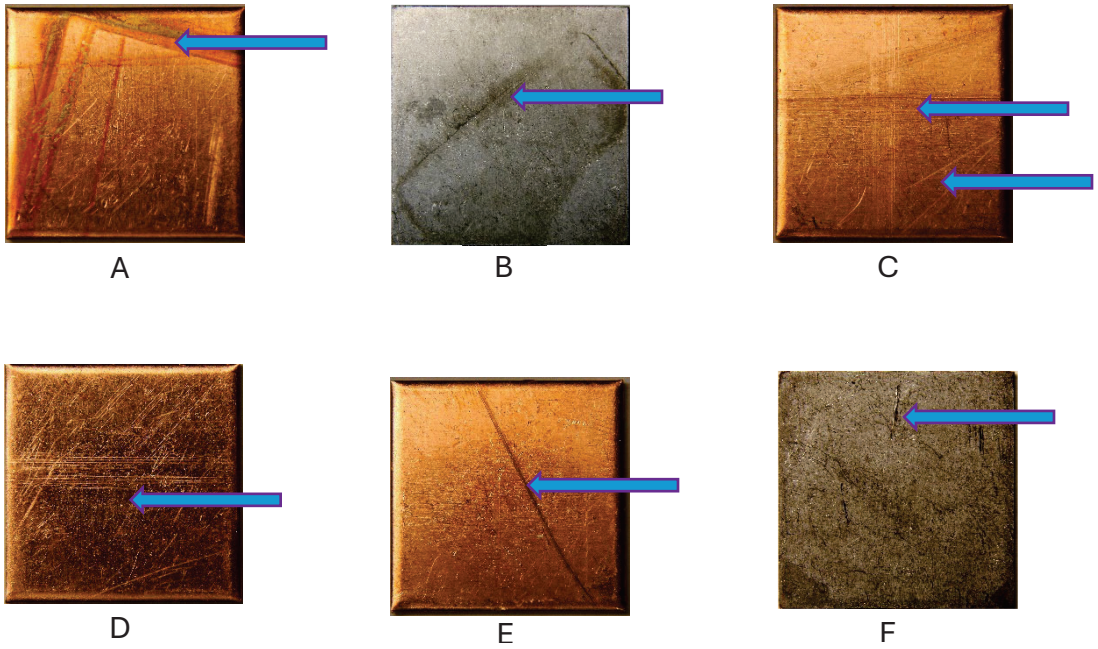
Table 1. Selective media and diagnostic characteristics for *P. aeruginosa* and *S. aureus* (see References 15a-b).

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 × 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 2

Examples of Failed Physically Screened Carriers



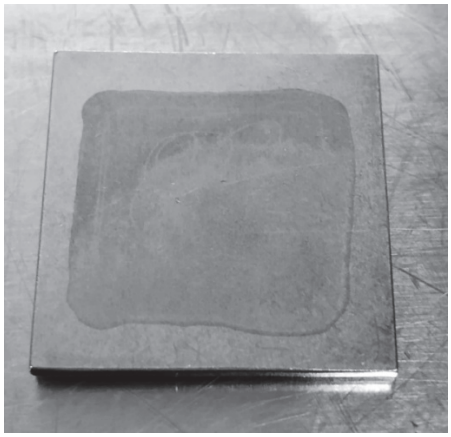
A and B fail due to discoloration of surface.

C and D fail due to surface scratches.

E and F fail due to deep gouge in surface.

Appendix 3

Example of a Dry Inoculated Stainless Steel Carrier



Appendix 4

Bacterial Neutralization Assay

A4.1 The neutralization of the product (unabraded) carriers is confirmed in triplicate by using product carriers in neutralizer, neutralizer only (without carriers), and PBS only (used to compare counts from the neutralizer and product carriers).

A4.2 NOTE: The neutralization assay can be performed prior to or concurrently with efficacy testing.

A4.3 **Preparation of the Test Organism**

A4.3a Refer to the preparation of test cultures (Section 12.5d) 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.

A4.3b **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.

A4.3b.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.

A4.3b.ii A calibration curve (OD @ 650 nm) may be used to estimate the number of viable organisms in *Test Suspension A*.

A4.3c **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.

A4.3c.i Ensure at least one preparation of *Test Suspension B* provides an average challenge of 20-200 CFU per 20 µL.

A4.4 **Neutralization Assay Components**

A4.4a ***Treatment 1: Neutralizer Effectiveness.*** Add a product 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 Section A4.5.

A4.4b ***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 Section A4.5.

A4.4c ***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 Section A4.5.

A4.5 **Processing and Recovery**

A4.5a Hold the mixtures from Appendix 4, Section 4 for 10±1 min at room temperature (21±3°C).

A4.5b At the conclusion of the holding period, vortex-mix each tube.

A4.5c Initiate filtration as soon as possible (e.g., within 30 min).

A4.5d 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).

A4.5e 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.

A4.5f 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.

A4.5g Incubate plates at 36±1°C for 48±4 h and count the colonies.

A4.5h Incubate plates with no growth or few colonies an additional 24±4 h and count the number of colonies.

A4.6 Data Analysis/Calculations

A4.6a 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.

A4.6b 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.

A4.6b.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.

A4.6c To verify effectiveness of the neutralizer, the average number of CFU in the **Neutralizer Effectiveness** treatment is at least 50% of the **Titer Control**.

A4.6c.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.

A4.6d If the criteria are not met, verify another neutralizer or mixture of neutralizers.

Appendix 5

Cytotoxicity Determination

A5.1 Prior to performing the neutralization assay, ensure the proposed neutralizer and product (unabraded) carriers used do not impact the quality of the cell line by performing the following:

A5.2 **Neutralizer Effect on Cell Line (for neutralizers other than CGM).**

A5.3 Add 0.5 mL of the proposed neutralizer to 4.5 mL CGM, 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.

A5.4 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).

A5.5 Incubate plate as appropriate and observe closely for cytotoxicity.

A5.6 If cytotoxicity is observed after at least 1 h, 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.

A5.7 If cell death occurs in under 1 h, the neutralizer cannot be tested.

A5.8 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 Reference 15.3 for further information on column filtration.

A5.9 **Neutralizer Plus Test Carrier Effect on Cell Line.**

A5.10 Add one product carrier (representative of Lot 1 testing) to 20 mL of neutralizer, equilibrated to 21±3°C, and vortex 2-3 s. Let this solution sit at room temperature for 10 min.

A5.11 Add 1.0 mL of this solution to 9 mL CGM, 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.

A5.12 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 test carrier and dilutions. Plate at least 8 wells for the 10^{0} dilution, 6 wells for the 10^{-1} dilution, and 4 wells 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.

A5.13 For highly toxic test chemicals, washing the cells with pre-warmed DPBS before the addition of CGM will help remove cytotoxicity.

A5.14 Have at least one well on each plate as a negative control (e.g., CGM).

A5.15 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.

A5.15a For the 10^0 dilution:

A5.15a.i On the day of the test, change two wells 1-2 h (1-h minimum) after the neutralized test chemical mixture was added to the cells.

A5.15a.ii Change two more additional wells 3-5 h after the neutralized test chemical mixture was added to the cells.

A5.15a.iii The following day, change one 1-2 h well, one 3-5 h well, and one previously unchanged well, plus one of the two previously changed wells.

A5.15b For the 10^{-1} dilution:

A5.15b.i On the day of the test, change two wells 3-5 h after the neutralized test chemical mixture was added to the cells.

A5.15b.ii The following day, change one 3-5 h well and one previous unchanged well.

A5.15c For the 10^{-2} dilution:

A5.15c.i On the day after the test, change one well.

A5.16 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.

A5.17 Score the cells as toxic or non-toxic in each in each test conditions.

A5.18 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.

A5.18a **Example:** In the 10^0 dilution, if the unchanged wells are toxic, but both the 1 h and 4 h media changes are non-toxic, change the media in the 10^0 dilutions after 4 h in all future testing.

A5.19 If cell death occurs in under 1 h, that test condition cannot be used.

A5.20 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 Reference 15.3 for further information on column filtration.

Appendix 6

Viral Neutralization Assay

A6.1 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 product (unabraded) carrier.

A6.2 NOTE: The neutralization assay can be performed prior to or concurrently with efficacy testing.

A6.3 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.

A6.4 **Prepare *Test Suspension A***. Dilute the virus stock suspension in CGM to achieve an average recovered concentration of approximately 3-4 logs (i.e., 1000-10000 virus particles) per vessel for the Titer Control sample. To achieve this, dilute the virus stock suspension through 10^{-4} (or as necessary).

A6.5 **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 min of preparation.

A6.6 **Neutralization Treatments**

A6.6a ***Treatment 1: Neutralizer Effectiveness.*** Add a product 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 6, Section 7.

A6.6b ***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 6, Section7.

A6.6c ***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 6, Section 7.

A6.6c.I Note: Steps should be conducted at timed intervals (e.g., 30 s) to ensure consistent time of contact.

A6.7 **Processing and Recovery**

A6.7a Hold the mixtures from Section 6 for 10±1 min at room temperature (21±3°C).

A6.7b 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).

A6.7b.I Initiate dilution and plating as soon as possible (e.g., within 5 min). Two analysts are recommended to perform vortexing and dilution steps to reduce holding time after vortexing.

A6.7b.II Perform serial dilutions of the samples for virus infectivity using the appropriate cell line – plate a minimum of 80% of the 10^0 vessel and all dilutions.

A6.7b.III For each well plated, add the maximum volume of the well (i.e., add 1 mL per well for a 24 well plate).

A6.7b.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 h, then remove and replace with fresh CGM.

A6.8 If cytotoxicity was observed in pre-neutralization testing, CGM may be removed from all wells in the affected dilutions at the appropriate time (1 h minimum), the wells washed with pre-warmed DPBS, and then the DPBS replaced with fresh CGM. Follow the same procedure for dilutions from the control carriers.

A6.9 Incubate test and control plates as appropriate for the test system.

A6.10 For the neutralizer to be considered effective:

A6.10a Ensure that the recovered virus in the **Titer Control** using *Test Suspension B* is between approximately 3-4 logs per vessel.

A6.10b 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.

A6.10c 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.

A6.11 All criteria in Section 10 must be met. If the criteria are not met, another neutralizer or mixture of neutralizers must be identified and verified.

Appendix 7

Recipe for Synthetic broth

A7.1 Solution A**:** Dissolve the following in 500 mL H_2O containing 18 mL 1 N NaOH:

A7.1a 0.05 g L-cystine

A7.1b 0.37 g DL-methionine

A7.1c 0.4 g L-arginine×HCl

A7.1d 0.3 g DL-histidine

A7.1e 0.85 g L-lysine×HCl

A7.1f 0.21 g L-tyrosine

A7.1g 0.5 g DL-threonine

A7.1h 1.0 g DL-valine

A7.1i 0.8 g L-leucine

A7.1j 0.44 g DL-isoleucine

A7.1k 0.06 g glycine

A7.1l 0.61 g DL-serine

A7.1m 0.43 g DL-alanine

A7.1n 1.3 g L-glutamic acid×HCl

A7.1o 0.45 g L-aspartic acid

A7.1p 0.26 g DL-phenylalanine

A7.1q 0.05 g DL-tryptophan

A7.1r 0.05 g L-proline

A7.2 Solution B: Dissolve the following in 500 mL H_2O:

A7.2a 3.0 g NaCl

A7.2b 0.2 g KCl

A7.2c g $MgSO_4$×7 H_2O

A7.2d 1.5 g KH_2PO_4

A7.2e 4.0 g Na_2HPO_4

A7.2f 0.01 g thiamine×HCl

A7.2g 0.01 g niacinamide

A7.3 Mix Solutions A and B, and steam sterilize 20 min at 121°C.

A7.4 Final pH should be 7.1±0.1.

Appendix 8

Carrier Distribution for Testing Copper-Containing Surface Products

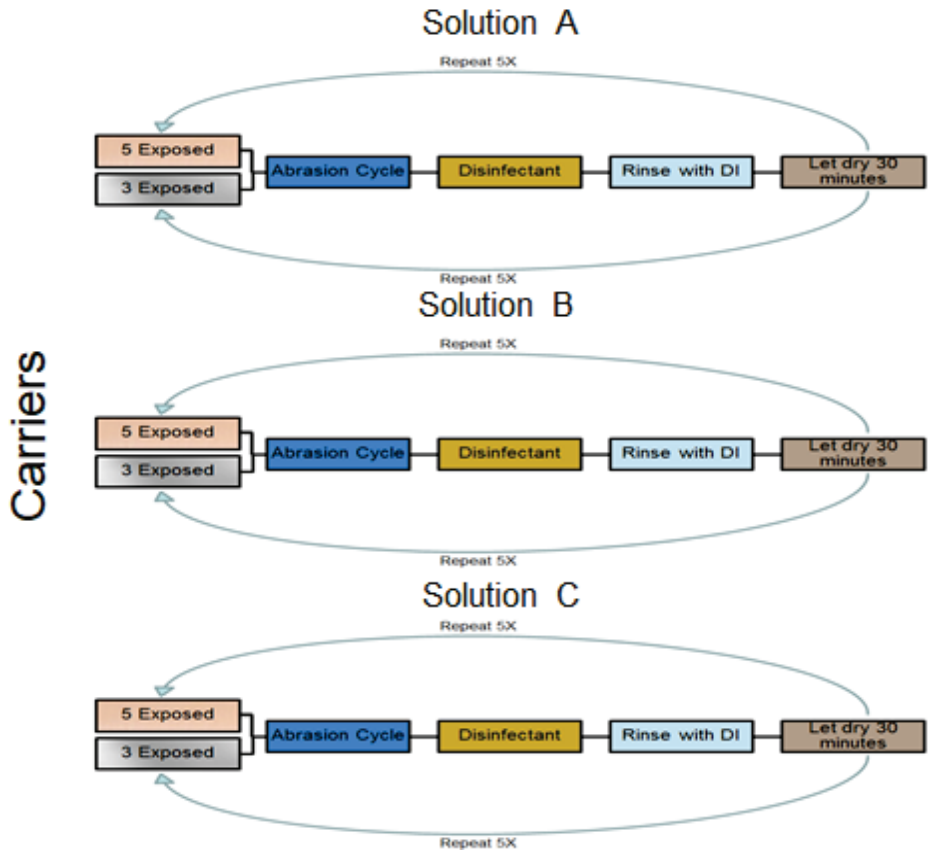
	Carrier Set	# of carriers per bacteria	# of carriers per virus
Lot 1	Product Set #1: Exposed Product	5 per exposure* (15 total)	5 per exposure* (15 total)
	Control Set #1: Exposed Stainless Steel	3 per exposure* (9 total)	3 per exposure* (9 total)
	Product Set #2: Unexposed Product	3	3
	Control Set #2: Unexposed Stainless Steel	3	3
	Total Carriers for Lot #1	30	30
Lot 2	Product Set #2: Unexposed Product	5	5
	Control Set #2: Unexposed Stainless Steel	3	3
	Total Carriers for Lot #2	8	8

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

NOTE: Control Set #2 can be shared for Lot 1 and Lot 2 if the residual product testing of both lots is performed concurrently (on the same test date).

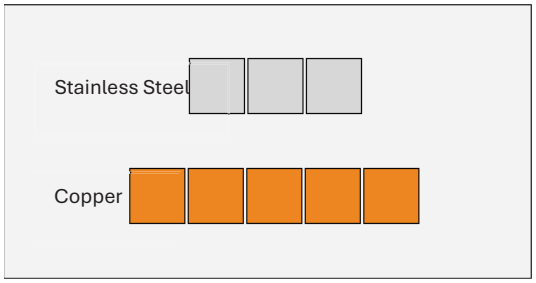
Appendix 9

Preparation for Physical Abrasion



Appendix 10

Recommended Abrasion Process – Carrier Configuration



Appendix 11

Carrier Exposure to Chemical Treatments

Treatment A	Treatment B	Treatment C
Sodium Hypochlorite (NaOCl)	Hydrogen peroxide	Quaternary ammonium
5 exposed product carriers	5 exposed product carriers	5 exposed product carriers
3 exposed stainless steel carriers	3 exposed stainless steel carriers	3 exposed stainless steel carriers

Appendix 12

Additional calculations for Lot #1

LOG DIFFERENCE BETWEEN UNEXPOSED AND EXPOSED STAINLESS STEEL				Outcome (Difference)
Difference between Unexposed Stainless Steel and Exposed Stainless Steel	Mean LD Unexposed Stainless Steel	–	Mean LD Exposed Stainless Steel: Solution A	≤ 0.5
	Mean LD Unexposed Stainless Steel	–	Mean LD Exposed Stainless Steel: Solution B	≤ 0.5
	Mean LD Unexposed Stainless Steel	–	Mean LD Exposed Stainless Steel: Solution C	≤ 0.5
LOG REDUCTION CALCULATIONS				**Outcome (LR)**
LR Unexposed Product	Mean LD Unexposed Stainless Steel	–	Mean LD Unexposed Product	≥ 3.0
LR Exposed Product	Mean LD Unexposed Stainless Steel	–	Mean LD Exposed Product: Solution A	≥ 3.0
	Mean LD Unexposed Stainless Steel	–	Mean LD Exposed Product: Solution B	≥ 3.0
	Mean LD Unexposed Stainless Steel	–	Mean LD Exposed Product: Solution C	≥ 3.0
LOG DIFFERENCE BETWEEN UNEXPOSED AND EXPOSED PRODUCT				**Outcome (Difference)**
Difference between Unexposed Product and Exposed Product	Mean LR Unexposed Product	–	Mean LR Exposed Product: Solution A	≤ 1.0
	Mean LR Unexposed Product	–	Mean LR Exposed Product: Solution B	≤ 1.0
	Mean LR Unexposed Product	–	Mean LR Exposed Product: Solution C	≤ 1.0

Appendix 13

Additional calculations for Lot #2

LOG REDUCTION CALCULATIONS				Outcome (LR)
LR Unexposed Product	Mean LD Unexposed Stainless Steel	–	Mean LD Unexposed Product	≥ 3.0

Appendix 14

Example Timing Scheme for Lot #1

Carrier #	Inoculation start time	Inoculation end time by	Contact time start (place in environmental chamber)	Contact time ends	Neutralizer carrier time start	Neutralizer carrier time end
1-30	00:00:00	00:10:00	00:10:00	01:10:00	01:10:00	01:20:00

Carriers 1-5: Exposed product (Treatment A), **Carriers 6-10**: Exposed product (Treatment B), **Carriers 11-15**: Exposed product (Treatment C), **Carriers 16-18**: Exposed stainless steel (Treatment A), **Carriers 19-21**: Exposed stainless steel (Treatment B), **Carriers 22-24**: Exposed stainless steel (Treatment C), **Carriers 25-27**: Unexposed product, **Carriers 28-30**: Unexposed stainless steel.

Appendix 15

Spearman-Karber $TCID_{50}$ Calculations

Negative Log_{10} ($TCID_{50}$/assayed volume) =

$$-Log_{10}(Initial\ plated\ dilution) - \left[\left(\left(\frac{\sum of\ \%\ infection\ at\ each\ dilution}{100}\right) - 0.5\right)(1)\right]$$

Log_{10} ($TCID_{50}$/1 mL):

$$Log_{10}(TCID_{50}/Volume\ Assayed) + Log_{10}(\frac{1\ mL}{Volume\ Assayed})$$

Log_{10} ($TCID_{50}$/Carrier) =

$$Log_{10}(TCID_{50}/1\ mL) + log_{10}(Total\ Volume\ of\ Neutralizer\ )$$

Log_{10} Reduction:

$$Log_{10}(TCID_{50}/Carrier\ of\ Plated\ Recovery\ Control) - Log_{10}(TCID_{50}/Carrier\ of\ Treated\ Carrier)$$

Note 1: All volumes used in the calculation should be in mL.

Note 2: Cytotoxic wells are considered positive for calculation purposes.