MICROBIOTEST PROTOCOL

EFFICACY EVALUATION OF CONTINUOUS BACTERIAL CONTAMINATION REDUCTION ON A COPPER ENHANCED HARD SURFACE SUPPLEMENTAL

Testing Facility
MICROBIOTEST
A Division of Microbac Laboratories, Inc.
105 Carpenter Drive
Sterling, VA 20164

Prepared for
Cupron Inc.
Suite 123
800 East Leigh Street
Richmond, VA 23219

January 3, 2012

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OBJECTIVE:

This test is designed to substantiate effectiveness claims for a substance containing copper with sanitizing claims intended to be registered with the Environmental Protection Agency as an inanimate hard surface other than those that come in contact with food or beverages. The test is consistent with the EPA Test Method for the Continuous Reduction of Bacterial Contamination on Copper Alloy Surfaces.

TESTING CONDITIONS:

A total of five replicates per challenge microorganism will be evaluated using carriers prepared from the copper enhanced hard surface. Two lots of the test surface will be evaluated. Prepared carriers of the test surface will be inoculated and re-inoculated based on the required regimen with *Pseudomonas aeruginosa*, Methicillin Resistant *Staphylococcus aureus*, and *Escherichia coli* O157:H7, held for the stipulated contact time(s), transferred to a neutralizing solution and mixed. Dilutions of the neutralizer will be plated, incubated and observed for growth.

MATERIALS:

A. Test materials supplied by the sponsor: (see last page for details).

Test carriers:	25 mm x 25 mm
Control carriers:	25 mm x 25 mm (containing no active)

The test materials will be tested as supplied by the sponsor unless directed otherwise by written instructions. All operations performed on the materials such as specialized storage conditions must be specified by the sponsor before initiation of testing.

The sponsor assures MICROBIOTEST, a Division of Microbac Laboratories, Inc. (MICROBIOTEST) testing facility management that the materials have been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

MICROBIOTEST will retain all unused materials for at least three months after completion of the test, then return them to the sponsor of the study or discard them in a manner that meets the approval of the safety officer of the laboratory.

B. Materials supplied by MICROBIOTEST including but not limited to:

1. Challenge microorganisms, required by EPA and the sponsor:
   a. *Pseudomonas aeruginosa*, ATCC 15442
   b. Methicillin Resistant *Staphylococcus aureus* (MRSA), ATCC 33592
   c. *Escherichia coli* O157:H7, ATCC 35150

2. Media and reagents:
   a. Tryptic Soy Broth (TSB)
   b. Neutralizer: 2X Letheen Broth
   c. Phosphate Buffer Saline dilution blanks (PBS)
   d. Tryptic Soy Agar (TSA)
   e. Heat-inactivated Fetal Bovine Serum (FBS)
   f. Triton X-100 solution (1% solution)
   g. Sterile deionized water
   h. 70-85% Isopropyl alcohol

3. Miscellaneous laboratory equipment and supplies.

4. Media, reagents and supplies for Antimicrobial Susceptibility Testing of MRSA:
   a. TSA containing 5% defibrinated sheep's blood (TSA+)
   b. 0.85% NaCl (SS)
   c. Mueller Hinton Agar (MHA)
   d. Control microorganism: *Staphylococcus aureus*, ATCC 25923
   e. 0.5% McFarland Standard
   f. Caliper measuring device
   g. 1 µg Oxacillin disc

TEST SYSTEM IDENTIFICATION:

All test and control tube racks will be labeled with microorganism, test agent (if applicable) and project number prior to initiation of the study and during incubation. Petri dishes will be labeled with microorganism prior to initiation of the study and microorganism and project number during incubation.

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EXPERIMENTAL DESIGN:

A. Inocula preparation:

Bacteria from stock cultures will be transferred into TSB and incubated at 35-37°C for 24±2 hours. Daily transfers will be made for at least three consecutive days (but no more than 10 days). For each transfer, tubes containing 10 mL of TSB will be inoculated using two loopfuls (4-mm inside diameter) of inoculum for each tube. A 48±4 hour culture will be used for the inocula on the day of testing.

The pellicle formed in the *Pseudomonas aeruginosa* culture will be aspirated before use.

Transfers more than 15 days away from the stock cultures will not be used for the inocula for the test.

For each microorganism, each culture will be thoroughly mixed on a vortex-mixer and allowed to settle. The upper two-thirds of each culture will be aspirated and used as the inoculum.

B. Addition of organic load:

To each prepared inocula, a 0.25 mL aliquot of FBS plus 0.05 mL1% Triton X-100 solution to 4.70 mL of bacteria suspension to yield a 5% FBS and 0.01% Triton X-100 soil load.

C. Test and Control Carrier preparation:

The test and control surfaces will be cleaned by submersion in 70-85% in Isopropyl alcohol, rinsed with sterile deionized water, and allowed to air dry. After drying completely, the carriers will be steam sterilized for 15 minutes at 121°C. The carriers will be allowed to cool and held at ambient room temperature until use. Prior to use, each carrier will be aseptically transferred into plastic Petri dishes (one dish for each carrier) matted with two pieces of filter paper using sterile forceps.

For each lot of the test material, per microorganism, five sets of with five replicate carriers per set will be prepared along with five sets per microorganism of the control material with three replicate carriers each for the primary aspects of the test. Additional surfaces will be prepared as required for remaining controls.

D. Test:

All test surfaces will be inoculated at staggered intervals with 5 µl of the challenge microorganism using a calibrated pipette. The inoculum will be spread to within approximately 1/8" of the edge of the carrier. This initial inoculation will be considered as "time zero".

The carriers will be dried at ambient conditions for the duration of exposure. The exposure period(s) begins with the initial "time-zero" inoculation.

The applicable sets not removed for quantitative recovery (see below) will be re-inoculated in the same manner at 3, 6, 9, 12, 15, 18, and 21 hours post "time-zero" inoculation.

The applicable sets for quantitative recovery will be removed at 2 (single inoculation), 6 (two inoculations), 12 (four inoculations), 18 (six inoculations), and 24 (8 inoculations) hours. At the conclusion of the applicable contact time for each set of surfaces, each carrier will be transferred to a jar containing 20 mL of neutralizer at the appropriate staggered intervals. Each jar will be sonicated for five minutes and then rotated by hand to mix. Within one hour after sonication, serial dilutions will be prepared using PBS (10^{-1} – 10^{-4}). Duplicate 1.0 mL aliquots from each jar/dilution (10^{0} – 10^{-4}) will be plated using TSA pour plates.

Plates will be incubated for 48±4 hours at 35-37°C, colonies will be counted and CFU/carrier calculated.

E. Controls:

1. Carrier quantitation control:

For each challenge microorganism, a parallel control will be run using the control carriers (surfaces) in the same manner as the test (inoculation and quantitative recovery) with the <u>exception that three replicates will be evaluated rather than five</u>. All plates will be incubated appropriately in the same manner as the test plates.

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2. Culture purity control:

   Each prepared culture will be streaked for isolation using TSA. All plates will be incubated in the same manner as the test plates. The isolated cultures will be observed for purity.

3. Organic soil sterility control:

   Duplicate 1.0 mL aliquots of the prepared organic soil will be plated in TSA pour plates. The plates will be incubated with the test plates observed for growth or no growth.

4. Inoculum confirmation counts control:

   Each prepared inoculum will be serially diluted using PBS and selected dilutions will be plated in duplicate using TSA pour plates. All plates will be incubated with the test plates.

5. Neutralizer sterility control:

   A single jar of containing the neutralizer will be incubated with the test plates. The neutralizer will be observed for growth or no growth.

6. Carrier sterility control:

   An uninoculated test (per lot) and control carrier will be subcultured into independent jars containing the neutralizer and incubated with the test plates. The neutralizer will be observed for growth or no growth.

7. Carrier viability control:

   For each challenge microorganism, a single inoculated control carrier will be subcultured into a jar containing the neutralizer and incubated with the test plates. The neutralizer jars will be observed for growth or no growth.

8. Neutralizer effectiveness control:

For each challenge microorganism, per lot of the test article, a single sterile test carrier will be neutralized in the same manner as the test (transferred into individual jars containing 20 mL of neutralizer. To each jar, a 1.0 mL aliquot of the diluted inoculum will be added to yield ≤100 CFU/mL in the neutralizer. The jar will be mixed and a 1.0 mL aliquot will be removed and plated in duplicate.

A numbers control will be performed in the same manner with the exception that a sterile control carrier will be used.

All plates will be incubated with the test plates.

9. Antimicrobial Susceptibility Testing of MRSA:

The prepared MRSA culture will be subcultured onto a TSA+ plate and the plate will be incubated for approximately 24 hours at 35-37°C. Following incubation, a suspension will be prepared by suspending growth from the TSA+ culture in SS to yield equivalent turbidity to a 0.5 McFarland Standard. This prepared suspension will be streaked onto MHA plate in a cross-hatch pattern and a 1 µg Oxacillin disc will be placed onto the center of the plate. The plate will be inverted and incubated for ≥ 24 hours at 35-37°C.

The same procedures will be conducted concurrently using the control microorganism, *Staphylococcus aureus*, ATCC 25923 to confirm the validity of the assay.

The interpretation of the zone of inhibitions (ZOI) will be based on established National Committee for Clinical Laboratory Standards (NCCLS) performance standards. As currently published, (NCCLS standard M100-S21) ZOI breakpoints must be ≤ 10 mm (rounded to the nearest whole mm) confirms resistance, 11-12 mm is considered intermediate resistance, and ≥ 13 mm confirms susceptibility.

10. Microorganism confirmation procedures:

A randomly selected colony from the carrier quantitation control plates, and if applicable, a randomly selected colony from a test plate will be confirmed by colony morphology and Gram stain according to extant SOPs. The same procedures will be performed using the culture purity control plates and the result regarding purity will be documented as well.

TEST ACCEPTANCE CRITERIA:

The test will be acceptable for evaluation of the test results if the neutralizer is effective and non-toxic. The study director may consider other causes that may affect test reliability and acceptance. There are no proposed statistical methods for this test.

- The average recovery for the Carrier Quantitation Control must be at least 2.0×10^4 CFU/carrier (for each quantitative recovery period).
- The CFU recovered for the neutralizer effectiveness controls should be within 1.0 $\log_{10}$ of the parallel neutralization confirmation control.
- The carrier sterility controls must exhibit no growth.
- The carrier viability controls must exhibit growth.
- The purity controls must demonstrate pure cultures.
- The organic soil sterility control must exhibit no growth.
- The neutralizer sterility control must exhibit no growth.
- For the Antimicrobial Susceptibility Testing: the test MRSA strain must exhibit resistance and the *Staphylococcus aureus* control strain (ATCC 25923) must exhibit susceptibility to Oxacillin.

PRODUCT EVALUATION CRITERIA:

According to EPA guidelines, the test agent meets effectiveness requirements, if the test results exhibit a minimum bacterial reduction of at least 90% over the corresponding Carrier Quantitation Controls at all recovery times over the 24 hour inoculation and exposure period.

DATA PRESENTATION:

The final report will include the following information in tabular form:

- The average colony-forming units (CFU)/carrier and percent reduction for each evaluation.
- The results for all the controls.

CONFIDENTIALITY:

All data generated at MICROBIOTEST are held in strictest confidence and are available only to the sponsor. In turn, no reference to the work, data, or MICROBIOTEST may be made public without the written consent of MICROBIOTEST.

REPORT FORMAT:

MICROBIOTEST employs a standard report format for each test design. Each final report provides the following information:

- Sponsor identification
- Test agent identification
- Type of test and project number
- Interpretation of results and conclusions
- Test results in tabular form
- Methods and evaluation criteria
- Quality Assurance and Compliance Statements

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned prior to initiation of the test. Resumes for the technical personnel are maintained and are available on request. This study will be conducted in the Applied Microbiology Laboratory at MICROBIOTEST, 105 Carpenter Drive, Sterling, Virginia 20164.

RECORDS TO BE MAINTAINED:

All raw data, protocol, protocol modifications, test agent records, final report, and correspondence between MICROBIOTEST and the sponsor will be stored in the archives at MICROBIOTEST, 105 Carpenter Drive, Sterling, Virginia 20164 or in a controlled facility off site.

All changes or revisions to this approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of any change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test agent; challenge microorganism used; media and reagent identification; and the type of neutralizers employed in the test will be addressed in a project sheet issued separately. The date the study director signs the protocol will be the initiation date. All project sheets will be forwarded to the study sponsor.

MISCELLANEOUS INFORMATION:

The following information is to be completed by sponsor before initiation of study:

A. Name and address: Cupron Inc.
Suite 123
800 East Leigh Street
Richmond, VA 23219

B. Test surface name*: CUPRON ENHANCED EOS HARD SURFACE GREY

Active ingredient: Copper oxide

Lot No. 1: _____

Lot No. 2: _____

*Note: the sponsor will also provide control coupons that will not contain any antimicrobial active ingredient (Cupron Control Hard Surfaces)

Inoculation intervals: "0 time", 3, 6, 9, 12, 15, 18, and 21 hours

Evaluated contact times: 2, 6, 12, 18, and 24 hours

Exposure temperature: Ambient room temperature 20±1C

C. Organic load – serum added to achieve 5% in the inoculum: ■ yes ☐ no

D. Precautions/storage – MSDS or certificate of analysis provided: ☐ yes ■ no

REPORT HANDLING: The sponsor intends to submit this information to: ■ US EPA ☐ US FDA ☐ Health Canada ☐ CAL DPR ☐ ARTG ☐ other: Internal Purposes

STUDY CONDUCT: ■ GLP ☐ non-GLP

PROTOCOL APPROVAL:
Sponsor Signature: _____ Date: 1/27/12
Alastair B. Monk, PhD

Study Director Signature: _____ Date: 02/29/12
Angela L. Hollingsworth

Date Issued: 03/12/12 Project Sheet No. 1 Page No. 1 Laboratory Project Identification No. 619-112

STUDY TITLE: EFFICACY EVALUATION OF CONTINUOUS BACTERIAL CONTAMINATION REDUCTION ON A COPPER ENHANCED HARD SURFACE - SUPPLEMENTAL	**STUDY DIRECTOR:** Angela L. Hollingsworth Signature 03/12/12 Date		
TEST AND CONTROL ARTICLES:	**LOT NO:**	**DATE RECEIVED:**	**DS NO.:**
Cupron Enhanced EOS Hard Surface Grey	05012064	03/07/12	C133
Cupron Enhanced EOS Hard Surface Grey	05112024	03/07/12	C134
Cupron Control Hard Surface	Not applicable	03/02/12 & 03/07/12	C122
PERFORMING DEPARTMENT(S): Applied Microbiology Laboratory	**STORAGE CONDITIONS:** Location: F4 ■ Dark ■ Ambient Room Temperature ☐ Desiccator ☐ Freezer ☐ Refrigerator ☐ Other:		

PROTECTIVE PRECAUTION REQUIRED: MSDS ☐Yes / ■ No

PHYSICAL DESCRIPTION: ■Solid ☐ Liquid ☐ Aerosol ☐ Other:

PURPOSE: See attached protocol. **AUTHORIZATION:** See client signature.

PROPOSED EXPERIMENTAL START DATE: 03/17/12 **TERMINATION DATE:** 03/20/12

CONDUCT OF STUDY: ☐ FDA ■ EPA ☐ R&D ■GLP ☐ GCP ☐ Other:

SPONSOR: Cupron Inc. 800 East Leigh Street, Suite 123 Richmond, VA 23219	**CONTACT PERSON:** Phone: E-mail:	Alastair B. Monk, PhD 804-381-5514 amonk@cupron.com

TEST CONDITIONS:

Challenge organism(s):	*Pseudomonas aeruginosa*, ATCC 15442 Methicillin Resistant *Staphylococcus aureus* (MRSA), ATCC 33592 *Escherichia coli* O157:H7, ATCC 35150
Active ingredient(s):	Copper oxide
Neutralizer(s):	Letheen Broth – 2X
Contact Time(s):	2, 6, 12, 18 and 24 hours
Contact Temperature(s):	Ambient (20±1°C)
Organic Load:	■ Yes / ☐ No (Per the protocol)
Incubation Time(s):	48±4 hours
Incubation Temperature(s):	35-37°C

Comments: The Miscellaneous Information section of the protocol did not include the specific lot numbers for the test and control articles. These identifiers are outlined above.

Date Issued: 03/28/12 Project Sheet No. 2 Page No. 1 Laboratory Project Identification No. 619-112

STUDY TITLE: EFFICACY EVALUATION OF CONTINUOUS BACTERIAL CONTAMINATION REDUCTION ON A COPPER ENHANCED HARD SURFACE - SUPPLEMENTAL	**STUDY DIRECTOR:** Angela L. Hollingsworth Signature 03/28/12 Date		
TEST AND CONTROL ARTICLES:	**LOT NO:**	**DATE RECEIVED:**	**DS NO.:**
Cupron Enhanced EOS Hard Surface Grey	05012064	03/07/12	C133
Cupron Enhanced EOS Hard Surface Grey	05112024	03/07/12	C134
Cupron Control Hard Surface	Not applicable	03/02/12 & 03/07/12	C122
PERFORMING DEPARTMENT(S): Applied Microbiology Laboratory	**STORAGE CONDITIONS:** Location: F4 ■ Dark ■ Ambient Room Temperature ☐ Desiccator ☐ Freezer ☐ Refrigerator ☐ Other:		

CONDUCT OF STUDY: ☐ FDA ■ EPA ☐ R&D ■GLP ☐ GCP ☐ Other:

SPONSOR:	Cupron Inc. 800 East Leigh Street, Suite 123 Richmond, VA 23219	**CONTACT PERSON:** Phone: E-mail:	Alastair B. Monk, PhD 804-381-5514 amonk@cupron.com
CO-SPONSOR:	EOS Surfaces, L.L.C. PO BOX 4146 Portsmouth, VA 23701	**CONTACT PERSON:** Phone: E-mail:	Kenneth G. Trinder, II 757-393-3671, ext. 4 kgt@eos-surfaces.com

EXPLANATION:

Protocol Amendment(s):

1. At the request of the original sponsor, Cupron Inc., a co-sponsor, EOS Surfaces, L.L.C. will be added for reporting purposes. EOS Surfaces, L.L.C. will be identified in the final report however all authorizations affiliated with the protocol (Protocol Amendment(s) and/or Deviation(s)), with the exception of this Amendment will be approved by Alastair Monk, PhD of Cupron Inc.

Date Issued: 03/31/12 Project Sheet No. 3 Page No. 1 Laboratory Project Identification No. 619-112

STUDY TITLE: EFFICACY EVALUATION OF CONTINUOUS BACTERIAL CONTAMINATION REDUCTION ON A COPPER ENHANCED HARD SURFACE - SUPPLEMENTAL	**STUDY DIRECTOR:** Angela L. Hollingsworth Signature 03/31/12 Date		
TEST AND CONTROL ARTICLES:	**LOT NO:**	**DATE RECEIVED:**	**DS NO.:**
Cupron Enhanced EOS Hard Surface Grey	05012064	03/07/12	C133
Cupron Enhanced EOS Hard Surface Grey	05112024	03/07/12	C134
Cupron Control Hard Surface	Not applicable	03/02/12 & 03/07/12	C122
PERFORMING DEPARTMENT(S): Applied Microbiology Laboratory	**STORAGE CONDITIONS:** Location: F4 ■ Dark ■ Ambient Room Temperature ☐ Desiccator ☐ Freezer ☐ Refrigerator ☐ Other:		

CONDUCT OF STUDY: ☐ FDA ■ EPA ☐ R&D ■GLP ☐ GCP ☐ Other:

SPONSOR:	Cupron Inc. 800 East Leigh Street, Suite 123 Richmond, VA 23219	**CONTACT PERSON:** Phone: E-mail:	Alastair B. Monk, PhD 804-381-5514 amonk@cupron.com
CO-SPONSOR:	EOS Surfaces, L.L.C. PO BOX 4146 Portsmouth, VA 23701	**CONTACT PERSON:** Phone: E-mail:	Kenneth G. Trinder, II 757-393-3671, ext. 4 kgt@eos-surfaces.com

EXPLANATION:

Protocol Amendment(s):

2. In reference to Section C on Page 4 of the protocol regarding the statement of "five sets of with five carriers per set will be prepared". This statement should have indicated "five sets with five carriers per set will be prepared".