US Environmental Protection Agency
Office of Pesticide Programs

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Protocol for the Evaluation of Bactericidal Activity of Hard, Non-porous Copper/Copper-Alloy Surfaces

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I. Overview

This document describes a testing protocol recommended by the Environmental Protection Agency (EPA) to support the registration of hard non-porous copper and copper alloy-based surface products with non-food contact surface sanitizer claims. The following items summarize the approach employed in this protocol to support these product claims:

- A detailed product characterization is recommended to provide information on the product's physical durability and chemical stability as they relate to the proposed use patterns. The durability assessment includes a 12 week abrasion and chemical exposure process.

- Efficacy testing involves the evaluation of two product production lots against *Staphylococcus aureus*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*, with the carriers from one of these lots having undergone the abrasion and chemical exposure process.

- An effective product is expected to achieve a 3 $\log_{10}$ reduction (LR) in viable bacteria (compared to the control) for all three microbes within a 1 hr contact period. Additional details on the performance standard are described in the Product Performance Data section.

Efficacy test results will be used to determine the sanitizing activity of the copper-containing surface product by comparing the reduction in viable bacteria on product carriers to the control carriers. If alterations to this protocol are deemed necessary by an applicant, the modified test protocol should be submitted to the EPA for review in advance of data generation with all requested changes to the procedure clearly identified. Note that this protocol is applicable to non-porous copper-containing surface products that are intended for indoor use only. Applicants interested in pursuing registrations with outdoor uses or for porous copper-containing surface products should consult with the EPA regarding protocol development and testing to support these uses. This procedure has been established for use with copper-containing materials; however, the procedure may also be appropriate to test other solid, non-food contact surfaces for antimicrobial activity.

Product performance testing is conducted on two production lots; one with exposed carriers and the second with unexposed carriers. The term "exposed" refers to carriers used in the physical and chemical assessment, while "unexposed" carriers have not been subjected to the physical and chemical assessment. Table 1 provides an overview of the

carrier testing requirements.

Table 1. Carrier distribution for testing of copper-containing surfaces*

Product Lot	Product Carriers	Control Carriers
Lot 1**	15 exposed carriers (5 for each of three chemical solutions)	3 unexposed product carriers and 3 unexposed stainless steel carriers
Lot 2***	5 carriers	3 stainless steel carriers

*Three microbes are evaluated for determining product efficacy (see Section IV. B)

** Product carriers exposed to abrasion and chemical solutions

***Product carriers not exposed to abrasion or chemical solutions

II. Product Characterization

Since both the physical durability and chemical stability of a hard non-porous antimicrobial product are critical to the level of efficacy performance over time, a profile of certain product characteristics should be submitted in support of registration. This section describes information that should be addressed in the product characterization submission. The information provided in this submission should pertain to the specific product and product components (as identified in the Confidential Statement of Formula), including all proposed formulation types and potential product variations. Attributes should include:

1. Define all product manufacturing and application processes, product compositions/ formulations, and proposed product use patterns/use sites. For products that involve the application of copper-containing material to a non-copper substrate for the purpose of forming a copper coating (either pre- or post-sale), describe each type of substrate material, in detail, that is proposed for use with the product. If the product proposes use with metal substrates, a discussion of metal substrate compatibility with the copper/copper-alloy component (coating) is recommended.

2. Describe the potential for physical disruption of the product surface (e.g., cracking, peeling, and chipping) resulting from normal use in relation to the proposed use patterns. The results of standardized surface hardness testing would be relevant information for most hard surface products. Describe the expected duration of use after product application/installation, and the potential for atypical physical or chemical challenges that could result from any of the proposed uses.

3. Describe the product surface characteristics including the thickness of the surface layer (if applicable), typical surface morphology, and any intentionally

manufactured features (e.g., gloss, matte). If nanostructures/nanomaterials are known to be present or are likely to be present on the product surface, a thorough description of these characteristics or components should be provided.

III. Abrasion and Chemical Exposure Process Overview

As noted above, this protocol includes a product assessment of the effects of mechanical abrasion and of exposure to certain chemical solutions. 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 cleaning materials (chemical solutions). A single abrasion and chemical exposure treatment involves six passes of an abrasive material against the product surface, followed by a 10 minute exposure of the product surface to a specific solution. These exposure treatments are to be performed on one production lot three times a day and five days a week for twelve consecutive weeks, resulting in a total of 180 total exposures (abrasion/chemical).

Specifically, the assessment involves the use of 15 copper product test carriers, 3 copper product control carriers, and 3 stainless steel control carriers from one production lot. The copper product control carriers and the stainless steel control carriers do not receive the abrasion and chemical solution exposures. The fifteen copper product test carriers are subjected to mechanical surface abrasion followed by exposure to one of three different chemical solutions (solutions A, B or C) in groups of 5 (see Table 2). Following the chemical exposure, the exposed carriers should be rinsed thoroughly with deionized or distilled water, air-dried and stored at room temperature until the next exposure cycle. Each group of 5 product test carriers should be uniquely identified and exposed to the same chemical solution for each exposure treatment during the 12 week process.

Table 2. Carrier exposure to chemicals

Product Lot	**Solution A** Sodium Hypochlorite (Bleach)	**Solution B** Hydrogen peroxide	**Solution C** EDTA/ phosphoric acid	**Controls** (do not receive abrasion and chemical exposures)
Lot 1	5 test carriers	5 test carriers	5 test carriers	3 copper product carriers and 3 stainless steel carriers

Product performance testing should be initiated within 2 weeks of completion of the 12 week exposure regimen. All carrier storage conditions (temperature and humidity range) should be included in the study report. As indicated in Section I, three groups of 15 product test carriers (one 15-carrier group per microbe) that have undergone the abrasion and chemical exposure process and 6 control carriers (21 total) are evaluated for efficacy

according to the laboratory methodology identified in Section IV. Each group (3) of 21 carriers is tested against one of the three test microbes identified in the method. In addition, testing is conducted on 5 product test carriers from an unexposed product lot (i.e., a second lot) and 3 stainless steel control carriers. Identification of product test carriers by the type of chemical solution used during the exposure process should be maintained throughout product performance testing.

A. Abrasion and Chemical Exposure Treatment Process

Carriers should be selected and prepared as described in Section IV (A) of this document for the abrasion and chemical exposure process. Note that all copper product test and control carriers must be cut from the relevant hard, non-porous copper-containing materials anticipated for final production. Individual carriers should be oriented with the copper treated surface side-up (i.e., the sanitizing copper-containing surface); this orientation should be maintained throughout the exposure treatment. All test and control carriers should be maintained under comparable conditions during each abrasion and chemical solution exposure treatment. The exposure treatment of the test carriers should be performed at room temperature.

The abrasion and chemical exposure treatment should be performed 3 times per day with each at least 2 hours between each treatment. As indicated, these daily exposure treatments should be performed 5 days per week for 12 consecutive weeks, after which a visual inspection of all carrier surfaces should be performed. Any visual changes to the product test carrier surfaces in comparison to the unexposed product controls (such as discoloration, pitting or the presence of scratches) should be recorded and included in the final study report submitted to EPA.

1. Chemical Solution Preparation

The treatment solutions to be applied during the chemical exposure process are identified below. All solutions should be clearly labeled, and new solutions should be prepared each day of treatment.

- **Solution A:** Solution A is a 3000 ± 150 ppm sodium hypochlorite solution prepared in distilled or deionized water. This solution should be formulated from an EPA-registered sodium hypochlorite product that allows spray application to hard surfaces. All details related to the source product and dilution process (if applicable) should be included in the study report. The final concentration of the solution should be verified and recorded.

- **Solution B:** Solution B should contain hydrogen peroxide (between 3.0% and 6.0%) and ethaneperoxoic acid as active ingredients. This solution should be an EPA-registered antimicrobial pesticide product that allows spray application to hard surfaces. The solution concentration for the ethaneperoxoic acid component is not

limited to a defined range, but should be indicated in the study report. All details related to the product selected and the dilution process (if applicable) should be included in the study report.

- **Solution C:** Solution C should contain between 5.0% and 5.2% ethylenediamine-tetraacetic acid, tetrasodium salt (CAS# 64-02-8) and phosphoric acid between 8.0% and 8.3%. Distilled or deionized water should be used as the diluent.

2. **Conducting the Abrasion Treatment**

- The abrasion exposure should be performed with a Gardco, Model D10V abrasion tester. A 3M Scotch-brite, General Purpose Hand Pad 7447 should be used as the abrasive material. Attach the pad to the abrasion tester as specified in the product use manual.

- The weight of the fully assembled abrasion boat should be between 1000 g and 1085 g.

- One abrasion cycle should consist of six (6) passes of the abrasive pad against the carrier test surface (the pad should contact the carrier surface six times).

- The abrasion tester speed should be set between 2.25 to 2.50 for a total surface contact time of approximately 6 seconds per treatment.

- Following the abrasion cycle, the carriers should be wiped with a clean, dry cloth and subjected to the chemical solution.

- Proceed to the chemical solution treatment

3. **Conducting the Chemical Solution Treatment**

- After preparing the chemical solutions (solutions A, B and C), place the test carriers, treatment surface up, on a flat surface (e.g., inside a Petri dish).

- Apply each chemical solution to 5 test carriers by spraying two to three pumps of the appropriate solution to each carrier surface (i.e., enough liquid to cover the carrier surface).

- Allow each test carrier to be in contact with the appropriate chemical solution for 10 mins at room temperature.

- After the 10 mins contact period, rinse thoroughly with distilled or deionized water, allow to air dry, and store at room temperature.

- Following the 180 abrasion-chemical exposures, rinse all carriers thoroughly with distilled or deionized water, allow to air dry, and store at room temperature. Proceed

to product performance testing.

IV. Test Methodology

A. Carriers.

For testing of three microbes, from one production lot prepare forty-five (45) *exposed* product test carriers (from copper-containing product), nine (9) *unexposed* product control carriers, and nine (9) stainless steel control carriers; and from a second production lot, prepare fifteen (15) *unexposed* product test carriers and nine (9) stainless steel control carriers. Extra carriers should be prepared for sterility assessment. The steel stock sheets used for the stainless steel control carriers should physically match the product carriers as best as possible with respect to thickness, degree of polish and/or brushed surface machining, etc. The composition of the copper-containing test carriers must be representative of the final product and meet the specifications for the target chemistry formulation. The chemical composition of the treated test product carriers must be documented.

1. Product test material and stainless steel stock material sheets should be die/machine cut into individual approximate 1in ×1in square carriers in similar fashion to minimize variation in size and cut edge artifacts.
2. Each carrier should be physically screened to insure uniformity. Carriers with visible surface or edge abnormalities (e.g., corrosion/rust, chipping, gouges or deep striations, etc.) should be discarded. *Note*: The screening should be conducted prior to the abrasion/chemical exposure.
3. Soak physically screened carriers in a suitable detergent solution (e.g., Liquinox) for 2-4 hrs to degrease and then rinse thoroughly in distilled or deionized water. Gently wipe with a clean lint free cloth and allow to completely dry. The rinsing should result in a surface free of residual detergent without any residual antimicrobial properties.
4. To prepare carriers for testing, immerse in 70% ethanol for 5-10 mins to decontaminate. Using sterile forceps remove individual carriers and place face up in matted, pre-sterilized Petri dishes (one carrier per dish). Allow carriers to dry in a Biological Safety Cabinet with lip open. Flaming, autoclaving, or exposure to UV radiation are not desirable techniques for sterilizing coupons and may alter the antimicrobial properties of the treated surfaces. Individual carriers should be oriented with the copper surface up; this orientation should be maintained.
5. To ensure absence of microbial contamination, randomly select a control and treated carrier from each batch and incubate in appropriate growth medium as a sterility control. No growth is the acceptance criterion.
6. Provide details of physical screening and sterility check along with vendor or source in the final report; coupon thickness and degree of surface brush or polish should also be reported.
7. Use cleaned sterile carriers (post exposure) within one week of preparation.
8. Copper coupons are considered single use; however, the stainless steel coupons may be re-used (clean and process accordingly prior to re-use).
9. Production lot (batch) identity must be maintained throughout the testing process.
10. Note: If a copper-containing material is applied (e.g., spray application) directly to

the surface of a porous or non-porous substrate to form the "antimicrobial product", then the application process and characteristics of the final deposition (e.g., thickness of copper material and the substrate) must be fully described and documented in the submission. The test carriers must be representative of the anticipated final product.

B. Test Cultures

1. The test microbes are *Staphylococcus aureus* (ATCC 6538), *Enterobacter aerogenes* (ATCC 13048) and *Pseudomonas aeruginosa* (ATCC 15442).
2. For methods for preparing stock cultures of *Staphylococcus aureus* and *Pseudomonas aeruginosa*, see current versions (2013) of the AOAC Use-dilution methods: 955.15 (*S. aureus*), and 964.02 (*P. aeruginosa*). For *E. aerogenes*, prepare stock cultures according to the Use-dilution method (955.15). Also, prepare *E. aerogenes* test suspensions using the method for *S. aureus*. Alternate preparation procedures may be used for test organisms not mentioned herein; however, the methodology must be clearly specified in the study protocol, and approved in advance.
    a. For *S. aureus* and *E. aerogenes*, defrost a single stock culture cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 µL of the thawed stock to a tube containing 10 mL Tryptic Soy Broth (TSB) and then vortex to mix. Incubate at 36±1°C for 18-24 hrs. Following incubation, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use.
    b. For *P. aeruginosa*, defrost a single cryovial at room temperature and briefly vortex to mix. Each cryovial should be single use only. Add 10 µL of the thawed stock to a tube containing 10 mL TSB and then vortex to mix. Incubate at 36±1°C for 18-24 hrs. Inspect culture prior to use. 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 to a new tube. Following removal of pellicle, use the broth culture to prepare a final test suspension. Briefly vortex the culture prior to use.

3. Dilute (in Phosphate Buffered Saline) or concentrate the culture appropriately to achieve the target carrier counts (a minimum 2.0 x 10^4 CFU/carrier). Centrifuge the 18-24 h broth cultures to achieve the desired level of viable cells on the dried carrier. Centrifuge at ~5,000 g_N for 20±5 mins and re-suspend the pellet in 10 mL Phosphate Buffered Saline (PBS). Note: Remove the supernatant without disrupting the pellet. 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 10 mL. If necessary, add 1 mL of PBS to the pellet to aid in the disaggregation.
4. Purity of the final test cultures (with soil load) should be determined by streak isolation on Tryptic Soy Agar (TSA) with 5% sheep's blood, or other appropriate plating medium, incubate (36±1°C for 24-48 hrs), examine for purity.
5. It is recommended that the titer of the final test culture (with soil load) be determined for informational purposes. Plate dilutions on TSA plates (or TSA with 5% sheep's blood) or other appropriate medium and incubate (36±1°C for 24-48 hrs) and enumerate. Count the number of colonies to determine the number of organisms per

mL (i.e., CFU/mL) of inoculum present at the start of the test.

C. Soil Load.

1. A soil load should be added to the final inoculum preparation to equal 5% fetal bovine serum and 0.01% Triton X-100. For example, add 0.25 mL aliquot of serum + 0.05 mL Triton X-100 to 4.70 mL final bacterial suspension to yield a 5% fetal bovine serum and 0.01% Triton X-100 soil load.

D. Efficacy Test Procedure

1. Evaluate fifteen (15) exposed product test carriers (from one production lot) with three (3) stainless steel control carriers and three (3) unexposed product control carriers against each of the three test organisms; and five (5) unexposed product test carriers and three (3) stainless steel control carriers from a second product lot against each of the three test organisms.
2. Control carriers should be performed concurrently with the test carriers. One set of control carriers (stainless steel) may be used for evaluating multiple surface lots against one test organism.
3. The exposure (contact time) of the inoculum to the carrier surface begins immediately upon inoculation; therefore, the contact time begins when final test suspension (with soil load) is deposited onto a sterile test carrier.
4. Record the initiation of the contact time and inoculate each sterile carrier at staggered intervals with 20 µL of final test culture using a calibrated pipette (a positive displacement pipette is desirable).
5. Spread the inoculum to within approximately 1/8 inch of the edge of each carrier, using a sterile transfer loop or the pipette tip, for example. Use an appropriate interval (e.g., 30 secs) to allow sufficient time for careful spreading of the inoculum.
6. The contact time begins immediately following carrier inoculation. Record the lab temperature and relative humidity during the one hour exposure period.
7. Allow carriers to remain in a horizontal position under ambient conditions with the lid on the Petri plate for 60±5 mins.
8. Following the exposure period, sequentially and aseptically transfer carriers to 20 mL of the appropriate neutralizer solution – this represents the 10^0 dilution.
9. Record the exposure period end time when the treated and control carriers are placed into the neutralizer solution.
10. After all the carriers have been transferred into the neutralizer, sonicate for 5 mins to suspend any survivors from the carriers, swirl to mix.
11. Within 30 minutes of sonication, prepare serial dilutions of the neutralized solution (10^0 dilution) out to 10^{-4} for the treated carriers. Transfer the control carriers to neutralizing subculture media and sonicate as for test carriers. Prepare serial dilutions of the neutralizing subculture medium and plate the appropriate dilutions in duplicate to yield countable numbers (30-300 colonies per plate). Incubate and enumerate with the treated carrier plates.
12. Plate in duplicate 1.0 mL or 0.1 mL using spread plating or pour plating technique on TSA plates (or TSA with 5% sheep blood).
13. Incubate the plates at 36±1°C for 48±4 hrs.
14. Following incubation, count colonies and record the results.

E. Study Controls

1. Purity Control. Perform a "streak plate for isolation" on TSA plates (or TSA with 5% sheep blood) for each final test culture, and following incubation examine in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism.
2. Soil Load Sterility Control. Streak plate or add a sample of the soil load to a growth medium, incubate, and visually examine for growth. The acceptance criterion for this study control is lack of growth.
3. Carrier Sterility Control. Add a representative un-inoculated test and control carrier to the neutralizing subculture medium. Incubate and examine for growth the subculture medium containing each carrier. The acceptance criterion for this study control is lack of growth.
4. Neutralizer Sterility Control. Incubate an unused tube of neutralizing solution and visually examine for growth. If the neutralizing solution does not support growth, then plate 1.0 mL or 0.1 mL using spread plating or pour plating technique on TSA plates (or TSA with 5% sheep blood). The acceptance criterion for this study control is lack of growth.

F. Neutralization Confirmation

1. Perform a neutralization confirmation control to demonstrate the neutralizer's ability to inactivate the test carrier. The neutralization of the test carriers is confirmed by using sterile test and control carriers and neutralizing as in the test procedure.
2. Add a sterile test carrier (one per production lot) to a tube of neutralizer solution (20 mL).
3. Hold the carrier in the neutralizing solution for approximately10 mins.
4. Add a 1.0 ml aliquot of a diluted suspension of the test organism yielding ≤200 CFU/ml of neutralizing subculture medium to the neutralizer, mix well. Hold for 10 mins. Duplicate plate a 1.0 mL or 0.1 mL aliquot of this mixed solution using spread plating or pour plating technique on TSA plates (or TSA with 5% sheep blood).
5. A numbers control is performed utilizing sterile stainless steel control carriers; process as indicated for the test carriers.
6. The resulting plates are incubated as in the test and enumerated. The acceptance criterion for this study is the difference between the treated and control counts should be ≤50%.

G. Product Performance Data

Impact of Abrasion and Chemical Exposure – Production Lot 1.

- Comparative visual observations should be used to identify any deleterious effects caused by the abrasion and chemical exposure for production lot 1; report findings in the study report.

- The effects of the abrasion and chemical exposure on mean log reduction should be

presented for production lot 1 – this is based on the mean log reduction for the *exposed* product carriers compared to the mean log reduction values for *unexposed* product carriers. The mean control counts associated with the stainless steel control carriers are used for the log reduction calculations.

- The mean log reduction values (i.e., per abrasion/chemical treatment per microbe) for the *exposed* product carriers compared to the *unexposed* product carriers should be within 0.5 logs; however, the mean log reduction for the *exposed* product carriers should not be less than the performance standard of 3 logs for any abrasion/chemical treatment group for any test microbe.

Production Lot 2. Mean log reduction data for production lot 2 should be calculated and presented by comparing viable counts for the 5 product carriers and 3 stainless steel control carriers.

Stainless Steel Control Counts Acceptance Criteria. The acceptance criterion for the control carriers is a minimum geometric mean of 2.0×10^4 CFU/carrier. All study controls must perform according to the criteria detailed in the study controls description section.

Product Efficacy. For the test substance to be considered a sanitizer, a ≥99.9% reduction (≥3 log reduction) in the numbers of each microbe (the difference between test product test carriers and the stainless steel control carriers) must be demonstrated following the exposure time (60 mins) for each lot.

H. Calculations/Data Analysis

- Calculate the mean log reduction in viable cells for each microbe for the following treatments: 1) *exposed* product carriers (per chemical) for production lot 1, 2) *unexposed* product control carriers (one 3-carrier set per microbe) for production lot 1, and 3) product carriers for production lot 2. Log reduction values are calculated based on the difference in log densities associated with the product test carriers compared to the stainless steel control carriers.

1. For determining the number of viable bacteria per carrier:

$$\text{CFU/carrier} = \frac{(\text{average number colonies/plate @ dilution}) \times (\text{dilution factor}) \times (\text{volume of neutralizer})}{(\text{Volume plated})}$$

2. For determining the geometric mean number of organisms surviving on three control carriers (unexposed copper or stainless steel) where X equals CFU/control carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2 + \text{Log}_{10}X_3}{3}$$

3. Example: For determining the geometric mean of number of organisms surviving on five product test carriers where Y equals CFU/test carrier:

$$\text{Geometric Mean} = \text{Antilog of } \frac{\text{Log}_{10}Y_1 + \text{Log}_{10}Y_2 + \text{Log}_{10}Y_3 + \text{Log}_{10}Y_4 + \text{Log}_{10}Y_5}{5}$$

(Adjust equation 3 above accordingly for three carriers for the unexposed product test carriers for lot 1)

4. % reduction = [(a-b)/a] × 100

   Where:

   a = geometric mean of the number of organisms surviving on the inoculated control carriers

   b = geometric mean of the number of organisms surviving on the test carriers.

5. Log_{10} Difference = (Log_{10} Stainless Steel Numbers Control) – (Log_{10} Product Test carriers)

V. Product Labeling

A. Label Claims

1. The following label claim is supported by the protocol:

   "This surface kills at least 99.9% of bacteria after a 1 hour contact time when maintained in accordance with the product care and use directions."

2. Claims are limited to indoor use of hard, non-porous copper/copper-alloy containing surfaces.

B. Required Label Language

1. Care and Use of Antimicrobial Copper Surfaces in Health Care Facilities:

   "Product Care and Use: Antimicrobial copper alloy surfaces must be cleaned and disinfected according to standard practice. Heath care facilities must maintain the product in accordance with infection control guidelines; users must continue to follow all current infection control practices, including those practices related to disinfection of environmental surfaces. This copper surface material has been shown to reduce microbial contamination, but does not necessarily prevent cross contamination. This product must not be waxed, painted, lacquered, varnished, or otherwise coated by any material."

2. Care and Use of Antimicrobial Copper Surfaces for Non-Health Care Facilities:

   "Product Care and Use: Routine cleaning to remove dirt and filth is necessary for standard hygiene and to assure the effective antibacterial performance of the antimicrobial copper surface. Cleaning agents typically used for environmental surfaces are permissible. The use of an antimicrobial copper surface does not replace standard good hygienic practices and/or infection control procedures. This product must not be waxed, painted, lacquered, varnished, or

otherwise coated by any material."