Study Title

Protocol for the AOAC International Use-Dilution Methods (955.14, 955.15, & 964.02)

Product Identity

DiOx+ DISINFECTANT STERILIZER

Data Requirement

40 CFR PART 158—DATA REQUIREMENTS
FOR PESTICIDES Subpart W—Antimicrobial Pesticides Guideline No. 810.2200

Author

Taylor Dreves
Microbiologist II

Study Completion Date

10-26-2021

Testing Facility

Q Laboratories
1930 Radcliff Drive
Cincinnati, OH 45204
(513) 471-1300

Laboratory Project Number (Study File)

QL 370181

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA section 10(d)(1)(A), (B), or (C).

Company: _____

Company Agent: _____Date: _____

_____ _____

Title Signature

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study meets the requirements of 40 CFR § 160.

SUBMITTER:

SIGNATURE: _____DATE: _____

SPONSOR:

SIGNATURE: _____DATE: _____

STUDY DIRECTOR:

SIGNATURE: _____ DATE: 10-26-21
Benjamin Bastin, B.S.
Associate Director, Microbiology Operations

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1.0 EFFICACY STUDY SUMMARY

STUDY TITLE:	**Protocol for the AOAC International Use-Dilution Methods (955.14, 955.15, & 964.02)**
LABORATORY PROJECT #:	QL 370181
GUIDELINE:	Guideline No. 810.2200 using Official Methods of Analysis of the AOAC International, Chapter 6, Disinfectants, Use-Dilution Methods (955.14, 955.15, & 964.02). Current edition. AOAC International, 2275 Research Blvd., Suite 300, Rockville, MD 20850 [Section 14.1, 14.2, and 14.3 of Appendix 1].
TESTING FACILITY:	Q Laboratories 1930 Radcliff Drive Cincinnati, OH 45204
STUDY DATES:	
STUDY INITIATION DATE:	06-09-2021
STUDY COMPLETION DATE:	10-26-2021
GLP COMPLIANCE:	Q Laboratories has developed and implemented a quality management system that enhances our ability to provide testing services that consistently meet client expectations and regulatory requirements. All testing was performed in accordance with EPA Good Laboratory Practice Standards (GLPS), as specified in 40 CFR Part 160. Periodic phase audits of the study were conducted by the Quality Assurance Unit to ensure testing compliance and a review of the final report by the QAU was conducted in accordance with 40 CFR, Part 160.35, subpart B.
TEST SUBSTANCE:	
DESCRIPTION:	DiOx+ DISINFECTANT STERILIZER
% ACTIVE INGREDIENT:	Chlorine Dioxide (ClO_2), 0.4 %
DILUTION:	½ oz or 15 mL or 1 tbsp of substance to 32 oz (946 mL) water
TEST CONDITIONS:	
SOIL LOAD:	5% fetal bovine serum
WATER:	Test substance is diluted in AOAC hard water solution prepared according to EPA SOP MB-30-02 [Section 14.4 of Appendix 1] to use-dilution.
CONTACT TIME:	3 minutes ± 5 seconds
TEMPERATURE:	Ambient Temperature (20 - 25 °C)
OTHER:	The inoculum applied includes 5% fetal bovine serum.

TEST RESULTS: Positive carriers/total carriers

Test Organism	Identification #	Test Results (form)		
		Lot 000136	Lot 00015	Lot 00016
Salmonella enterica	ATCC* 10708	0	0	0
Staphylococcus aureus	ATCC 6538	0	0	0
Pseudomonas aeruginosa	ATCC 15442	0	0	0

*American Type Culture Collection

CONTROL RESULTS:

The control carriers for *S. aureus* and *P. aeruginosa* were between 1.0×10^6 to 1.0×10^7 CFU/carrier. The control carriers for *S. enterica* were between 1.0×10^5 to 1.0×10^6 CFU/carrier. Growth occurred in all viability control tubes. Growth did not occur in any of the sterility tubes. Neutralization was considered adequate and meet the specification in Section 13.0 of Appendix 1. For media quality controls, comparable growth acceptance was within 50 - 200 %. No growth occurred in the media sterility control. No disrupted pellicles of *P. aeruginosa* test culture were used. No contamination occurred in the subculture tubes.

CONCLUSION:

Based on the results presented in this study report, the test article met the performance standard: (0) positive carriers out of sixty (60) for each lot, when tested against *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella enterica*. The performance standard listed in 810.2200 for *S. aureus* and *P. aeruginosa* is no more than three positive carriers out of 60 per test. The performance standard for *S. enterica* is no more than one positive carrier out of 60 per test. All testing was performed in accordance with EPA Good Laboratory Practice Standards (GLPS), as specified in 40 CFR Part 160. Periodic phase audits of the study were conducted by the Quality Assurance Unit to ensure testing compliance and a review of the final report by the QAU was conducted in accordance with 40 CFR, Part 160.35, subpart B.

2.0 QUALITY ASSURANCE STATEMENT

Study Title: Protocol for the AOAC International Use-Dilution Methods (955.14, 955.15, & 964.02)

Study #: QL 370181

In accordance with the Good Laboratory Practice Standards (EPA 40 CFR § 160), quality assurance audits of this study were conducted and reported to management and the study director as listed below:

Phase Inspected	Date of Inspection	Date Reported to Study Director	Date Reported to Management
In Process-Assay	ALS 6-30-21	ALS 10-26-21	ALS 10-26-21
In Process-Calculation of Results	ALS 10-14-21	ALS 10-26-21	ALS 10-26-21
Final Report	ALS 10-19-21	ALS 10-26-21	ALS 10-26-21

Cynthia Hauck 10-26-21
Date
Quality Assurance Unit, Q Laboratories

COMPLIANCE STATEMENT

This study meets the requirements for 21 CFR part 58.

The following analysts participated in the study:

Taylor Drews	Dave Brooks
Madison Sanseppe	Tea Thomas

Study Director:
Q Laboratories

B. Bastin 10.26.21
Benjamin Bastin Date

3.0 **STUDY PERSONNEL**

STUDY DIRECTOR: SIGNATURE: 10.26.21
Benjamin Bastin, B.S.
Associate Director, Microbiology Operations

LEAD ANALYST: SIGNATURE: 10.26.21
Taylor Dreves
Microbiologist II

4.0 STUDY REPORT

STUDY TITLE: Protocol for the AOAC International Use-Dilution Methods (955.14, 955.15, & 964.02)

SPONSOR: Boon Industries Inc.
110 Spring Hill Drive, Suite #16
Grass Valley, CA 95959

TEST FACILITY: Q Laboratories
1930 Radcliff Drive
Cincinnati, OH 45204

TEST SUBSTANCE IDENTIFICATION

TEST SUBSTANCE NAME: DiOx+ DISINFECTANT STERILIZER, CAS# 10049-04-4, Chlorine Dioxide (ClO_2), 0.4 %

LOT/BATCH NUMBER(S):
LOT 000136, Date Manufactured 04.02.21
LOT 00015, Date Manufactured 06.01.21 Batch 21.07.27, *Note: It was ensured lot was aged 60 days prior to testing*
LOT 00016, Date Manufactured 06.08.21, Batch 21.07.28

DESCRIPTION OF TEST SUBSTANCE:
Active: Chlorine Dioxide (ClO_2), 0.4 %
Color: Yellow-green liquid
Viscosity: 0.984 cP (centipoise) at 25 °C
Storage specification is cap closed tight, away from light, preferably refrigerated, expiration date is not specified.

CHEMICAL CHARACTERIZATION: The identity, solubility, stability, strength, purity, and chemical composition was provided by the Study Sponsor and is attached as Appendix 2.

STUDY INITIATION DATE: 06-09-2021
EXPERIMENTAL START DATE: 06-15-2021
EXPERIMENTAL END DATE: 10-12-2021
STUDY COMPLETION DATE: 10-26-2021

STUDY OBJECTIVE: The objective of the study was to document the antimicrobial efficacy of the test article against *Pseudomonas aeruginosa* American Type Culture Collection (ATCC) 15442, *Salmonella enterica* ATCC 10708, and *Staphylococcus aureus* ATCC 6538 based on the guidance provided in the Official Methods of Analysis of the AOAC International, Chapter 6, Disinfectants, Use-Dilution Methods (955.14, 955.15, & 964.02). Current edition.

AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417 [Section 14.1, 14.2, and 14.3 of Appendix 1] and US Environmental Protection Agency Office of Pesticide Programs, SOP Number MB-05-16, Standard Operating Procedure for AOAC Use Dilution Method for Testing Disinfectants [Section 14.5 or Appendix 1].

TEST METHOD: The design of this evaluation was based on the guidance provided in the Official Methods of Analysis of the AOAC International, Chapter 6, Disinfectants, Use-Dilution Methods (955.14, 955.15, & 964.02). Current edition. AOAC International, 2275 Research Blvd., Suite 300, Rockville, MD 20850 [Section 14.1, 14.2, and 14.3 of Appendix 1] and US Environmental Protection Agency Office of Pesticide Programs, SOP Number MB-05-16, Standard Operating Procedure for AOAC Use Dilution Method for Testing Disinfectants [Section 14.5 of Appendix 1].

TEST SYSTEM/STRAINS: The test system was polished stainless-steel cylinders inoculated and allowed to dry with a single organism of the following:

Bacterial strains:
Staphylococcus aureus ATCC 6538
Pseudomonas aeruginosa ATCC 15442
Salmonella enterica serovar Choleraesuis ATCC 10708

5.0 **STUDY MATERIALS**

MEDIA

Neutralizer: Letheen Broth (LBR) modified with sodium thiosulfate
Dehydrated Powder Ingredients:

Proteose Peptone No. 3	10.0 g/L
Beef Extract	5.0 g/L
Sodium Chloride	5.0 g/L
Polysorbate 80	5.0 g/L
Lecithin	0.7 g/L
Sodium Thiosulfate	10.0 g/L

Preparation:

Suspend 25.7 g of Letheen Broth powder and 10.0 g of Sodium Thiosulfate powder in 1000 mL of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Dispense 10 mL into 16 x 150 mm test tubes and sterilize in an autoclave at 121 °C for 15 minutes. Final pH is 7.0 ± 0.2 at 25 °C. Store up to 3 months at room temperature.

Phosphate Buffered Saline (PBS)

Ingredients:

Dulbecco's Phosphate Buffered Saline 10x (DPBS 10x)	100 mL
Deionized Water	1000 mL
1M HCl	as needed

Preparation:

Suspend 100 mL of DPBS 10x in 1000 mL of DI water. Mix thoroughly and adjust pH to 7.1 ± 0.2 using 1M HCl. Sterilize in an autoclave at 121 °C for 15 minutes. Final pH is 7.1 ± 0.2 at 25 °C. Store up to 3 months at room temperature.

Tryptic Soy Agar (TSA)
Dehydrated Powder Ingredients:

Pancreatic digest of Casein	15 g/L
Papaic digest of Soybean	5 g/L
Sodium Chloride	5 g/L
Agar	15 g/L

Preparation:

Measure 1000 mL of DI water into a graduated cylinder and transfer approximately half the water to a flask. Weigh 40 g of dehydrated Tryptic Soy Agar and add to the water in the flask. Mix briefly and add the remaining water. Sterilize in an autoclave at 121 °C for 15 minutes. Final pH is 7.3 ± 0.2 at 25 °C. Store up to 2 months at room temperature.

Sterile Deionized (DI) Water
Ingredients:

Deionized Water	as needed

Preparation:

Measure desired volume of DI water into a suitable autoclavable vessel. Sterilize in an autoclave at 121 °C for 15 minutes. Store up to 3 months at room temperature.

Synthetic Broth (SB) (Commercially available from Fisher Scientific Cat No. NC0493363)
Dehydrated Powder Ingredients:

L-Cystine	0.050 g/L
DL-Methionine	0.370 g/L
L-Arginine	0.400 g/L
DL-Histidine	0.300 g/L
L-Lysine	0.850 g/L
L-Tyrosine	0.210 g/L
DL-Threonine	0.500 g/L
DL-Valine	1.000 g/L
L-Leucine	0.800 g/L
DL-Isoleucine	0.440 g/L
Amino acetic acid	0.060 g/L
DL Serine	0.610 g/L
DL-Alanine	0.430 g/L
L-Glutamic acid	1.300 g/L
L-Aspartic acid	0.450 g/L
DL-Phenylalanine	0.260 g/L
DL-Tryptophan	0.050 g/L
L-Proline	0.050 g/L
Sodium chloride	3.000 g/L
Potassium chloride	0.200 g/L
Magnesium sulphate	0.050 g/L
Monopotassium phosphate	1.500 g/L
Disodium phosphate	4.000 g/L
Thiamine hydrochloride	0.010 g/L
Nicotinamide	0.010 g/L

Preparation:

Suspend 16.9 g in 1000 mL DI water. Heat if necessary to dissolve the medium completely. Dispense 10 mL amounts in 20 x 150 mm culture tubes and sterilize in an autoclave at 121 °C for 15 minutes. Cool to room temperature and just before use, aseptically add 0.1 ml of 10% sterile dextrose solution. Final pH is 7.1 ± 0.2 at 25 °C. Store up to 2 months (without dextrose added) at 2 - 8°C.

Tryptic Soy Agar with 5% Sheep Blood (SBA) (Commercially available from BD, PN 221261)
Ingredients:

Pancreatic digest of Casein	14.5 g/L
Papaic digest of Soybean Meal	5 g/L
Sodium Chloride	5 g/L
Agar	14 g/L
Growth Factors	1.5 g/L
Defibrinated Sheep blood	5%

Preparation:

Available as pre-poured plates.

REAGENTS

Soil Load:
Fetal Bovine Serum (Commercially available from Fisher Scientific Cat No. SH3008802HI) at a 5% concentration in the inoculum suspension.

Hard Water:
AOAC hard water was used to dilute the Test Substance.

EQUIPMENT

Transfer Loops 23 AWG, platinum 4 mm loop fused on 75 mm shaft, bent at a 30° angle
Incubator Temperature Range 36 ± 1 °C
Incubator Thermometer, NIST Traceable
Steam Autoclave
Vortex Mixer
Calibrated, Traceable Minute/Second Timer
Refrigerator 2 - 8 °C
Refrigerator Thermometer, NIST Traceable
Adjustable Pipettor 20 µL – 200 µL and 100 µL – 1000 µL capacity
Reichert Quebec® Colony Counter
Hand Tally
Biological Safety Cabinet (BSC)
Metal Forceps
Timer
Spectrophotometer
Ultrasonic Cleaner
Specialized glassware
    For disinfectant, use autoclavable 25 x 100 mm tubes (Bellco Glass Inc., Vineland, NJ). For glassware used to prepare test chemical, refer to SOP MB-22.
    Available from Bellco Glass Inc., Vineland, NJ
Wire Hook
    For carrier transfer.
    Make 3 mm right angle bend at end of 50 - 75 mm nichrome wire No. 18 B&S gauge.

6.0 TEST METHOD

PREPARATION OF TEST SUBSTANCE

The test substance was diluted ½ oz or 15 mL or 1 tbsp of substance to 32 oz (946 mL) AOAC hard water, prepared according to EPA SOP MB-30-02 [Section 14.4 of Appendix 1].

PREPARATION OF TEST SYSTEM/STRAINS

Seed-lot culture maintenance techniques were followed to ensure the viable microorganisms used for inoculation are not more than five passages removed from the original master seed lot.
The test microorganism cultures were prepared as follows:
SBA was used as the propagation medium for cultures from a Q Laboratories frozen stock culture stored at -70 °C for 18 - 24 h at 36 ± 1 °C.
After initial incubation, an isolated colony was picked to SB, vortexed (*S. aureus* and *S. enterica*) and incubated at 36 ± 1 °C for 24 ± 2 h.

Note: One daily transfer was required prior to the inoculation of a final test culture. Daily cultures could be subcultured for up to 5 days; each daily culture may be used to generate a test culture.

To generate test cultures, a sufficient number of 50 mL conical tubes containing 10 mL SB were inoculated with 10 µL per tube of the 24 h culture, then vortexed to mix.
Cultures were incubated 48 - 54 h at 36 ± 1 °C. The 48 - 54 h test culture was not shaken.
For *P. aeruginosa*, the pellicle was removed at the top of the broth tube by aspirating with a pipette. The broth was transferred to a sterile test tube. The pellicle at the bottom of the culture tube was avoided during the transfer.
For *S. aureus*, *S. enterica*, and *P. aeruginosa*, using a vortex-style mixer, mix the 48 - 54 h test cultures for 3 - 4 s and let stand 10 min at room temperature before continuing.
The upper portion (upper ¾) of each culture was removed leaving behind any debris or clumps and transferred to a sterile flask; each culture was pooled in its respective flask and swirled to mix.
The OD was measured and recorded at 650 nm. Sterile SB medium was used to calibrate the spectrophotometer.
An aliquot (about 10 mL) of the final test culture was transferred into a sterile tube for carrier inoculation.
To achieve mean carrier counts within the target concentration of 10^5 - 10^6, the final test culture was diluted with sterile broth.
An aliquot of about 10 mL of the final test culture was transferred into a sterile tube for carrier inoculation.

Note: The test culture for carrier inoculation was used within 30 minutes.

Note: To achieve mean carrier counts within the appropriate range (see Section 13.0), the final test culture was diluted (e.g., one part culture plus one part sterile broth) prior to the addition of the organic soil to the inoculum using the sterile culture medium used to generate the final test culture (e.g., synthetic broth). If diluted test culture for carrier inoculation was used, it was used within 30 min.

An appropriate amount of organic soil was added make achieve a 5 % solution and swirled to mix.

Carrier inoculation:
Test culture was dispensed in 20 mL portions into sterile 25 x 150 mm test tubes.
Carriers, 20, were aseptically transferred into each of the tubes containing the test culture.
Carriers were repositioned as necessary to ensure complete coverage with the test culture.
Carriers were allowed to remain in the inoculum for 15 ± 2 min.
Following the carrier exposure period, carriers were removed individually from the inoculum using a flamed nichrome wire hook, each carrier was briefly tapped against the side of the tube to remove excess culture and placed vertically on end in sterile Petri dishes matted with 2 layers of sterile Whatman No. 2 filter paper. The inoculum was not removed from the tube in advance of removing carriers. Carriers did not touch or fall over in the Petri dish. No more than 12 carriers were placed in a Petri dish. Lid was placed on Petri dish.
Carriers were dried in an incubator at 36 ± 1 °C for 40 ± 2 min. The timed activities of carrier inoculation were recorded on the appropriate master data sheet. All carriers were exposed to disinfectant within two hours of drying.
At least 80 carriers were inoculated; 60 carriers are required for testing, 6 for control carrier counts, and 1 for viability control.

EXPOSURE CONDITIONS

Contact time: 3 minutes ± 5 seconds
Temperature: 25 ± 1 °C
Quantity of test substance delivered: 10 mL (± 0.5 mL)
Carriers were transferred using alternating 75 mm long 18 GA nichrome wire needles with a 3 mm right angle bend at end.
Test substance was prepared per EPA SOP MB-22-05, Disinfectant Product Preparation [Section 14.7 of Appendix 1] and used within 3 hours of preparation.
The test substance was diluted ½ oz or 15 mL or 1 tbsp of substance to 32 oz (946 mL) AOAC hard water, prepared according to EPA SOP MB-30-02 [Section 14.4 of Appendix 1].

TEST SYSTEM RECOVERY

Test Procedure:
The carriers were transferred sequentially from the Petri dish to the test tubes containing the disinfectant at appropriate intervals (e.g., 30 second intervals).
One carrier was added per tube and the tube was swirled using 2 - 3 gentle rotations before placing it back in the water bath. The carrier was added within 5 seconds of the specified time for a contact time of 3 minutes ± 5 seconds.
Using alternating hooks, the hooks were flame-sterilized and allowed to cool after each carrier transfer. When lowering the carriers into the disinfectant tubes, neither the carrier itself nor the tip of the wire hook touched the interior sides of the tube. If the interior sides of the tube were touched, the carrier was repeated.
Following the exposure time, the carriers was sequentially transferred into subculture/neutralizer media. The carrier was removed from the disinfectant with a sterile hook, tapped against the interior sides of the tube to remove the excess disinfectant, and transferred into the subculture tube within ± 5 s. Tapping the carrier was avoided against the upper third of the tube. Contact of the carrier to the interior sides of the subculture tube was avoided during transfer.

The subculture tube was recapped and shaken thoroughly. Tubes were incubated at 36 ± 1 °C for 48 ± 2 h.
A secondary subculture tube was not deemed necessary to achieve complete neutralization.
The subculture tubes were thoroughly shaken after all of the carriers had been transferred.
Subculture tubes were incubated 48 ± 2 h at 36 ± 1 °C.
Timed events were recorded on the appropriate master data sheet.

Results:
Each tube was gently shaken prior to recording results. Results were recorded as + (growth) or 0 (no growth) as determined by presence or absence of turbidity, on the appropriate master data sheet.
Viability controls were used for comparative determination of a positive tube.
A minimum of 20 % of the remaining negative tubes were assayed for the presence of the test microbe using isolation streaks on TSA. Preliminary results were recorded and isolation streaks were conducted at 48 ± 2 h. Negative tubes were incubated for an additional 24 hours to confirm the results.

Confirmatory Steps for Test Microbes:
Confirmatory steps listed in Section 11.10 of Appendix 1 were not necessary.

7.0 PROTOCOL CHANGES

PROTOCOL AMENDMENTS

No protocol amendments were necessary.

PROTOCOL DEVIATIONS

No protocol deviations were reported.

8.0 CONTROLS

PREPARATION OF CONTROL(S)

Neutralization:
Sterile carriers were used for this assay.
The neutralization was performed in advance of product testing to verify that the prescribed neutralizer was suitable for the efficacy evaluation. The two test scenarios were conducted concurrently to determine an appropriate approach for performing the product efficacy evaluation:
First Scenario: carriers were exposed to the disinfectant and transferred into the neutralizer subculture medium (primary tube). No secondary subculture medium transfers were conducted. The neutralizer tubes containing the carriers were inoculated with a test organism suspension to deliver 5 - 100 CFU/mL.
Second Scenario: carriers were exposed to the disinfectant and transferred into the neutralizer subculture medium (primary tube); in addition, the carriers were subsequently

transferred to a secondary subculture medium (secondary tube). Tubes were inoculated with a test organism suspension to deliver 5 - 100 CFU/mL.
The purpose of the two-scenario approach was to determine if the prescribed neutralizer for the disinfectant is sufficient to support growth.
Inoculum Preparation:
Inocula were prepared as described in Section 11.3 of Appendix 1.
Inoculum Enumeration:
Ten-fold serial dilutions of the inocula were prepared by pipetting 1 mL of the final test culture into 9 mL tubes of PBS. Four dilutions were used, (e.g., 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) to inoculate the neutralizer. The target number of cells was 5 - 100 CFU/mL. This concentration was achieved in one of the two highest dilutions.
To estimate CFU/mL, each of the four dilution tubes was briefly mixed by vortex and 0.1 mL plated in duplicate on TSA.
The dilution and plating information were recorded on the appropriate master data sheet.
Plates (inverted) were incubated at 36 ± 1 °C for up to 48 ± 2 h and colony counts were recorded.
Plates that had colony counts over 300 were labeled as too numerous to count (TNTC). The counts were recorded on the appropriate master data sheet.
Product Sample Preparation:
The product was prepared as described in Section 11.6 of Appendix 1.
Carrier Preparation:
The carriers were prepared as described in Section 11.4 of Appendix 1.
First Scenario: Neutralizer - Primary Subculture Treatment Only:
Four dried carriers (with broth culture added) were required per organism. The carrier type used for test were used.
The product was applied to the carriers as described in Section 11.7 of Appendix 1.
Four carriers per organism were exposed to the disinfectant for the specified contact time in the same manner as in product efficacy testing. The carrier transfer information was recorded on the appropriate master data sheet.
After the last carrier of a set (4 total carriers) had been treated with disinfectant, and the contact time was complete, carriers were aseptically transferred in order in a timed fashion into tubes containing the specified neutralizer, in the same manner as product efficacy testing. Excess liquid was drained from the carrier prior to the transfer. This set of neutralizer tubes (4 total tubes) represented the Neutralizer-Primary Subculture Treatment. Refer to Section 10.11 of Appendix 1 for treatment inoculation.
Proceeded immediately with the Second Scenario.
Second Scenario: Neutralizer Subculture Treatment Plus Secondary Subculture Treatment:
Four dried carriers (with broth culture added) were used per organism. The same carrier type was used required for the specific test.
The product was applied to the carriers according to specific instruction provided.
Four carriers per organism were exposed to the disinfectant for the specified contact time in the same manner as product efficacy testing. The carrier transfer information was recorded on the appropriate master data sheet.
After the last carrier of a set (4 total carriers) had been treated with the disinfectant, and the contact time was complete, carriers were aseptically transferred in order in a timed fashion into tubes containing the specified neutralizer, in the same manner as in product efficacy

testing. Excess liquid was drained from the carrier prior to the transfer. This set of neutralizer tubes (4 total tubes) represented the Neutralizer-Primary Subculture Treatment.
Following the last carrier transfer into the neutralizer tube, both First and Second Scenario neutralizer tubes were incubated at room temperature for 30 - 45 min. Then, for the Second Scenario, each carrier was transferred in order into a culture tube containing the secondary subculture medium. This portion of the assay was not timed. This set of tubes (4 total tubes) represented the Secondary Subculture Treatment. Refer to Section 10.11 of Appendix 1 for treatment inoculation.
Neutralization Controls:
Inoculated controls:
The Neutralizer-Primary Inoculated Control contained four tubes of fresh, unexposed (to disinfectant) neutralizer-primary media.
The Secondary Subculture Inoculated Control contained four tubes of secondary subculture media.
The preparation of each medium was the same as used in the treatments. Refer to Section 10.11 of Appendix 1 for treatment inoculation.
Uninoculated controls:
Neutralizer-Primary and Secondary Subculture Uninoculated Controls. One tube each of uninoculated neutralizer and secondary subculture media was incubated with the other tubes.
Sterility of carriers was confirmed concurrently with testing: an uninoculated carrier was added to a tube of 10 - 20 mL LBR and incubated at 36 ± 1 °C for 3 - 10 days.

Treatment Inoculation:
After Section 10.9.5 of Appendix 1, all tubes were inoculated concurrently using Tables 1, 2, and 3.

Table 1: First Scenerio: Inoculation of Treatment Group with Dilutions of the Test Organism*

Treatment	Dilutions Added			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Neutralizer-Primary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL

*1×10^{-4} through 1×10^{-7}; based on an approx. starting suspension of 10^8 to 10^9 CFU/mL

Table 2: Second Scenerio: Inoculation of Treatment Group with Dilutions of the Test Organism*

Treatment	Dilutions Added			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Neutralizer-Primary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL
Secondary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL

*1×10^{-4} through 1×10^{-7}; based on an approx. starting suspension of 10^8 to 10^9 CFU/mL

Table 3: Controls: Inoculation of Control Groups with Dilutions of the Test Organism*

Controls		Dilutions Added			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
Inoculated Controls (media performance)	Neutralizer - Primary	0.1 mL	0.1 mL	0.1 mL	0.1 mL
	Secondary Subculture				

*1×10^{-4} through 1×10^{-7}; based on an approx. starting suspension of 10^8 to 10^9 CFU/mL

Tubes were shaken thoroughly. All tubes were incubated for 48 ± 2 h at 36 ± 1 °C.
Recording Results and Confirmation Testing:
Results were recorded as + (growth/turbidity) or 0 (no growth) on the appropriate master data sheet.
For each treatment and control group, a minimum of one positive tube was Gram stained per treatment. The tube with the highest dilution showing growth (inoculated with the dilution with fewest CFU/mL delivered) was selected.
Confirmation results were recorded on the appropriate master data sheet.
Interpretation of Results:
Plate count data. One of the four dilutions plated provided counts within the approximate target range, 5 - 100 CFU/mL.
Controls. Growth in the Secondary Subculture Inoculated Control verified the presence of the test microbe, performance of the media, and provided a basis for comparison of growth in the neutralizer and subculture treatment tubes. Growth in the Neutralizer-Primary Inoculated Control was comparable to the Secondary Subculture Inoculated Control as the neutralizer was the same as the secondary subculture media.
The Neutralizer-Primary Uninoculated Control and Secondary Subculture Uninoculated Control tubes were used to determine sterility and showed no growth for the test to be valid.

Treatments. The occurrence of growth in the Neutralizer-Primary Subculture and Secondary Subculture Treatment tubes were used to assess the effectiveness of the neutralizer.
Efficacy Evaluation based on Neutralization Results:
Results from the First Scenario indicated effective neutralization; therefore, the efficacy evaluation was conducted using only the neutralizer subculture tubes (i.e., primary tubes).
Data Analysis/Calculations:
Plate counts were enumerated and used to calculate the CFU/mL concentration added to each tube based on the average of countable plates. TNTC was applied for plates with counts above 300 CFU.
To calculate the average CFU/mL per dilution added to each tube, the plate counts were added for each plate within the dilution and divided by two.
Counts from 0 through 300 were used in the calculations.

Sterility and Viability Controls:
Sterility control. One sterile, uninoculated carrier was placed into a tube of neutralizing subculture broth. Tube was incubated with the efficacy test. Results were reported as + (growth), or 0 (no growth) as determined by presence or absence of turbidity. Growth did not occur in the tube. Results were recorded on the appropriate master data sheet.
Viability control. 1 dried inoculated untreated carrier(s) were placed into separate tubes of the neutralizing subculture broth (if primary and secondary media are different). Tubes were incubated with the efficacy test. Results were reported as + (growth) or 0 (no growth) as determined by presence or absence of turbidity. Growth occurred in both tubes. Results were recorded on the appropriate master data sheet.
Results were interpreted as described in Section 13.0.
Confirmatory Steps for Test Microbes:
For *S. aureus*, the presence of the test microbe would be confirmed in a minimum of four positive carriers, if present, per test.
For *P. aeruginosa*, a minimum of seven positive carriers would be confirmed, if present, per test.
For *S. enterica*, a minimum of three positive carrier would be confirmed, if present, per test.
For tests with fewer positives than indicated above for each microbe, each positive carrier would be confirmed.
If the test with ≥ 20 positive carriers, a minimum of 50 % of the positives would have been confirmed.
The presence of the test microbe was confirmed using the Bruker MALDI Biotyper® microbial identification system.

9.0 STUDY ACCEPTANCE CRITERIA

STUDY REQUIREMENTS

1) The mean Log Density (LD) for control carriers falls inside the specified range.
   a. The mean LD for carriers inoculated with *S. aureus* and *P. aeruginosa* must be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6) and not above 7.0 (corresponding to a geometric mean density of 1.0×10^7).
   b. The mean LD for carriers inoculated with *S. enterica* must be at least 5.0 (corresponding to a geometric mean density of 1.0×10^5) and not above 6.0 (corresponding to a geometric mean density of 1.0×10^6).
   c. Refer to United States Environmental Protection Agency (EPA)Series 810 for guidance on retesting scenarios.
2) Growth should occur in all viability control tubes.
3) Growth should not occur in any of the sterility tubes.
4) Neutralization is considered adequate if the recovery of organisms from meets the specification Section 10.14 of Appendix 1.
5) For media quality controls, comparable growth acceptance will be within 50 - 200 %. Sterility acceptance is no growth.
6) Use of a disrupted *Pseudomonas aeruginosa* pellicle prepared in Section 11.3 of Appendix 1 invalidates the *P. aeruginosa* data.
7) Contamination in subculture tubes deems the test invalid.
8) Performance Objective:
   a. In order to demonstrate disinfection against each organism, the performance standard described in Section 11.11 of Appendix 1.

10.0 DATA ANALYSIS

CALCULATIONS

Calculate plate count averages using the following equation:

$$PC1 = Plate\ Count\ 1$$
$$PC2 = Plate\ Count\ 2$$
$$DF = Dilution\ Factor$$
$$PCA = Plate\ Count\ Average$$
$$PCA = \frac{[(PC1 \times DF) + (PC2 \times DF)]}{2}$$

Calculate geometric mean using the following equation:

$$\Pi = Geometric\ mean$$
$$n = number\ of\ values$$
$$\mathcal{X}i = values\ to\ average$$
$$\left(\prod_{i=1}^{n} \mathcal{X}i\right)^{\frac{1}{n}}$$

A logarithmic transformation measuring surviving control counts for each microorganism was performed.

11.0 STUDY RETENTION

Data Retention
All testing data, protocol, protocol modifications, test material records, the final report, and correspondence between Q Laboratories and the sponsor are stored in the archives at Q Laboratories, 1930 Radcliff Drive, Cincinnati, Ohio 45204, according to Q Laboratories SOP 20-ADMN-ISO-008, for a period of at least seven (7) years.

Specimen Retention
All unused test material is offered for return to the Study Sponsor at the expense of Study Sponsor. If not desired by Study Sponsor, all unused test material will be dispose of within 30 days following the study completion.

12.0 STUDY RESULTS

Control and Neutralization Results (Tables 1-2)

Based on the results reported in Table 1, the mean Log Density (LD) for control carriers fell inside the specified ranges. The mean LD for carriers inoculated with *S. aureus* and *P. aeruginosa* were at least 6.0 (corresponding to a geometric mean density of 1.0×10^6) and not above 7.0 (corresponding to a geometric mean density of 1.0×10^7). The mean LD for carriers inoculated with *S. enterica* were at least 5.0 (corresponding to a geometric mean density of 1.0×10^5) and not above 6.0 (corresponding to a geometric mean density of 1.0×10^6).

Based on the results reported in Table 2, the First Scenario indicated effective neutralization, and the efficacy evaluation was conducted using only the neutralizer subculture tubes (i.e., primary tubes).

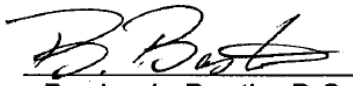
Study Results (Table 3)

Based on the results presented in Table 3, the test article met the performance standard: (0) positive carriers out of sixty (60) for each lot, when tested against *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella enterica*.

13.0 **STUDY CONCLUSION**

Based on the results presented in this study report, the test article met the performance standard: (0) positive carriers out of sixty (60) for each lot, when tested against *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Salmonella enterica*. The performance standard listed in AOAC Guideline No. 810.2200 for *S. aureus* and *P. aeruginosa* is no more than three positive carriers out of 60 per test. The performance standard for *S. enterica* is no more than one positive carrier out of 60 per test. The control carriers for *S. aureus* and *P. aeruginosa* were between 1.0×10^6 to 1.0×10^7 CFU/carrier. The control carriers for *S. enterica* were between 1.0×10^5 to 1.0×10^6 CFU/carrier. Growth occurred in all viability control tubes. Growth did not occur in any of the sterility tubes. Neutralization was considered adequate and meet the specification in Section 10.14 of Appendix 1. For media quality controls, comparable growth acceptance was within 50 - 200 %. No growth occurred in the media sterility control. No disrupted pellicles of *P. aeruginosa* test culture were used. No contamination occurred in the subculture tubes. All testing was performed in accordance with EPA Good Laboratory Practices Standards (GLPS), as specified in 40 CFR Part 160. Periodic phase audits of the study were conducted by the Quality Assurance Unit to ensure testing compliance and a review of the final report by the QAU was conducted in accordance with 40 CFR, Part 160.35, subpart B.

REPORT SUBMITTED BY:



Benjamin Bastin, B.S.
Associate Director, Microbiology Operations

10-26-21
Study Completion Date

TABLE 1: Carrier Control Results

TEST ORGANISM	DATE PERFORMED	PRE-TEST RESULT (CFU/carrier)	POST-TEST RESULT (CFU/carrier)
S. enterica	6-25-2021	1.3E+06	1.1E+06
S. aureus	6-25-2021	1.4E+06	1.4E+06
P. aeruginosa	6-30-2021	1.5E+06	1.4E+06
S. enterica	10-08-2021	1.4E+06	1.3E+06
S. aureus	10-08-2021	1.4E+06	1.3E+06
P. aeruginosa	10-08-2021	1.2E+06	1.4E+06
S. enterica	10-08-2021	1.4E+06	1.5E+06
S. aureus	10-08-2021	1.4E+06	1.5E+06
P. aeruginosa	10-08-2021	1.4E+06	1.2E+06

TABLE 2: Neutralization Results

NEUTRALIZATION CONFIRMATION					
SAMPLE ID	**TEST ORGANISM**	**DATE PERFORMED**	**INOCULUM (CFU/mL)**	**No. SUBCULTURE TUBES TESTED**	**RESULTS**
Lot 000136	*S. enterica*	6-15-2021	4.9E+06	1	+[1]
	S. aureus	6-15-2021	3.2E+05	1	+
	P. aeruginosa	6-18-2021	5.8E+07	1	+
Lot 00015*	*S. enterica*	8-10-2021	4.7E+07	1	+
	S. aureus	8-10-2021	6.3E+07	1	+
	P. aeruginosa	8-10-2021	3.7E+07	1	+
Lot 00016	*S. enterica*	10-08-2021	6.2E+07	1	+
	S. aureus	10-08-2021	4.6E+07	1	+
	P. aeruginosa	10-08-2021	4.5E+07	1	+

*Lot aged 60 days prior to testing
[1]Results were reported as + (growth)

TABLE 3: Test Results

TEST ORGANISM	IDENTIFICATON #	TEST RESULTS		
		Lot 000136	**Lot 00015***	**Lot 00016**
S. enterica	ATCC* 10708	0/60[1]	0/60	0/60
S. aureus	ATCC 6538	0/60	0/60	0/60
P. aeruginosa	ATCC 15442	0/60	0/60	0/60

*Lot aged 60 days prior to testing
[1]0/60 indicates (0) positive carriers out of sixty (60) for each lot.

Appendix 1 - Study Protocol

Study Title

Protocol for the AOAC International Use-Dilution Methods (955.14, 955.15, & 964.02)

Product Identity

DiOx+ DISINFECTANT STERILIZER

Data Requirement

40 CFR PART 158—DATA REQUIREMENTS
FOR PESTICIDES Subpart W—Antimicrobial Pesticides Guideline No. 810.2200

Author

Dane Brooks
Microbiology R&D Project Manager

Study Sponsor

Boon Industries Inc.
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Grass Valley, CA 95959

Testing Facility

Q Laboratories
1930 Radcliff Drive
Cincinnati, OH 45204
(513) 471-1300

Protocol Number (Study File)

QL 370181

Proposal Number

QL 21025-1A

Preparation Date

October 28, 2021

Study Director

Benjamin Bastin, B.S., Associate Director of Microbiology Operations

Proposed Experimental Start Date

06-08-2021

Proposed Experimental End Date

06-30-2021

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List of Tables

1.0 EFFICACY STUDY SUMMARY

1.1 STUDY TITLE: Protocol for the AOAC International Use-Dilution Methods (955.14, 955.15, & 964.02)

1.2 LABORATORY PROJECT #: QL 370181

1.3 GUIDELINE: Guideline No. 810.2200 using Official Methods of Analysis of the AOAC International, Chapter 6, Disinfectants, Use-Dilution Methods (955.14, 955.15, & 964.02). Current edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417 [Section 14.1, 14.2, and 14.3].

1.4 TESTING FACILITY: Q Laboratories
1930 Radcliff Drive
Cincinnati, OH 45204

1.5 TEST SUBSTANCE:
- 1.5.1 **DESCRIPTION:** DiOx+ DISINFECTANT STERILIZER
- 1.5.2 **% ACTIVE INGREDIENT:** Chlorine Dioxide (ClO_2), 0.4 %
- 1.5.3 **DILUTION:** ½ oz or 15 mL or 1 tbsp of substance to 32 oz (946 mL) water

1.6 TEST CONDITIONS:
- 1.6.1 **SOIL LOAD:** 5% fetal bovine serum
- 1.6.2 **WATER:** Test article is diluted in AOAC hard water solution prepared according to EPA SOP MB-30-02 [Section 14.4] to use-dilution.
- 1.6.3 **CONTACT TIME**: 3 minutes ± 5 seconds
  - 1.6.3.1 **TEMPERATURE:** Ambient Temperature (20 - 25 °C)
  - 1.6.3.2 **OTHER:** The inoculum applied includes 5% fetal bovine serum.

2.0 STUDY OBJECTIVE

2.1 The objective of the study is to document the antimicrobial efficacy of the test article against *Pseudomonas aeruginosa* American Type Culture Collection (ATCC) 15442, *Salmonella enterica* ATCC 10708, and *Staphylococcus aureus* ATCC 6538 based on the guidance provided in the Official Methods of Analysis of the AOAC International, Chapter 6, Disinfectants, Use-Dilution Methods (955.14, 955.15, & 964.02). Current edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417 [Section 14.1, 14.2, and 14.3] and US Environmental Protection Agency Office of Pesticide Programs, SOP Number MB-05-16, Standard Operating Procedure for AOAC Use Dilution Method for Testing Disinfectants [Section 14.5].

3.0 SCOPE

3.1 This test method should be performed only by those trained in microbiological techniques.

3.2 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this protocol with the exception of the test article dilution instructions.

3.3 This protocol may involve hazardous materials, operations and equipment. This protocol does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this protocol to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use. Q Laboratories will utilize SOP No. 20-ADMN-POLI-003A.

3.4 All testing will be performed in accordance with EPA Good Laboratory Practices Standards (GLPS), as specified in 40 CFR Part 160. Periodic phase audits of the study will be conducted by the Quality Assurance Unit to ensure testing compliance and a review of the final report by the QAU will be conducted in accordance with 40 CFR, Part 160.35, subpart B.

4.0 TEST METHOD

4.1 The design of this evaluation is based on the guidance provided in the Official Methods of Analysis of the AOAC International, Chapter 6, Disinfectants, Use-Dilution Methods (955.14, 955.15, & 964.02). Current edition. AOAC International, Suite 500, 481 North Frederick Avenue, Gaithersburg, MD 20877-2417 [Section 14.1, 14.2, and 14.3] and US Environmental Protection Agency Office of Pesticide Programs, SOP Number MB-05-16, Standard Operating Procedure for AOAC Use Dilution Method for Testing Disinfectants [Section 14.5].

5.0 TEST SYSTEM/ORGANISMS

5.1 The test system will be polished stainless steel cylinders inoculated and allowed to dry with a single organism of the following:

5.1.1 *Staphylococcus aureus* ATCC 6538

5.1.2 *Pseudomonas aeruginosa* ATCC 15442

5.1.3 *Salmonella enterica* serovar Choleraesuis ATCC 10708

Note: Appropriate laboratory safety conditions will be employed while working with enriched culture suspensions. These conditions will include, but are not limited to, the use of appropriate PPE (including disposable gloves, beard nets, hair nets, and lab coats), Biological Safety Cabinets, and protective eyewear.

6.0 TERMS AND CONDITIONS

6.1 Test article characterization as to the content, stability, etc., is the responsibility of the Study Sponsor. The test articles shall be characterized by the sponsor prior to the completion of the study.

7.0 TEST ARTICLE IDENTIFICATION, CHARACTERIZATION, AND HANDLING

7.1 Test Article Name - DiOx+ DISINFECTANT STERILIZER
7.2 Lot Numbers (As identified by the Study Sponsor)
  7.2.1 LOT 000136, Date Manufactured 04.02.21
  7.2.2 LOT 00015, Date Manufactured 06.01.21
  7.2.3 LOT 00016, Date Manufactured 06.08.21
7.3 Active Ingredient Type & Concentration - Chlorine Dioxide (ClO_2), 0.4 %
7.4 Diluent - AOAC hard water
7.5 Dilution - ½ oz or 15 mL or 1 tbsp of substance to 32 oz (946 mL) water
7.6 Manufacture Date – April 2nd, 2021
7.7 Expiration Date – October 2nd, 2021
7.8 Inert Control Article - Phosphate Buffered Saline
7.9 Lot Number(s) - To be specified in the final study report
7.10 Active Ingredient Type & Concentration - NaCl (0.9%)
7.11 Manufacture Date - To be specified in the final study report
7.12 Expiration Date - To be specified in the final study report

7.13 Special Handling Requirements – DiOx+ is sensitive to light and exposure to air. Product is to be kept in a cool dark place.

Test article characterization as to content, stability, etc., is the responsibility of the Study Sponsor. The test articles shall be characterized by the Sponsor prior to the completion of this study.

Test articles are handled as follows:

7.14 The test articles are stored at ambient (room) temperature under fluorescent lighting or in a cabinet.
7.15 The test articles are shaken or otherwise mixed well immediately prior to use (if applicable).
7.16 The test articles are handled safely in accordance with the chemical risks they may pose, stated in the MSDS or by the Study Sponsor during the course of pre-study communication.

8.0 STUDY PARAMETERS

8.1 Test Microorganisms - *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 15442, and *Salmonella enterica* ATCC 10708
8.2 Artificial Soil Load - 5% fetal bovine serum
8.3 Negative Control Article - None
8.4 Inert Control Article (Used for Initial Numbers Control) - Phosphate Buffered Saline
8.5 Number of Tests Comprising the Study - 540
8.6 Test Replicates - 60 replicates per organism per lot
8.7 Test Article Form - Liquid concentrate
8.8 Test Temperature - Ambient (room temperature)
8.9 Contact Time(s) - 3 minutes ± 5 seconds

8.10 Neutralization Broth - Letheen Broth (LBR) modified with sodium thiosulfate

8.11 Proposed Experimental Start Date: 06-02-2021
8.12 Proposed Experimental Termination Date: 06-30-2021

9.0 STUDY MATERIALS

9.1 Media:

9.1.1 Neutralizer: LBR modified with sodium thiosulfate

Dehydrated Powder Ingredients:
Proteose Peptone No. 3 ..... 10.0 g/L
Beef Extract ..... 5.0 g/L
Sodium Chloride ..... 5.0 g/L
Polysorbate 80 ..... 5.0 g/L
Lecithin ..... 0.7 g/L
Sodium Thiosulfate ..... 10.0 g/L

Preparation:
Suspend 25.7 g of Letheen Broth powder and 10.0 g of Sodium Thiosulfate powder in 1000 mL of purified water. Mix thoroughly. Heat with frequent agitation and boil for 1 minute to completely dissolve the powder. Dispense 10 mL into 16 x 150 mm test tubes and sterilize in an autoclave at 121 °C for 15 minutes. Final pH is 7.0 ± 0.2 at 25 °C. Store up to 3 months at room temperature.

9.1.2 Phosphate Buffered Saline (PBS)

Ingredients:
Dulbecco's Phosphate Buffered Saline 10x (DPBS 10x) .....100 mL
Deionized Water.....1000 mL
1M HCl .....as needed

Preparation:
Suspend 100 mL of DPBS 10x in 1000 mL of DI water. Mix thoroughly and adjust pH to 7.1 ± 0.2 using 1M HCl. Sterilize in an autoclave at 121 °C for 15 minutes. Final pH is 7.1 ± 0.2 at 25 °C. Store up to 3 months at room temperature.

9.1.3 Tryptic Soy Agar (TSA)

Dehydrated Powder Ingredients:
Pancreatic digest of Casein ..... 15 g/L
Papaic digest of Soybean ..... 5 g/L
Sodium Chloride ..... 5 g/L
Agar ..... 15 g/L

Preparation:
Measure 1000 mL of DI water into a graduated cylinder and transfer approximately half the water to a flask. Weigh 40 g of dehydrated Tryptic Soy Agar and add to the water in the flask. Mix briefly and add the remaining water. Sterilize in an autoclave at 121 °C for 15 minutes. Final pH is 7.3 ± 0.2 at 25 °C. Store up to 2 months at room temperature.

9.1.4 Sterile Deionized (DI) Water

Ingredients:

Deionized Water	as needed

Preparation:
Measure desired volume of DI water into a suitable autoclavable vessel. Sterilize in an autoclave at 121 °C for 15 minutes. Store up to 3 months at room temperature.

9.1.5 Synthetic Broth (SB) (Commercially available from Fisher Scientific Cat No. NC0493363)

Dehydrated Powder Ingredients:

L-Cystine	0.050 g/L
DL-Methionine	0.370 g/L
L-Arginine	0.400 g/L
DL-Histidine	0.300 g/L
L-Lysine	0.850 g/L
L-Tyrosine	0.210 g/L
DL-Threonine	0.500 g/L
DL-Valine	1.000 g/L
L-Leucine	0.800 g/L
DL-Isoleucine	0.440 g/L
Amino acetic acid	0.060 g/L
DL Serine	0.610 g/L
DL-Alanine	0.430 g/L
L-Glutamic acid	1.300 g/L
L-Aspartic acid	0.450 g/L
DL-Phenylalanine	0.260 g/L
DL-Tryptophan	0.050 g/L
L-Proline	0.050 g/L
Sodium chloride	3.000 g/L
Potassium chloride	0.200 g/L
Magnesium sulphate	0.050 g/L
Monopotassium phosphate	1.500 g/L
Disodium phosphate	4.000 g/L
Thiamine hydrochloride	0.010 g/L
Nicotinamide	0.010 g/L

Preparation:

Suspend 16.9 g in 1000 mL DI water. Heat if necessary to dissolve the medium completely. Dispense 10 mL amounts in 20 x 150 mm culture tubes and sterilize in an autoclave at 121 °C for 15 minutes. Cool to room temperature and just before use, aseptically add 0.1 ml of 10% sterile dextrose solution. Final pH is 7.1 ± 0.2 at 25 °C. Store up to 2 months at 2 - 8°C.

9.1.6 Tryptic Soy Agar with 5% Sheep Blood (SBA) (Commercially available from BD, PN 221261)

Ingredients:
Pancreatic digest of Casein ..... 14.5 g/L
Papaic digest of Soybean Meal..... 5 g/L
Sodium Chloride..... 5 g/L
Agar ..... 14 g/L
Growth Factors..... 1.5 g/L
Defibrinated Sheep blood..... 5%

Preparation:
Available as pre-poured plates.

Note: Substitutions of equivalent media made be made without sponsor approval. As appropriate, media are purchased sterile or sterilized via autoclaving.

9.2 Equipment
- 9.2.1 Transfer Loops 23 AWG, platinum 4 mm loop fused on 75 mm shaft, bent at a 30° angle
- 9.2.2 Incubator Temperature Range 36 ± 1 °C
- 9.2.3 Incubator Thermometer, NIST Traceable
- 9.2.4 Steam Autoclave
- 9.2.5 Vortex Mixer
- 9.2.6 Calibrated, Traceable Minute/Second Timer
- 9.2.7 Refrigerator 2 - 8 °C
- 9.2.8 Refrigerator Thermometer, NIST Traceable
- 9.2.9 Adjustable Pipettor 20 µL – 200 µL and 100 µL – 1000 µL capacity
- 9.2.10 Reichert Quebec® Colony Counter, or equivalent
- 9.2.11 Hand Tally
- 9.2.12 Biological Safety Cabinet (BSC)
- 9.2.13 Metal Forceps
- 9.2.14 Timer
- 9.2.15 Spectrophotometer
- 9.2.16 Ultrasonic Cleaner
- 9.2.17 Specialized glassware
  - 9.2.17.1 For disinfectant, use autoclavable 25 x 100 mm tubes (Bellco Glass Inc., Vineland, NJ). For glassware used to prepare test chemical, refer to SOP MB-22.
  - 9.2.17.2 Available from Bellco Glass Inc., Vineland, NJ
- 9.2.18 Wire Hook
  - 9.2.18.1 For carrier transfer.

9.2.18.2 Make 3 mm right angle bend at end of 50 - 75 mm nichrome wire No. 18 B&S gage.

Note: Substitutions of equivalent equipment may be made without sponsor approval. As appropriate, equipment/apparatus are purchased sterile or sterilized via autoclaving.

9.3 Materials

9.3.1 Test tubes
9.3.2 Glass beaker
9.3.3 Serological Pipettes
9.3.4 20 - 200 µL and 100 - 1000 µL Micropipette tips
9.3.5 50 mL Conical Tubes
9.3.6 Disposable Petri dishes, 100 x 15 mm
9.3.7 Disposable L-shaped plastic spreaders
9.3.8 Whatman™ Qualitative Filter Paper: Grade 2 Circles
9.3.9 Carriers

9.3.9.1 Polished stainless steel cylinders, 8 ± 1 mm outer diameter, 6 ± 1 mm inner diameter, 10 ± 1 mm length; type 304 stainless steel, SS 18 - 8.
9.3.9.2 Physical screening, cleaning, and storage of carriers will be prepared according to EPA SOP MB-03-07 [Section 14.6].
9.3.9.3 Use only carriers that pass bioscreening. Bioscreen carriers according to EPA SOP MB-03-07 [Section 14.6].
9.3.9.4 Available from Fisher Scientific catalog number 07-907-5Q.

Note: Substitutions of equivalent materials may be made without sponsor approval. As appropriate, materials are purchased sterile or sterilized via autoclaving.

10.0 NEUTRALIZATION

10.1 Sterile carriers are used for this assay.
10.2 Perform the neutralization in advance of product testing to verify that the prescribed neutralizer is suitable for the efficacy evaluation. Concurrently conduct two test scenarios to determine an appropriate approach for performing the product efficacy evaluation:

10.2.1 First Scenario: expose carriers to the disinfectant and transfer them into the neutralizer subculture medium (primary tube). No secondary subculture medium transfers are conducted. Inoculate the neutralizer tubes containing the carrier with a test organism suspension to deliver 5 - 100 CFU/mL
10.2.2 Second Scenario: expose carriers to the disinfectant and transfer them into the neutralizer subculture medium (primary tube); in addition, subsequently transfer the carriers to a secondary subculture medium (secondary tube). Inoculate tubes with a test organism suspension to deliver 5 - 100 CFU/mL.

10.3 The purpose of the two-scenario approach is to determine if the prescribed neutralizer for the disinfectant is sufficient to support growth.

10.4 Inoculum Preparation:
- 10.4.1 Prepare inoculum as described in Section 11.3.

10.5 Inoculum Enumeration:
- 10.5.1 Prepare serial ten-fold dilutions of the inoculum by pipetting 1 mL of the final test culture into 9 mL of PBS. Use four dilutions, (e.g., 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) to inoculate the neutralizer (primary tubes) and subculture medium (secondary tubes). The target number of cells is 5 - 100 CFU/mL; this level should be seen in one of the two highest dilutions.
- 10.5.2 To estimate CFU/mL, plate 0.1 mL of each of the four dilutions in duplicate on TSA. Briefly vortex each dilution tube prior to plating. Plates must be dry prior to incubation.
- 10.5.3 Record the dilution and plating information on the appropriate master data sheet.
- 10.5.4 Incubate plates (inverted) at 36 ± 1 °C for up to 48 ± 2 h and record colony counts. Plates that have colony counts over 300 are labeled as too numerous to count (TNTC). Record the counts on the appropriate master data sheet.

10.6 Product Sample Preparation:
- 10.6.1 Prepare the product as described in Section 11.6.

10.7 Carrier Preparation:
- 10.7.1 Prepare the carriers as described in Section 11.4.

10.8 First Scenario: Neutralizer - Primary Subculture Treatment Only:
- 10.8.1 Requires four dried carriers (with broth culture added) per organism. Use the carrier type required for the specific test.
- 10.8.2 Apply the product to the carriers as described in Section 11.7.
- 10.8.3 Per organism, expose four of the carriers to the disinfectant for the specified contact time in the same manner as product efficacy testing. Record the carrier transfer information on the appropriate master data sheet.
- 10.8.4 After the last carrier of a set (4 total carriers) has been treated with disinfectant, and the contact time is complete, aseptically transfer carriers in order in a timed fashion into tubes containing the specified neutralizer, in the same manner as product efficacy testing. Drain excess liquid from the carrier prior to the transfer. This set of neutralizer tubes (4 total tubes) represents the Neutralizer-Primary Subculture Treatment. Refer to Section 10.11 for treatment inoculation.
- 10.8.5 Proceed immediately with the Second Scenario.

10.9 Second Scenario: Neutralizer Subculture Treatment Plus Secondary Subculture Treatment:
- 10.9.1 Requires four dried carriers (with broth culture added) per organism. Use the carrier type required for the specific test.
- 10.9.2 Apply the product to the carriers according to specific instruction provided
- 10.9.3 Per test organism, expose four of the carriers to the disinfectant for the specified contact time in the same manner as product efficacy

testing. Record the carrier transfer information on the appropriate master data sheet.

10.9.4 After the last carrier of a set (4 total carriers) has been treated with the disinfectant, and the contact time is complete, aseptically transfer carriers in order in a timed fashion into tubes containing the specified neutralizer, in the same manner as product efficacy testing. Drain excess liquid from the carrier prior to the transfer. This set of neutralizer tubes (4 total tubes) will represent the Neutralizer-Primary Subculture Treatment.

10.9.5 Following the last carrier transfer into the neutralizer tube, incubate both First and Second Scenario neutralizer tubes at room temperature for 30 - 45 min. Then, for the Second Scenario, transfer each carrier in order into a culture tube containing the secondary subculture medium. This portion of the assay is not timed. This set of tubes (4 total tubes) represents the Secondary Subculture Treatment. Refer to Section 10.11 for treatment inoculation.

10.10 Controls:

10.10.1 Inoculated controls:

10.10.1.1 The Neutralizer-Primary Inoculated Control contains four tubes of fresh, unexposed (to disinfectant) neutralizer-primary media.

10.10.1.2 The Secondary Subculture Inoculated Control contains four tubes of secondary subculture media.

10.10.1.3 It is highly desirable that the preparation of each medium be the same as used in the treatments. Refer to Section 10.11 for treatment inoculation.

10.10.2 Uninoculated controls:

10.10.2.1 Neutralizer-Primary and Secondary Subculture Uninoculated Controls. Incubate one tube each of uninoculated neutralizer and secondary subculture media with the other tubes.

10.10.3 Confirm sterility of carriers in advance or concurrently with testing: add an uninoculated carrier to a tube of 10 - 20 mL LBR and incubate at 36 ± 1 °C for 3 - 10 days.

10.11 Treatment Inoculation:

10.11.1 After Section 10.9.5, inoculate all tubes concurrently using Tables 1, 2, and 3.

Table 1: First Scenerio: Inoculation of Treatment Group with Dilutions of the Test Organism*

Treatment	Dilutions Added			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Neutralizer-Primary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL

*1×10^{-4} through 1×10^{-7}; based on an approx. starting suspension of 10^8 to 10^9 CFU/mL

Table 2: Second Scenerio: Inoculation of Treatment Group with Dilutions of the Test Organism*

Treatment	Dilutions Added			
	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Neutralizer-Primary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL
Secondary Subculture Treatment	0.1 mL	0.1 mL	0.1 mL	0.1 mL

*1×10^{-4} through 1×10^{-7}; based on an approx. starting suspension of 10^8 to 10^9 CFU/mL

Table 3: Controls: Inoculation of Control Groups with Dilutions of the Test Organism*

Controls		Dilutions Added			
		10^{-4}	10^{-5}	10^{-6}	10^{-7}
Inoculated Controls (media performance)	Neutralizer - Primary	0.1 mL	0.1 mL	0.1 mL	0.1 mL
	Secondary Subculture				
Sterility Controls	Neutralizer - Primary	0.1 mL	0.1 mL	0.1 mL	0.1 mL
	Secondary Subculture				

*1×10^{-4} through 1×10^{-7}; based on an approx. starting suspension of 10^8 to 10^9 CFU/mL

10.11.2 Shake tubes thoroughly. Incubate all tubes for 48 ± 2 h at 36 ± 1 °C.

10.12 Recording Results and Confirmation Testing:

10.12.1 Record results as + (growth/turbidity) or 0 (no growth) on the appropriate master data sheet.

10.12.2 For each treatment and control group, Gram stain a minimum of one positive tube per treatment. Select the tube with the highest dilution showing growth (inoculated with the dilution with fewest CFU/mL delivered).

10.12.3 Record confirmation results on the appropriate master data sheet.

10.13 Interpretation of Results:

10.13.1 Plate count data. One of the four dilutions plated should provide counts within the approximate target range, 5 - 100 CFU/mL/

10.13.1.1 Note: The lack of complete neutralization of the product of the neutralizer itself may be masked when high level of inoculum is added to the subculture tubes.

10.13.2 Controls. Growth in the Secondary Subculture Inoculated Control verifies the presence of the test microbe, performance of the media, and provides a basis for comparison of growth in the neutralizer and subculture treatment tubes. *No growth or only growth in tubes which received high levels of inoculum (e.g., a dilution with plate counts which are TNTC) indicates poor media performance*. Growth in the Neutralizer-Primary Inoculated Control should be comparable to the Secondary Subculture Inoculated Control if the neutralizer is the same as the secondary subculture media.

10.13.2.1 There may be cases when the neutralizer (primary tubes) is significantly different from the secondary subculture media. In these cases, growth may not be comparable to the Secondary Subculture inoculated Control.

10.13.2.2 The Neutralizer-Primary Uninoculated Control and Secondary Subculture Uninoculated Control tubes are used to determine sterility and must show no growth for the test to be valid.

10.13.3 Treatments. The occurrence of growth in the Neutralizer-Primary Subculture and Secondary Subculture Treatment tubes are used to assess the effectiveness of the neutralizer.

10.13.3.1 First Scenario: The neutralizer itself may exhibit bacteriostatic activity against the test microbe. *No growth or growth only in tubes which received a high titer of inoculum (e.g., the dilution with plate counts which are TNTC) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions. For the neutralizer to be deemed effective, growth must occur in the Neutralizer Primary Subculture Treatment tubes which received a lower titer of inoculum (e.g., 5-100 CFU/mL).*

10.13.3.2 Second Scenario: The neutralizer itself or in combination with the recovery (subculture) medium may exhibit bacteriostatic activity against the test microbe. *No growth or growth only in tubes which received a high titer of inoculum (e.g., the dilution with plate counts which are TNTC) indicates poor neutralization and/or presence of bacteriostatic properties of the neutralizer or neutralizer-disinfectant interactions. For the neutralizer to be deemed effective, growth must occur in the Secondary Subculture Treatment tubes which received a lower titer of inoculum (e.g., 5 100 CFU/mL).*

10.14 Efficacy Evaluation based on Neutralization Results:

10.14.1 If results from the First Scenario indicate effective neutralization, conduct the efficacy evaluation using only the neutralizer subculture tubes (i.e., primary tubes).

10.14.2 If results from the First Scenario (Neutralizer-Primary Subculture Treatment only) are inconclusive and/or indicate that a bacteriostatic effect from the neutralizer or neutralizer-disinfectant interaction is present, evaluate results from the Second Scenario to determine if the Secondary Subculture tube provide appropriate neutralization.

10.14.3 If the Second Scenario is deemed effective, conduct the efficacy evaluation using both subculture media tubes (i.e., primary and secondary tubes).

10.14.4 If results from the Second Scenario (Neutralizer-Primary Subculture Treatment tubes and Secondary Subculture Treatment tubes) are inconclusive and/or indicate that a bacteriostatic effect from the neutralizer or neutralizer-disinfectant interaction is present, assay an alternative neutralizer prior to conducting the efficacy evaluation. The alternative neutralizer may not be specified in the test parameters.

10.15 Data Analysis/Calculations:

10.15.1 Enumerate plate counts and calculate CFU/mL added to each tube based on the average of countable plates. Apply TNTC for counts above 300 CFU.

10.15.2 To calculate the average CFU/mL per dilution added to each tube, add the plate counts for each plate within the dilution and divide by two.

10.15.3 Use counts from 0 through 300 in the calculations.

11.0 TEST DESCRIPTION

11.1 Preparation of carriers:

11.1.1 Physical screening, cleaning, and storage of carriers will be prepared according to EPA SOP MB-03-07 [Section 14.6].

11.1.2 Use only carriers that pass bioscreening. Bioscreen carriers according to EPA SOP MB-03-07 [Section 14.6].

11.2 Preparation of test substance (article):

11.2.1 Dilute the test substance ½ oz or 15 mL or 1 tbsp of substance to 32 oz (946 mL) AOAC hard water, prepared according to EPA SOP MB-30-02 [Section 14.4].

11.3 Test Microorganism Preparation:

Seed-lot culture maintenance techniques are followed to ensure the viable microorganisms used for inoculation are not more than five passages removed from the original master seed lot.

11.3.1 The test microorganism cultures will be prepared as follows:

11.3.1.1 Propagate on SBA from a Q Laboratories frozen stock culture stored at -70 °C for 18 - 24 h at 36 ± 1 °C.

11.3.1.2 After initial incubation, pick an isolated colony to SB, vortex (*S. aureus and S. enterica*) and incubate at 36 ± 1 °C for 24 ± 2 h.

Note: One daily transfer is required prior to the inoculation of a final test culture. Daily cultures may be subcultured for up to 5 days; each daily culture may be used to generate a test culture.

11.3.1.3 To generate test cultures, inoculate a sufficient number of 50 mL conical tubes containing 10 mL SB with 10 µL per tube of the 24 h culture, then vortex to mix.

11.3.1.4 Incubate 48 - 54 h at 36 ± 1 °C. Do not shake the 48 - 54 h test culture.

11.3.1.5 For *P. aeruginosa,* remove the pellicle at the top of the broth tube by aspirating with a pipette. Transfer the broth to a sterile test tube. Avoid transferring the pellicle at the bottom of the culture tube. Presence of pellicle in the final culture makes it unusable for testing.

11.3.1.6 For *S. aureus*, *S.enterica*, and *P. aeruginosa,* using a vortex-style mixer, mix the 48 - 54 h test cultures for 3 - 4 s and let stand 10 min at room temperature before continuing.

11.3.1.7 Remove the upper portion (upper ¾) of each culture leaving behind any debris or clumps, and transfer to a sterile flask; pool each culture in its respective flask and swirl to mix.

11.3.1.8 Measure and record the OD at 650 nm. Use sterile SB medium to calibrate the spectrophotometer.

11.3.1.9 Transfer an aliquot (about 10 mL) of the final test culture into a sterile tube for carrier inoculation.

11.3.1.10 To achieve mean carrier counts within the target concentration of 10^5 - 10^6, the final test culture may be diluted with sterile broth.

11.3.1.11 Transfer an aliquot of about 10 mL of the final test culture into a sterile tube for carrier inoculation.

Note: Use the test culture for carrier inoculation within 30 minutes.

Note: To achieve mean carrier counts within the appropriate range (see Section 13.0), the final test culture may be diluted (e.g., one part culture plus one part sterile broth) prior to the addition of the organic soil to the inoculum using the sterile culture medium used to generate the final test culture (e.g., synthetic broth). Use the diluted test culture for carrier inoculation within 30 min.

Note: Concentration of the final test culture may be used in the event the bacterial titer in the final test cultures is too low (OD ≤ 0.2). Concentration may be achieved using centrifugation (e.g., 5,000 g for 20 min) and resuspending the pellet in the appropriate volume of the sterile final test culture medium necessary to meet the carrier count range. Use the concentrated test culture for carrier inoculation within 30 min.

11.3.1.12 Add appropriate amount of organic soil make achieve a 5 % solution. Swirl to mix.

11.4 Carrier Inoculation:

11.4.1 Aliquot 20 mL portions of test culture into sterile 25 x 150 mm test tubes.

11.4.2 Drain the water from the carriers. Aseptically transfer 20 carriers into each of the tubes containing the test culture. The test culture must completely cover the carriers; reposition carriers as necessary to ensure coverage. Alternatively, siphon off the water from the carriers and add 20 mL test culture directly to the carriers without transferring.

11.4.3 Allow carriers to remain in the inoculum for 15 ± 2 min.

11.4.4 Following the carrier exposure period, remove carriers individually from the inoculum using a flamed nichrome wire hook, briefly tap each carrier against the side of the tube to remove excess culture, and place on end in vertical position in sterile Petri dish matted with 2 layers of Whatman No. 2 (or equivalent) sterile filter paper. Do not remove inoculum from the tube in advance of removing carriers. Ensure that carriers do not touch or fall over in the Petri dish. Place no more than 12 carriers in a Petri dish. Place lid on Petri dish.

11.4.5 Dry carriers in incubator at 36 ± 1 °C for 40 ± 2 min. Record the timed carrier inoculation activities on the appropriate master data sheet. Expose all carriers to disinfectant within two hours of drying.

11.4.6 Inoculate at least 80 carriers; 60 carriers are required for testing, 6 for control carrier counts, and 1 for viability control as listed in Table 1.

Note: Vortex-mix the inoculum periodically during the inoculation of carriers.

11.5 Enumeration of Viable Bacteria from Control Carriers:

11.5.1 Select one carrier of 6 Petri dishes, assay dried carriers in 2 sets of three carriers, one set immediately prior to conducting the efficacy test and one set immediately following the test. There are 3 carriers are enumerated prior to initiating the test procedure and 3 carriers are enumerated after the test procedure.

11.5.2 Place each inoculated dried carrier into a tube containing 10 mL of LBR and sonicate in an ultrasonic cleaner for 1 min ± 5 s. Record the time of sonication activities on the appropriate master data sheet.

11.5.3 For sonication, place tubes into an appropriately sized glass beaker with tap water to the level of the LBR in the tubes. Place the beaker in an ultrasonic cleaner so that the water level in the beaker is even with the water level fill-line on the tank. Fill the tank with tap water to the water level fill-line. Hold the beaker so that it does not touch the bottom of the tank and all 3 liquid levels (inside the test tubes, inside the beaker, and inside the tank) are approximately the same.

11.5.4 After sonication, briefly mix and make serial ten-fold dilutions in 9 mL dilution blanks of PBS. Briefly vortex and plate 0.1 mL aliquots of appropriate dilutions in duplicate on TSA using spread plating. Plate appropriate dilutions to achieve colony counts in the range of 30 - 300 colony forming units (CFU) per plate. Spread inoculum evenly over the surface of the agar. Plates must be dry prior to incubation. If the serial dilutions are not made and plated immediately, keep the sonicated tubes at 0 - 5 °C until this step can be done. Complete the dilutions and plating within 2 h after sonication.

Alternatively, pool the LBR from the tubes with the carriers and briefly vortex for each set of three carriers. Serially dilute and plate 0.1 mL aliquots of the pooled media (30 mL).

11.5.5 Incubate plates (inverted) at 36 ± 1 °C for up to 48 ± 2 h.

11.5.6 Count colonies. Plates that have colony counts over 300 will be reported at TNTC. Record counts on the appropriate master data sheet.

Note: At a minimum, conduct a culture purity check (isolation streak) using suspension from one dilution tube of one carrier or pooled set.

11.6 Disinfectant Sample Preparation:

11.6.1 Prepare disinfectant sample per EPA SOP MB-22-05, Disinfectant Product Preparation [Section 14.7].

11.6.2 Equilibrate the water bath and allow it to come to 25 ± 1 °C. Prepare the disinfectant dilutions within 3 hours of performing the assay unless test parameters specify otherwise. Record the time of disinfectant preparation on the appropriate master data sheet.

11.6.3 Dispense 10 mL aliquots of the disinfectant into 25 x 100 mm test tubes, one tube per carrier. Place tubes in the equilibrated water bath for approximately 10 min to allow disinfectant to come to specified temperature. Record the temperature of the water bath before and after testing on the appropriate master data sheet.

11.7 Test Procedure:

11.7.1 Sequentially transfer the carriers from the Petri dish to the test tubes containing the disinfectant at appropriate intervals (e.g., 30 second intervals).

11.7.2 Add one carrier per tube and swirl the tube using 2 - 3 gentle rotations before placing it back in the water bath. Add carrier within ± 5 seconds of the specified time for a contact time of 3 minutes ± 5 seconds.

11.7.3 Using alternating hooks, flame-sterilize the hook and allow it to cool after each carrier transfer. When lowering the carriers into the disinfectant tubes, neither the carrier itself nor the tip of the wire hook can touch the interior sides of the tube. If the interior sides of the tube are touched, repeat the carrier.

11.7.4 Following the exposure time, sequentially transfer the carriers into subculture/neutralizer media. Remove the carrier from the disinfectant with a sterile hook, tap it against the interior sides of the tube to remove the excess disinfectant, and transfer it into the subculture tube within ± 5 s. Avoid tapping the carrier against the upper third of the tube. Avoid contact of the carrier to the interior sides of the subculture tube during transfer.

11.7.5 Recap the subculture tube and shake thoroughly. Incubate at 36 ± 1 °C for 48 ± 2 h.

11.7.6 If a secondary subculture tube is deemed necessary to achieve neutralization, then transfer the carrier from the primary tube to a secondary tube of sterile medium after a minimum of 30 ± 5 min at room temperature from the end of the initial transfer. Within 25 - 60 min of the initial transfer, transfer the carriers using a sterile wire hook to a second subculture tube. Move the carriers in order but the movements do not have to be timed. Thoroughly shake the subculture tubes after all of the carriers have been transferred. Incubate both the primary and secondary subculture tubes 48 ± 2 h at 36 ± 1 °C.

11.7.7 Record timed events on the appropriate master data sheet.

11.8 Sterility and Viability Controls:

11.8.1 Viability controls. Place 1 (or 2) dried inoculated untreated carrier(s) into separate tubes of the neutralizing subculture broth (if primary and secondary media are different). Incubate tubes with the efficacy test. Report results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity. Growth should occur in both tubes. Record results on the appropriate master data sheet.

11.8.2 Sterility controls. Place one sterile, uninoculated carrier into a tube of neutralizing subculture broth. Incubate tube with the efficacy test. Report results as + (growth), or 0 (no growth) as determined by

presence or absence of turbidity. Growth should not occur in the tube. Record results on the appropriate master data sheet.

11.9 Results:

11.9.1 Gently shake each tube prior to recording results. Record results as + (growth) or 0 (no growth) as determined by presence or absence of turbidity, on the appropriate master data sheet.

11.9.2 For a test with secondary subculture tubes, a positive result in either the primary or secondary subculture tube is considered a positive result for a carrier set.

11.9.3 Specialized neutralizer/subculture medium such as Dey/Engley broth will not show turbidity; rather the presence of pellicle at the surface of the medium (for *P. aeruginosa*) or a color change to the medium (yellow for growth of *S. aureus* or *S. enterica*) must be used to assess the results as a positive or negative outcome.

11.9.3.1 Use viability controls for comparative determination of a positive tube.

11.9.3.2 If the product passes the performance standard, a minimum of 20 % of the remaining negative tubes will be assayed for the presence of the test microbe using isolation streaks on TSA. Record preliminary results and conduct isolation streaks at 48 ± 2 h, however, continue to incubate negative tubes for up to an additional 24 hours to confirm the results.

11.10 Confirmatory Steps for Test Microbes:

11.10.1 For *S. aureus*, confirm the presence of the test microbe in a minimum of four positive carriers, if present, per test.

11.10.2 For *P. aeruginosa*, confirm a minimum of seven positive carriers. If present, per test.

11.10.3 For *S. enterica*, confirm a minimum of three positive carrier sets, if present, per test.

11.10.4 For tests with fewer positives than indicated above for each microbe, confirm each positive carrier.

11.10.5 For any test with ≥ 20 positive carriers, confirm a minimum of 50 % of the positives.

11.10.6 If secondary subculture tubes are used and both primary and secondary subculture tubes are positive in a carrier set, select only the secondary subculture tube for microbe confirmation.

11.10.7 To confirm the presence of the test microbe, use Gram staining, solid media, and biochemical evaluation (i.e., Vitek 2 Compact).

11.10.7.1 Streak isolate growth from a positive subculture medium tube onto TSA or SBA, and appropriate selective medium. Incubate media plates 24 ± 2 h at 36 ± 1 °C and record the results. Examine colonies on plates for morphology and characteristics of the test organism (conforming to the morphology in Bergey's Manual).

11.10.7.2 Reference Bergey's Manual of Systematic Bacteriology Volumes 1 and 2 [Section 14.8 and 14.9] for typical diagnostic characteristics of the test microbes (Gram stain reactions, cell morphology, and colony characteristics on solid media).

11.10.7.3 Alternatively, isolates may be analyzed using Bruker's MALDI Biotyper®.

11.10.7.4 If confirmatory testing determines that the identity of the unknown was not the test organism, annotate the positive entry (+) on the results sheet to indicate a contaminant was present.

11.11 Performance Standard:

11.11.1 The performance standard for *S. aureus* is 0 - 3 positive carriers out of sixty.

11.11.2 The performance standard for *P. aeruginosa* is 0 - 6 positive carriers out of sixty.

11.11.3 The performance standard for *S. enterica* is 0 - 2 positive carriers out of sixty.

11.11.4 If replicated testing is required for any microbe, conduct testing with that microbe on independent test days.

11.12 Re-use of Stainless Steel Carriers:

11.12.1 After use, autoclave all carriers using a validated and appropriate sterilization cycle. Carriers for which test results were negative may be reused after cleaning. Carriers that are positive are re-cleaned and screened biologically (see SOP MB-03-07, Section 14.6) before re-use. These carriers may be reused if the biological screening test results is no growth. The extra inoculated carriers, positive control, and those used for carrier counts may be autoclaved, re-cleaned, and used again.

11.13 Media Quality Controls:

11.13.1 Media quality will be verified using Q Laboratories Standard Operating Procedure 10-CGMP-METH-009G.

12.0 DATA ANALYSIS/CALCULATIONS

12.1 Calculate plate count averages using the following equation:

$$PC1 = Plate\ Count\ 1$$
$$PC2 = Plate\ Count\ 2$$
$$DF = Dilution\ Factor$$
$$PCA = Plate\ Count\ Average$$
$$\mathrm{PCA} = \frac{[(PC1 \times DF) + (PC2 \times DF)]}{2}$$

12.2 Calculate geometric mean using the following equation:

$$\Pi = Geometric\ mean$$
$$n = number\ of\ values$$
$$\mathcal{X}i = values\ to\ average$$
$$\left(\prod_{i=1}^{n} \mathcal{X}i\right)^{\frac{1}{n}}$$

12.3 A logarithmic transformation measuring surviving control counts for each microorganism will be performed.

13.0 STUDY ACCEPTANCE CRITERIA

13.1 Study Requirements:
- 13.1.1 The mean Log Density (LD) for control carriers falls inside the specified range.
  - 13.1.1.1 The mean LD for carriers inoculated with *S. aureus* and *P. aeruginosa* must be at least 6.0 (corresponding to a geometric mean density of 1.0×10^6) and not above 7.0 (corresponding to a geometric mean density of 1.0×10^7).
  - 13.1.1.2 The mean LD for carriers inoculated with S. enterica must be at least 5.0 (corresponding to a geometric mean density of 1.0×10^5) and not above 6.0 (corresponding to a geometric mean density of 1.0×10^6).
  - 13.1.1.3 Refer to Series 810 for guidance on retesting scenarios.
- 13.1.2 Growth should occur in all viability control tubes.
- 13.1.3 Growth should not occur in any of the sterility tubes.
- 13.1.4 Neutralization is considered adequate if the recovery of organisms from meets the specification Section 12.14.
- 13.1.5 For media quality controls, comparable growth acceptance will be within 50 - 200 %. Sterility acceptance is no growth.
- 13.1.6 Use of a disrupted *Pseudomonas aeruginosa* pellicle prepared in Section 11.3 invalidates the *P. aeruginosa* data.
- 13.1.7 Contamination in subculture tubes deems the test invalid.

13.2 Performance Objective:
- 13.2.1 In order to demonstrate disinfection against each organism, the performance standard described in Section 11.11.

14.0 REFERENCES

14.1 Official Methods of Analysis. Method 955.14 - *Salmonella enterica*. Posted March 2013. AOAC INTERNATIONAL, Gaithersburg, MD.

14.2 Official Methods of Analysis. Method 955.15 - *Staphylococcus aureus*. Posted March 2013. AOAC INTERNATIONAL, Gaithersburg, MD.

14.3 Official Methods of Analysis. Method 964.02 - *Pseudomonas aeruginosa*. Posted March 2013. AOAC INTERNATIONAL, Gaithersburg, MD.

14.4 US Environmental Protection Agency Office of Pesticide Programs, SOP Number MB-30-02, Standard Operating Procedure for Preparation of hard water and other diluents for preparation of antimicrobial products. https://www.epa.gov/sites/production/files/2019-08/documents/mb-30-02.pdf (Accessed 06-03-2021)

14.5 US Environmental Protection Agency Office of Pesticide Programs, SOP Number MB-05-16, Standard Operating Procedure for AOAC Use Dilution Method for Testing Disinfectants. https://www.epa.gov/sites/production/files/2020-02/documents/mb-05-16.pdf (Accessed 06-03-2021)

14.6 US Environmental Protection Agency Office of Pesticide Programs, SOP Number MB-03-07, Standard Operating Procedure for Screening of Polished Stainless Steel Penicylinders, Porcelain Penicylinders, and Glass Slide Carriers Used in Disinfectant Efficacy Testing. https://www.epa.gov/sites/production/files/2018-01/documents/mb-03-07.pdf (Accessed 06-03-2021)

14.7 US Environmental Protection Agency Office of Pesticide Programs, SOP Number MB-22-05, Standard Operating Procedure for Preparation and Sampling Procedures for Antimicrobial Test Substances. https://www.epa.gov/sites/production/files/2020-02/documents/mb-22-05.pdf (Accessed 06-03-2021)

14.8 Krieg, Noel R. and Holt, John G. 1984. Bergey's Manual of Systematic Bacteriology Volume 1. Williams & Wilkins, Baltimore, MD*. P. aeruginosa* p. 164*, S. enterica* p. 447.

14.9 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.

15.0 FINAL REPORT

15.1 A final validation report will be prepared upon completion of the study, including a tabularized summary of data and a description of results of the study.

16.0 RECORDS TO BE MAINTAINED

16.1 All testing data, protocol, protocol modifications, test material records, the final report, and correspondence between Q Laboratories and the sponsor will be stored in the archives at Q Laboratories, 1930 Radcliff Drive, Cincinnati, Ohio 45204, according to Q Laboratories SOP 20-ADMN-ISO-008, for a period of at least seven (7) years.

17.0 QUALITY COMPLIANCE

17.1 Q Laboratories has developed and implemented a quality management system that enhances our ability to provide testing services that consistently meet client expectations and regulatory requirements. All testing will be performed in accordance with EPA Good Laboratory Practices Standards (GLPS), as specified in 40 CFR Part 160. Periodic phase audits of the study will be conducted by the Quality Assurance Unit to ensure testing compliance and a review of the final report by the QAU will be conducted in accordance with 40 CFR, Part 160.35, subpart B.

18.0 PROTOCOL MODIFICATIONS

18.1 During the testing phase, changes to the protocol may be required. The study sponsor will be notified immediately of any modifications to the protocol. Approval of the modifications is required before any additional analysis is

conducted. The modifications will be added to the protocol as an amendment and approved by both the study director and study sponsor.

19.0 PRODUCT DISPOSAL

19.1 All unused test devices will be offered for return to the Study Sponsor at the expense of Study Sponsor. If not desired by Study Sponsor, all unused test material to be disposed of within 30 days following the study completion.

20.0 ACCEPTANCE OF STUDY PROTOCOL

20.0 **ACCEPTANCE OF STUDY PROTOCOL**

Protocol for the AOAC International Use-Dilution Methods (955.14, 955.15, & 964.02)

Q Laboratories (Testing Facility)
1930 Radcliff Drive
Cincinnati, OH 45204

Study Director: _____ 6.9.21
Benjamin J Bastin, B.S. — Date
Associate Director, Microbiology Operations

Boon Industries Inc.
110 Spring Hill Drive, Suite #16
Grass Valley, CA 95959

_____ Justin M. Gonzalez 06/09/21
Representative — Date

Chief Executive Officer
Title

Appendix 2 - Material Safety Data Sheet

DiOx+ MATERIAL SAFETY DATA SHEET

SECTION 1: PRODUCT AND COMPANY IDENTIFICATION

Product Name:	Chlorine Dioxide Aqueous Solution (ClO_2) < 0.4%
Recommended Use:	Biocide
MSDS Number:	98018-4000
Manufacturer:	Boon Industries Inc., 110 Spring Hill Drive, Suite 16, Grass Valley, Ca. 95945
Product Information:	1 (530) 648-1333
Emergency Number:	1 (800) 222-1222

SECTION 2: HAZARDS IDENTIFICATION

GHS Classification:	Skin Irritation:	Category 2
	Eye Irritation:	Category 2B
	Acute Toxicity – Inhalation:	Category 4
Hazard Statements:	Causes Skin Irritation Causes Eye Irritation Harmful if Inhaled	
Signal Word:	Warning	
Pictogram:		
Unclassified Hazards:	None	
Ingredients with Unknown Toxicity:	None	
Carcinogenicity:	None of the components present in this material at concentrations equal to or greater than 0.1% are listed by IARC, NTP, or OSHA as a carcinogen.	

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SECTION 3: COMPOSITION/INFORMATION ON INGREDIENTS

Active Ingredient(s):	Chemical Name:	Chlorine Dioxide
	CAS#:	10049-04-4
	Molecular Formula:	ClO_2
	Concentration:	0.4% (4,000ppm)
Inert Ingredient(s):	Chemical Name:	Water
	CAS#:	7732-18-5
	Molecular Formula:	H_2O
	Concentration:	>99.6% (> 996,000 ppm)

SECTION 4: FIRST AID MEASURES

Skin Contact: Prolonged contact of concentrated solutions of the material (<1000ppm) may be highly irritating. Take off contaminated clothing and shoes immediately. Wash off with plenty of water and mild soap. If burning or irritation persists, consult a physician.

Eye Contact: Flush eyes gently with large amounts of water while holding eyelids apart. If there is visual difficulty or if symptoms persist, seek medical attention.

Inhalation: If symptoms such as shortness of breath or trouble breathing develop, immediately move to fresh air. Seek medical attention and keep person quiet and warm. Provide the injured party with oxygen. If not breathing, administer artificial respiration.

Ingestion: Call a Poison Control center or a doctor for treatment advice. First Aid is normally not required when small amounts of material have been ingested. If symptoms develop DO NOT induce vomiting. Have the person drink large quantities of water or milk immediately. DO NOT give anything by mouth to an unconscious person.

Note to Physician: Probable mucosal damage may contraindicate the use of gastric lavage.

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SECTION 5: FIRE FIGHTING MEASURES

NFPA Rating:	Health – 1 Flammability – 0 Reactivity – 1	
Flammable Properties:	Flash Point: Fire and Explosion Hazard: Extinguishing media:	Does not flash Not a fire or explosion hazard Water
Explosive Limit:	Chlorine Dioxide is not explosive. Chlorine Dioxide Gas may spontaneously decompose at concentrations above 10%. Chlorine Dioxide Gas may explode with violent force at concentrations of 30% or greater in the air at standard temperature and pressure.	
Firefighting Instructions:	Wear self-contained breathing apparatus (SCBA) with a full-face piece operated in the "positive pressure demand" setting. Wear appropriate chemically resistant protective gear.	

SECTION 6: ACCIDENTIAL RELEASE MEASURES

Safeguards:	Evacuate personnel to safe areas. Avoid inhalation. Notify proper authorities of any runoff, as required.
Spill Clean Up:	Prevent runoff to sewers, streams, lakes or other bodies of water. Dilute with water. Absorb liquid with absorbent material like sand, earth, clay, floor absorbent, or other absorbent material and move to containers. Rinse the area with water.
Note:	Review the HANDLING AND STORAGE section along with FIREFIGHTING MEASURES before clean-up. Use appropriate PERSONAL PROTECTIVE EQUIPMENT during clean-up.

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DiOx+ MATERIAL SAFETY DATA SHEET

SECTION 7: HANDLING AND STORAGE

Handling: Prevent the accumulation of chlorine dioxide gas by using only in well-ventilated areas. Avoid inhalation or contact with skin, eyes and clothing. Wear protective gloves to avoid skin contact. Wear protective eye wear to avoid eye contact.

Storage: Store away from children. Store in a cool dark place away from direct sunlight or heat. Only store in the container it is shipped in and authorized by the manufacturer for storage. Do not expose the material to freezing temperatures. Keep away from strong acids or oxidizing agents. Do not heat the material in excess of 140°F. Above 140°F the gas concentration in the headspace of the container may reach unstable concentrations.

SECTION 8: EXPOSURE CONTROLS / PERSONAL PROTECTION

OSHA (PEL): The OSHA permissible exposure limit for ClO2 gas in the air is 0.1ppm (0.3 mg/m3) as an eight-hour time weighted average. This limit is the same for NIOSH and ACGIH.

NIOSH & ACGIH: NIOSH & ACGIH have established short term exposure limits at 0.3ppm (0.83 mg/m3) for periods not to exceed 15 minutes. Short term exposure limits should not be repeated more than four times per day with at least sixty-minute intervals in between exposures.

Engineering Controls: Ensure adequate mechanical ventilation, especially in confined areas.

Eye Protection: Wear coverall splash-proof face and eye protection when the possibility exists for face contact due to splash or spray. Safety glasses should be in compliance with OSHA regulations.

Skin Protection: Whenever there is the possibility for skin contact, wear as appropriate impervious gloves, pants, boots, apron, and hood.

Respiratory Protection: Ensure adequate ventilation and monitoring to maintain OSHA permissible exposure limits below 0.1ppm. Monitor to assess the proper level of respiratory protection necessary. Refer to requirements established in 29 CFR 1910.134 for the facility's respiratory protection program. Wear a NIOSH/MSHA approved apparatus for leaks and emergencies with concentrations that exceed 5ppm.

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SECTION 9: PHYSICAL AND CHEMICAL PROPERTIES

Appearance & Color:	Yellow-green liquid	
Odor & Odor Threshold:	Slight Chlorine Odor	0.1ppm threshold
pH:	2 -8	
Freezing Point:	0°C (32°F)	
Boiling Point:	100°C (212°F)	
Density:	8.41 LB/GAL	
Specific Gravity:	1 – 1.01	
Water Solubility:	Complete	
Flash Point:	Not Applicable	
Flammability:	Not Applicable	
Vapor Pressure:	Not Established	
Viscosity:	0.984 cP (centipoise) at 25°C	

SECTION 10: STABILITY AND REACTIVITY

Stability:	Stable and non-reactive under normal temperatures, storage and use conditions. Decomposes on heating and exposure to light.
Hazardous Reactions:	Contact with reducing agents, acids, organic materials, oxidizing agents will release toxic gases of chlorine and/or chlorine dioxide. Material does not undergo hazardous polymerization.
Incompatability:	Avoid strong acids, chlorinated compounds, oxidizing agents, and reducing agents. Avoid exposure to light, metals, sulfur compounds, carbon monoxide, excessive heat, phosphorous, mercury and organic materials.
Conditions to Avoid:	Do not store the material at or below freezing (32°F). The solution should not be heated above 140°F.

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SECTION 11: TOXICOLOGICAL INFORMATION

Chlorine Dioxide exposure routes are typically through the respiratory system by the inhalation of vapors, skin and eye contact as well as ingestion. The solution is unlikely to cause serious eye irritation or injury. The vapor is a mucous membrane and respiratory tract irritant.

CHLORINE DIOXIDE (SOLUTION)

Repeated Dose Toxicity:	Inhalation Multiple species Target Organs: Respiratory system Respiratory tract irritation, inflammation, lung damage Oral – drinking water Multiple species Target Organ: Blood Abnormal decrease in number of red blood cells., Abnormal decrease in red blood cell hemoglobin (hemoglobinemia)
Oral LD50:	94 mg/kg, rat
Inhalation LC50:	32 ppm, rat Target Organs: Respiratory Tract Symptoms: Respiratory tract irritation
Mutagenicity:	Did now show mutagenic effects in animal experiments. Did not cause genetic damage in cultured bacterial cells.
Reproductive Effects:	Evidence suggest that the solution is not a reproductive toxin in animals even at very high exposure levels.
Teratogenicity:	Animal testing has shown effects on embryo-fetal development at levels below those causing maternal toxicity. Reduced growth and behavioral effects in offspring.
Cancer Effects:	This solution is not listed as a carcinogen by the National Toxicology Program, or the Occupational Safety and Health Administration, the International Agency for Research on Cancer, The United States Environmental Protection Agency or the American Conference of Industrial Hygienists.

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SECTION 12: ECOLOGICAL INFORMATION

Chlorine Dioxide (Solution) Biodegradability:	Readily biodegradable and degradable.
Aquatic Toxicity Chlorine Dioxide (Solution):	96 H LC50: Pimephales promelas (fathead minnow) 0.02 mg/l 48 H LC50: Daphnia magna (Water flea) 0.026 mg/l

SECTION 13: DISPOSAL CONSIDERATIONS

Waste Disposal:	Disposal of all materials should be in accordance with all applicable Federal, State, and local rules, regulations and requirements.
Environmental Hazards:	Used or empty containers should be recycled or disposed of at an approved waste handling site.

SECTION 14: TRANSPORT INFORMATION

Transportation of this solution should be in accordance with all applicable Federal, State, and local rules, regulations and requirements. Reference the rules and regulations of the US Department of Transportation, including all applicable packaging and labeling requirements.

DoT Information:	Reference UN 1760 Regulated as a hazardous material when shipped by motor vehicle or rail car.
Hazard Label:	CORROSIVE
Technical Name: **Proper Shipping Name:**	0.4% Chlorine Dioxide Aqueous Solution Corrosive Liquid, N.O.S.
Class:	Class 8 – Corrosive[1]
Packaging Group:	III (must not ship or store in metal containers)

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[1] DiOx+ is a Class 8 – Corrosive material only because it is corrosive to aluminum and steel. It is not highly corrosive to skin. Some packaging may react dangerously or be degraded by the solution. Product must be packaged and shipped in the original containers from the manufacturer.

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SECTION 15: REGULATORY INFORMATION

EPA FIFRA Information:	This solution is a registered pesticide product with the Environmental Protection Agency (EPA Registration Number 98018-01).
SARA 313 Regulated Chemicals:	Chlorine Dioxide CAS# 10049-04-4 0.4% (4,000ppm)
CERCLA RQ:	40 CFR 302.4(a)
SARA 302 Components:	40 CFR 355 Appendix A
OSHA:	Process Safety Management 29 CFR 1910
California Prop 65:	This product does not contain any chemicals known to the State of California to cause cancer, birth defects, or any other reproductive harm.
Toxic Substance Control Act:	No known restrictions

SECTION 16: OTHER INFORMATION

DISCLAIMER: The information provided in this material safety data sheet is correct to the best of our knowledge and believed to be accurate. However, NO WARRANTY IS EXPRESSED OR IMPLIED REGARDING THE ACCURACY OF ANY OF THE INFORMATION, ORIGINATED BY THE COMPANY OR BY OTHERS. NO WARRANTY OR GUARANTEE OF ANY OTHER KIND, EXPRESS OR IMPLIED, IS MADE REGARDING PERFORMANCE, SAFETY, SUITABILITY, STABILITY OR OTHERWISE. BOON INDUSTRIES INC., ASSUMES NO RESPONSIBILITY TO INJURY TO THE OPERATOR OR OTHERS NEARBY CAUSED BY THE MATERIAL IF REASONABLE SAFETY PROCEDURES ARE NOT ADHERED TO AS STIPULATED IN THE MATERIAL SAFETY DATA SHEET. Operators assume risk in their use of the material. Those receiving this material safety data sheet are advised to confirm, in advance of any need, that the information is current, applicable, and suitable to their circumstances.

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