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ASTM Efficacy of Sanitizers for Inanimate, Hard, Nonporous, Non-Food Contact Surfaces

By
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&
Kent Oostra

For

Synergy Americas, Inc
127 Stream Road
Winterport, ME 04496
Reference Nu: 21-16912

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FINAL STUDY REPORT

Study Title
ASTM E1153-14 Efficacy of Sanitizers Recommended for Inanimate, Hard, Nonporous Non-Food Contact Surfaces

Product Identity
Synergy Envirotab 4g: 400ppm in 1 Liter of water
Lots: 2020/12-05, 2020/12-06, 2020/12-07
Final Concentration: 100ppm

Test Microorganisms
Staphylococcus aureus ATCC 6538
Enterobacter aerogenes ATCC 13048

Test Guidelines
U. S. EPA Guideline 8.10.2300

Data Requirements
ASTM E1153-14

Author
Katherine Sandoval
Molecular Biology Director

Study Completion Date
10/07/2021

Performing Laboratory
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Study Sponsor
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Laboratory Reference Number
21-16912 68744

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STATEMENT OF NO DATA CONFIDENTIALITY

No claim of confidentiality, on any basis whatsoever, is made for any information contained in this document. I acknowledge that information not designated as within the scope of FIFRA section 10(d)(1)(A), (B), or (C) and which pertains to a registered or previously registered pesticide is not entitled to confidential treatment and may be released to the public, subject to the provisions regarding disclosure to multinational entities under FIFRA 10(g).

Company: Exact Scientific Services, Inc.

Agent/Submitter: Katherine Sandoval

Title: Director of Molecular Biology

Date: November 01, 2021

Signature: Katherine Sandoval

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The following is a detailed description of all differences between the practices used in the study and those required by 40 CFR part § 160.

Records concerning test substance characteristics (i.e., composition, purity, stability, strength, solubility) are maintained by the Study Sponsor.

Study Director

Company: Exact Scientific Services, Inc.

Name: Kent Oostra

Title: CEO

Signature: Kent Oostra Date: 1/20/2023

Study Sponsor

Company: Synergy Americas, Inc.

Name: Michael R. Martin

Signature: [signature] Date: 1/25/2023

Study Submitter

Company: Synergy Americas, Inc.

Name: Michael R. Martin

Title: Senior Consultant

Signature: [signature] Date: 1/25/2023

QUALITY ASSURANCE STATEMENT

The following quality assurance audits were conducted in accordance with Good Laboratory Practice Standards outlined in 40 CFR § 160 and reported to management and the Study Director:

Phase Inspected	Date Inspected	Date Reported to Study Director	Date Reported to Management
Protocol	08/18/2021	08/18/2021	08/25/2021
In Process (Test)	09/21/2021	10/04/2021	10/07/2021
Draft Report	10/04/2021	10/07/2021	10/07/2021
Final Report	10/07/2021	10/07/2021	11/01/2021

PERSONNEL INVOLVED IN THE STUDY

Analyst

Name: Joleen Gouette

Title: Molecular Biology Technician

Study Director

Name: Katherine Sandoval

Title: Director of Molecular Biology

Professional or Supervisory Personnel

Name: Kent Oostra

Title: CEO

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FINAL STUDY REPORT SUMMARY

Study Title:	ASTM E1153-14
Study Identification Number:	21-16912 68744
Test Method:	ESS 6.2.53; ASTM E1153-14
Test Microorganisms:	*Staphylococcus aureus* ATCC 6538 *Enterobacter aerogenes* ATCC 13048
Test Substance:	Synergy Envirotab, 4g Lots: 2020/12-05, 2020/12-06, 2020/12-07
Test Substance Dilution:	1 tablet dissolved in 1000mL Water (Charge Solution) 25mL Charge Solution in 75mL AOAC Synthetic hard Water Final concentration 100 ppm
Carrier Type:	Nonporous Test Surface, Pre-Cleaned Borosilicate Glass Squares, 25mm x 25mm x 2mm slides
Number of Carriers Per Lot:	*Staphylococcus aureus*: 5 *Enterobacter aerogenes:* 5
Contact Time:	4 Minutes
Exposure Temperature:	20±1ºC
Neutralizer:	Letheen Broth containing 19.5% Polysorbate 80 Neogen, Sigma SKU 1104050500
Study Objective:	This test is designed to determine effectiveness for a product to be used as a sanitizer. It measures the potential of the test material to sanitize hard, non-porous, non-food contact surfaces contaminated with bacteria.
Study Dates:	Study Initiation:08/17/2021 Experimental Start: 09/21/2021 Experimental End: 09/28/2021 Study Completion: 10/07/2021
Media and Reagents:	Deionized Water, Sterile and Non-Sterile AOAC Synthetic Hard Water Phosphate Buffered Dilution Water (PBW) Letheen Broth with Tween 80 (LB) Tryptic Soy Broth (TSB) Blood Agar with Esculin (BEA) 3M Rapid Aerobic Count Petrifilm Gram Stain Reagents
Test Synopsis:	Specified number of carriers per organism per test lot were inoculated with a known concentration of microorganism. Carriers were treated with test substance for client specified contact time. Carriers were neutralized after exposure to test substance. Neutralizing broth was enumerated to determine percent reduction in microorganisms.

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Study Results:

Test Date	Microorganism	Percent Reduction (%)			Control Slide Carrier Geometric Mean Density
		Lot: 2020/12-05	Lot: 2020/12-06	Lot: 2020/12-07	
09/21/2021	*E. aerogenes*	N/A	>99.999	N/A	2.839×10^{12}
09/27/2021	*E. aerogenes*	>99.999	N/A	>99.999	9.916×10^{9}
09/21/2021	*S. aureus*	N/A	>99.999	N/A	1.618×10^{8}
09/27/2021	*S. aureus*	>99.999	N/A	>99.999	1.920×10^{12}

TEST PROCEDURES

Test Substance Preparation

Test substance was prepared per submitter instructions:

Prepare a $400mgL^{-1}$ Chlorine Dioxide Charge Solution, reduced to working concentration of 100ppm solution in AOAC Hard Water. See calculations below:

Equation: C1V1=C2V2

$$100 \text{ ppm} * 100 \text{ mL} = 400 \text{ ppm} * X \text{ mL}$$

$$\frac{100 * 100}{400} = 25 \text{ mL}$$

i Dissolve one tablet in 1000 mL water with little to no headspace.
ii Allow tablet to fully dissolve approximately 1 hour.
iii Invert container several times to ensure complete mixing.
iv Dilute charge solution by mixing 25mL charge solution with 75mL AOAC Hard Water.
v Store in a cool, dark environment. Use within 3 hours of preparation.
vi Test with exposure time of 4 minutes.

ASTM E1153-14 Procedures

See appendix 1: ESS 6.1.53

PROTOCOL AMENDMENTS

None. Initial version performed.

PROTOCOL DEVIATIONS

ASTM E1153-14 Protocol was followed directly with the following exceptions.

i Exposure time of 4 minutes was used per client request, opposed to protocol exposure time of 5 minutes.

STUDY CONTROLS

Viability Control:

For each test lot, an inoculated, untreated carrier was collected and suspended in 25mL of neutralizing broth alongside all test carriers.

Positive Control:

For each test lot, three inoculated carriers were collected and exposed to 5mL of sterile DI water for duration of exposure period and suspended in 20mL neutralizing broth. This was performed for each organism.

Negative Process Control:

For each test lot, three uninoculated carriers were treated alongside test carriers to ensure no contamination occurred during any test procedures.

Neutralization Subculture Sterility Controls:

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Four primary neutralizing subculture media tubes and four secondary neutralizing subculture media tubes were incubated alongside test carriers.

Media Sterility Controls:
All media lots went through quality control testing including inoculation with test organisms to ensure growth promotion, sterility evaluation, and pH monitoring.

Test Microorganism Purity Control:
Test cultures used in this study were sub-cultured onto blood agar and visually examined for purity prior to evaluation.

Confirmation of Positive:
Positive tubes with turbidity/growth were confirmed via microscopic exam and/or colony morphology on selective agar plates.

ACCEPTANCE CRITERIA

i Test microorganisms must demonstrate a concentration geometric mean $\geq 7.5 \times 10^5$.
ii Neutralizing subculture must provide growth when inoculated with <100 cfu/mL.
iii Sterility controls must produce no growth.
iv Viability controls must produce growth.
v Media sterility controls must produce no growth.
vi Test microorganism must demonstrate pure target organism.
vii Test substance must produce $\geq$99.9% reduction for all organisms evaluated.

Retest Guidance
i If inoculum geometric mean is $> 7.5 \times 10^5$, and all negative controls are acceptable, no retest is necessary.
ii If inoculum geometric mean is $< 7.5 \times 10^5$, retest is required.
iii If a test fails and inoculum geometric mean is $< 7.5 \times 10^5$, retest is not required.
iv If inoculum geometric mean are within target range and all controls are acceptable, but test fails, retest may not be conducted.

CALCULATIONS AND STATISTICAL ANALYSIS

The following calculations were used in this study to evaluate efficacy of testing procedures and product.

- i cfu/mL of microbial counts: colony forming units * 10^{-x}, where x is the countable dilution used.
- ii Surviving organism per carrier: cfu/mL * total volume used to suspend carrier.
- iii Log Calculation: Log_{10} (cfu/mL* 10^{-x})
- iv Antilog: $10^{(\text{Average } Log_{10}(\text{surviving organism per carrier}))}$
- v Geometric Mean (GM): Antilog (Average Log_{10}(surviving organism per carrier))
- vi Percent Reduction: $\frac{(\text{GM control} - \text{GM test}) \times 100}{\text{GM control}}$

STUDY RECORD AND TEST SUBSTANCE RETENTION

Study Record Retention

The original study report, protocol, and raw data will be maintained at Exact Scientific Services for a minimum of 5 years following the study completion date. After this retention time, records may be destroyed. Study sponsor may request copy of report at any time within the retention period.

All facility records pertaining to quality assurance and employee training will be maintained at Exact Scientific Services for a minimum of three years.

Test substance will be retained for a minimum of 30 days following the study completion date. Test substance may be returned to study sponsor upon request after this time. Arrangements may be made for extended storage period, if necessary, upon request.

RESULTS

Neutralizer Subculture Study

Table 1: Neutralization Confirmation Study for *S. aureus*

Product Lot: 2020/12/07	Dilutions Tested			
Treatment	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Product Test: Neutralizer Primary Subculture Treatment				
Primary Subculture Treatment	+	+	+	+
Confirmation				Confirmed
Product Test: Neutralizer Secondary Subculture Treatment				
Primary Subculture Treatment	+	+	+	+
Secondary Subculture Treatment	+	+	+	+
Confirmation				Confirmed
Inoculated Control: Neutralizer Primary Subculture Treatment				
Primary Subculture Treatment	+	+	+	+
Confirmation				Confirmed
Inoculated Control: Neutralizer Secondary Subculture Treatment				
Primary Subculture Treatment	+	+	+	-
Secondary Subculture Treatment	+	+	+	+
Confirmation				Confirmed
Negative Control: Neutralizer Primary Subculture Treatment				
Primary Subculture Treatment	-	-	-	-
Negative Control: Neutralizer Secondary Subculture Treatment				
Primary Subculture Treatment	-	-	-	-
Secondary Subculture Treatment	-	-	-	-
	Geometric Mean			
Inoculum Numbers Control	10^{-4}	10^{-5}	10^{-6}	10^{-7}
S. aureus	730	110	10	1
Confirmation				Confirmed

ASTM E1153 Method

Table 2: Inoculum Numbers Controls

Test Date	Test Organism	Test Substance Lot	Average Inoculum cfu/mL	Average Surviving Organisms Per Control Carrier
09/21/2021	*E. aerogenes*	2020/12/05	4.50×10^{12}	2.95×10^{12}
09/27/2021	*E. aerogenes*	2020/12/06	1.50×10^{12}	1.04×10^{10}
09/27/2021	*E. aerogenes*	2020/12/07	1.50×10^{12}	1.04×10^{10}
09/21/2021	*S. aureus*	2020/12/05	1.10×10^{13}	1.76×10^{8}
09/27/2021	*S. aureus*	2020/12/06	5.40×10^{12}	2.10×10^{12}
09/27/2021	*S. aureus*	2020/12/07	5.40×10^{12}	2.10×10^{12}

Table 3: Percent Reduction

Test Date	Test Organism	Test Substance Lot	Geometric Mean Test Carriers	Geometric Mean Control Carriers	Percent Reduction
09/21/2021	*E. aerogenes*	2020/12/05	4.3600	2.839×10^{12}	>99.999%
09/27/2021	*E. aerogenes*	2020/12/06	1.904	9.916×10^{9}	>99.999%
09/27/2021	*E. aerogenes*	2020/12/07	0.000	9.916×10^{9}	>99.999%
09/21/2021	*S. aureus*	2020/12/05	0.000	1.618×10^{8}	>99.999%
09/27/2021	*S. aureus*	2020/12/06	1.904	1.920×10^{12}	>99.999%
09/27/2021	*S. aureus*	2020/12/07	0.000	1.920×10^{12}	>99.999%

STUDY CONCLUSION

Test Substance Synergy Enviortab (lots 2020/12/05, 2020/12/06, 2020/12/07) was tested against *Staphylococcus aureus* and *Enterobacter aerogenes*. A total of 6 inoculated slide carriers were exposed to each lot of the test substance with a contact time of 4 minutes. The slide carriers were then neutralized in letheen broth.

Following 4-minute contact time, Synergy Envirotab demonstrated a greater than 99.999% rate of kill for *S. aureus* and *E. aerogenes* for all three lots tested.

Comparison validation study was conducted comparing DI Water to AOAC hard water. The concentration of chlorine was compared and were found to be equivalent. This indicates the effectiveness of the product does not change between AOAC Hard Water and DI Water.

Under the protocol conducted and the acceptance criteria provided, all lots tested meet requirements stated in the U.S. EPA Product Performance Test Guidelines OCSPP 810.2300: Sanitizers for Use on Inanimate Non-Food Contact Surfaces, Guidance for Efficacy Testing.

This study was conducted in compliance with the approved protocol, all experimental controls met the established acceptance criteria.

REFERENCES

ASTM E1153-14 Standard Test Method for Efficacy of Sanitizers Recommended for Inanimate, Hard, Nonporous Non-Food Contact Surfaces

EPA MB-22 Disinfectant Sample Preparation

EPA MB-17 Neutralization Confirmation

U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines OCSPP 810.2300: Sanitizers for Use on Hard Surfaces—Efficacy Data Recommendations September 2012.
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APPENDIX 1

ESS 6.2.53 ASTM Efficacy of Sanitizers Recommended for Inanimate, Hard, Nonporous Non-Food Contact Surfaces Standard Operating Procedures

Sections Included in this Document:

1. Purpose
2. Scope
3. Responsibilities
4. Background
5. References
6. Materials/Equipment
7. Procedure
8. Records/Results
9. Quality Control
10. Definitions
11. Safety
12. Attachments
13. Appendix

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1. Purpose

1.1. The purpose of this test is to determine the antimicrobial efficacy of sanitizers against bacterial microorganisms on pre-cleaned, inanimate hard, nonporous, non-food contact surfaces.

2. Scope

2.1. This method applies to liquid-based sanitizers for on pre-cleaned, inanimate hard, nonporous, non-food contact surfaces.

3. Responsibilities

3.1. Analyst:
- 3.1.1. Follow this procedure
- 3.1.2. Enter results in Laboratory Information Management System (LIMS)
- 3.1.3. Submit a draft for review and approval

3.2. Director(s):
- 3.2.1. Review data
- 3.2.2. Approve data in LIMS
- 3.2.3. Submit results to client

4. Background

4.1. This method was developed by the American Society for Testing and Materials (ASTM) to determine efficacy of both dilutable and ready to use sanitizers for the purpose of certification through the U.S. Environmental Protection Agency (EPA). This method directly reflects the ASTM method.

5. References

5.1. ASTM E1153-14- Efficacy of Sanitizers Recommended for Inanimate, Nonporous Non-Food Contact Surfaces

5.2. EPA MB-22 Disinfectant Sample Preparation

5.3. EPA MB-17 Neutralization Confirmation

5.4. EPA OCSPP 810.2300- Sanitizers for Use on Hard Surfaces

6. Materials and Equipment

6.1. Instruments

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6.1.1. Refrigerator (4 ± 2 °C)
6.1.2. Incubator (37 ± 2 °C)
6.1.3. Humidity controlled incubator (37±2ºC, 40-41%RH)
6.1.4. Hot air oven (≥180ºC)
6.1.5. Vortex
6.1.6. Spectrophotometer

6.2. Equipment
6.2.1. Sterile glass tubes
6.2.2. Sterile pipettes (2 mL, 10 mL, 25 mL)
6.2.3. Sterile transfer loop, 4m inside diameter
6.2.4. Digital Timer
6.2.5. Thermometers
6.2.6. Pipette bulb or equivalent
6.2.7. Forceps
6.2.8. Sterile 50mL centrifuge tubes
6.2.9. Flaming apparatus- 70-95% ethanol and flame
6.2.10. Sterile stainless steel penicylinders
6.2.10.1. Carriers, polished stainless steel penicylinders, 8±1mm outer diameter, 6±mm inner diameter, 10±1mm length; type 304 stainless steel, SS 18-8 (S&L Aerospace Metals, or Fischer Scientific Cat. No. 07-907-5Q).
6.2.11. Nonporous Test surface, pre-cleaned
6.2.11.1. Borosilicate Glass Squares, 25mm by 25mm by 2mm slides

6.3. **Reagents**
6.3.1. Phosphate Buffered Water (PBW)
6.3.2. Letheen Broth with Tween 80 (LB)
6.3.3. Tryptic Soy Broth (TSB)
6.3.4. Blood Agar with Esculin (BEA)
6.3.5. DI Water, Sterile and Non-Sterile
6.3.6. 3M Aerobic Plate Count petrifilm
6.3.7. Gram Stain Reagents
6.3.8. Disinfectant
6.3.8.1. BTC 835:50 % n-alkyl (50 % C14, 40 % C12, 10 % C16) Dimethyl Benzyl Ammonium Chloride
6.3.9. Test Product- prepared according to manufacturer's instructions

7. Procedure

7.1. **Stainless Steel Penicylinder Carrier Screening for Neutralizing Confirmation**

This procedure describes the preparation of stainless steel penicylinder carriers for the AOAC Neutralization Confirmation Method. This procedure is applicable to stainless steel penicylinder carriers only. For any alternative carrier, see EPA SOP MB-03-07.

7.1.1. Physical Screening
7.1.1.1. Visually screen polished stainless-steel carriers.
7.1.1.2. Discard carriers that fail physical screening due to visible damage (dull, chipped, dented, or gouged).
7.1.1.3. Place carriers that pass physical screening in a container and label with date and "Physically Screened"

7.1.2. Cleaning
7.1.2.1. Soak the physically screened carriers overnight (approx. 12 hr.) in 1N NaOH.
7.1.2.2. Rinse several (3-4) times with tap water.

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7.1.2.3. Collect a portion of the last rinse water and add 2-3 drops of 1% phenolphthalein.

7.1.2.4. If any NaOH remains, the phenolphthalein turns pink, indicating the need for additional rinsing. Continue to rinse the carriers until the addition of phenolphthalein to the collected portion of the rinse water does not produce a color change (to pink).

7.1.2.5. Rinse twice more with DI water. Allow carriers to air dry and store in a closed container marked with date and "Cleaned Carriers/Not Biologically Screened."

7.1.3. <u>Biological Screening</u>

7.1.3.1. Place the cleaned carriers into 25×150 mm test tubes, 20 per tube.

7.1.3.2. Cover the carriers with DI water and cap.

7.1.3.3. Autoclave at 121 ºC for 20 min; cool and store at room temperature.

7.1.3.4. Perform Use-Dilution testing (see section 7.5) on each carrier using the following parameters:

7.1.3.5. *Staphylococcus aureus*, 500 ppm solution of BTC 835 prepared using sterile deionized water, 20±1 ºC, no organic soil, ten-minute exposure period, and letheen broth as the neutralizer. Use primary subculture tubes only.

7.1.3.6. Select Control Carriers

a. Select one (1) carrier from each petri dish for controls.
b. Three (3) pre-carrier controls- used to enumerate initial carrier bacterial concentrations.
c. Three (3) post-carrier controls- used to enumerate carrier bacterial concentrations upon method completion.
d. One (1) viability control carrier- untreated, inoculated carrier.

7.1.3.7. Pre-Carriers: Aseptically transfer three carriers to sterile tubes with 10mL of letheen broth.

a. Sonicate for one minute according to diagram 1 in appendix (13.3). Make sure water and letheen broth levels are even.
b. After sonication, mix and make four ten-fold serial dilutions into 9 mL PBS tubes.
c. Plate dilutions 10-1, -3, -5 in duplicate.
d. Incubate plates at 36±1ºC for 48±2 hours.

7.1.3.8. Post-Carriers: Aseptically transfer three carriers to sterile, empty tubes for later use.

a. Upon study completion, repeat steps for pre-carriers above.

7.1.3.9. Viability Control: Aseptically transfer one inoculated carrier into tube containing 10mL letheen broth.

a. Keep control at 2-5 °C until study is complete. Incubate tube with all other tubes.

7.1.3.10. Autoclave and repeat procedures in section 7.1 for all failed carriers.

7.1.3.11. Autoclave all passing carriers separately.

7.1.3.12. Re-wash according to procedure 7.12.

7.1.3.13. These carriers are to be collected.

a. After drying, store in sterile, sealed container marked as "Sterile Pooled Carriers".

7.1.4. <u>Preparing for Testing</u>

7.1.4.1. Remove required number of cleaned carriers from the "Sterile Pooled Carriers".

7.1.4.2. Place carriers into 25x150mm test tube (20 per tube).

7.1.4.3. Cover carriers in tubes with DI water.

7.1.4.4. Autoclave at 121 °C for 20 minutes.

7.1.4.5. Cool and store at room temperature.

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7.1.4.6. After testing, all negative carriers and those used as controls, clean according to section7.1.2 and return to master pool.

a. Any carriers giving positive result, must be re-screened according to section 7.1.

7.2. **Glass Slide Carrier Preparation for ASTM E1153-14 Testing**

This procedure describes the preparation borosilicate glass square carriers for the ASTM E1153-14 method. This procedure is applicable to borosilicate glass square slide carriers only. For any alternative carrier, see ASTM E1153-14.

7.2.1. Physical Screening

7.2.1.1. Visually screen glass slide carriers.

7.2.1.2. Discard carriers that fail physical screening due to visible damage (dull, chipped, dented, or gouged).

7.2.2. Cleaning

7.2.2.1. Dip slide carriers in 70-95% ethanol.

7.2.2.2. Rinse with DI water.

7.2.2.3. Air Dry.

7.2.3. Sterilizing

7.2.3.1. Place the cleaned slide carriers into large foil dish.

7.2.3.2. Place in hot air oven for $\geq$2 hours at $\geq$180ºC.

7.2.3.3. Place into sterile petri dish and cover using sterile techniques.

7.3. **Inoculum Preparation**

This procedure describes how to prepare the inoculum to be used in any of the methods in this procedure.

7.3.1. ATCC Culture Maintenance

7.3.1.1. ATCC microorganism strains are to be obtained in either Kwik Stik dehydrated pellets or freeze-dried dehydrated pellet vials.

7.3.1.2. Organisms are to be logged into the Microorganism Logbook to record received date, organism, ATCC number, lot number, expiration date, and hydration date.

7.3.1.3. Once re-hydrated per manufacturer instructions, all bacterial microorganisms are to be plated onto BEA to ensure purity and incubated in TSB at 35 °C (or appropriate temperature) for 24 hours.

7.3.1.4. Bacterial Cultures may be stored in TSB and/or isolated onto BEA and wrapped in parafilm for one month in refrigerated conditions (4 ± 2 °C).

a. Bacterial cultures may be subcultured up to three transfers.

7.3.2. *Staphylococcus aureus* ATCC 6538 for Neutralizing Confirmation Procedures (Section 7.5)

7.3.2.1. From culture plate, use a sterile swab to harvest colonies and disperse into 9 mL PBW.

7.3.2.2. Using a spectrophotometer, set to read percent transmittance at 530 nm wavelength.

7.3.2.3. Dilute or concentrate bacterial suspension as appropriate to obtain 78-80 % transmittance

a. This is an estimated cell concentration of 1.0 x 10^9 cfu/mL.

7.3.2.4. Use serial dilutions into 9 mL PBW to obtain target concentration as described in the method.

7.3.3. *Staphylococcus aureus* ATCC 6538 and *Enterobacter aerogenes* ATCC 13048 for ASTM E1153-14 Procedures (Section 7.6)

7.3.3.1. From bacterial stock cultures grown in TSB, no more than one month old, inoculate tube containing 10mL of TSB and incubate for 24± 2 hours at 35°C-39°C.

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7.3.3.2. Using a 4mm inside diameter transfer loop, transfer a loopful of culture into fresh broth.
   a. Incubate 24± 2 hours at 37± 2 °C. (Transfer #1).

7.3.3.3. Using a 4mm inside diameter transfer loop, transfer a loopful of transfer #1 into fresh broth.
   a. Incubate 24± 2 hours at 37± 2 °C. (Transfer #2).

7.3.3.4. Using a 4mm inside diameter transfer loop, transfer a loopful of transfer #2 into fresh broth.
   a. Incubate 51± 3 hours at 37± 2 °C. (Transfer #3).

7.3.3.5. Use serial dilutions if necessary to ensure final bacterial concentration target of $\geq 7.5 \times 10^5$ organisms per control square survive.

7.4. **Product Preparation**

This procedure describes the preparation of the product to be used in the ASTM E1153-14 method. This can be prepared according to client supplied product preparation instructions. Sterile water or AOAC Synthetic Hard Water (see 7.3.3) may be used depending on the product. AOAC Synthetic Hard Water Solution 1 and 2 (see 7.3.1 and 7.3.2) must be prepared to make the AOAC Synthetic Hard Water.

7.4.1. AOAC Synthetic Hard Water Solution 1

7.4.1.1. Dissolve 7.94 g $MgCl_2$ (anhydrous) and 18.50 g CaCl2 in boiled deionized water and bring to a volume of 250 mL volumetrically.

7.4.1.2. Sterilize by membrane filtration. Used for the preparation of hard water at various concentrations.

7.4.2. AOAC Synthetic Hard Water Solution 2

7.4.2.1. Dissolve 14.01 g $NaHCO_3$ in boiled deionized water and bring to a volume of 250 mL volumetrically.

7.4.2.2. Sterilize by membrane filtration. Used for the preparation of hard water at various concentrations.

7.4.3. Preparation of 400 ppm AOAC Synthetic Hard Water

7.4.3.1. Per 1 L: Add 4 mL of AOAC Synthetic Hard Water Solution 1 (1 mL for each 100-ppm hardness desired) and 4 mL of AOAC Synthetic Hard Water Solution 2 to a 1L volumetric flask and bring to volume with sterile deionized water.
   a. If preparing concentration other than 400 ppm, per 1 L preparation, use 1mL of AOAC Synthetic Hard Water Solution 1 (per 100 ppm hardness desired) and 4 mL AOAC Synthetic Hard Water solution 2.
   b. Bring to volume using sterile deionized water in a 1 L volumetric flask and continue with steps 7.3.3.2 and 7.3.3.3 below.

7.4.3.2. Measure the pH of the hard water sample. The pH should be between 7.6 and 8.0 at room temperature. If necessary, adjust the pH using 1 N NaOH or 1N HCl.

7.4.3.3. Filter sterilize the hard water using a 0.2 µm filter unit.

7.5. **Neutralizing Confirmation**

This procedure describes the evaluation of the effectiveness of neutralizers specified for sanitizer efficacy testing using the ASTM E1153-14 method. This is a quantitative approach to determine the effectiveness of the neutralizer itself to neutralize the effects of the sanitizer product being tested, using the same conditions specified for product testing (water hardness, pH, neutralizer, contact time, temperature). It is imperative that this method be performed prior to ASTM E1153-14 testing as the product tested must be completely neutralized post exposure time.

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For this method, use pre-screened penicylinder from the sterile master pool. You will need 8 test carriers, and a negative carrier control per test organism.

7.5.1. Product Preparation

7.5.1.1. Prepare product according to section 7.4 and manufacturer instruction.

7.5.1.2. Use for testing within three hours of preparation.

7.5.2. Inoculum Preparation

7.5.2.1. Prepare inoculum according to section 7.3.2 to obtain a culture suspension of approximately 1.0 x 10^9 cfu/mL.

7.5.2.2. Prepare ten-fold serial dilutions by inoculating 1 mL of test culture into 9 mL of PBW, 7 times. Use the 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} dilutions for this method.

7.5.2.3. Obtain numbers control by plating 0.1 mL of each of the four dilutions in duplicate on TSA. Incubate plates at 36 ± 1 °C for 48 ± 2 hours.

7.5.2.4. Use within 30 minutes or refrigerate at 4 ± 1 °C for up to two hours.

7.5.3. Neutralizing Subculture Procedures

7.5.3.1. Start with a total of 38 tubes containing 10mL each of neutralizing broth:

a. 4 – Primary Subculture Treatment
b. 8 – Secondary Subculture Treatment
c. 4 – Positive Primary Subculture Control
d. 8 – Positive Secondary Subculture Control
e. 4 – Negative Primary Subculture Control
f. 8 – Negative Secondary Subculture Control

7.5.3.2. Apply product to 8 test carriers according to the specific instructions provided by the manufacturer.

a. Use 4 test carriers per product, per one test organism for each of the primary and secondary subculture treatment.
b. Be sure to start contact time at 30s- or 60s-time intervals to allow enough time for transfer.

7.5.3.3. After specified contact time, aseptically remove carrier from product and allow to drain, in order so that each carrier has been exposed to product for the exact specified time.

7.5.3.4. Place carrier in a timed fashion into neutralizing broth.

7.5.3.5. Incubate at room temperature for 30-45 minutes.

7.5.3.6. Put aside the 4 primary test carriers.

7.5.3.7. In order, Transfer each of the secondary subculture carriers to the second tube containing 10 mL neutralizing broth. This step is not timed.

7.5.4. Controls

7.5.4.1. Uninoculated Controls

a. Neutralizer-primary and secondary subculture negative controls: a minimum of 1 tube each of uninoculated neutralizer and secondary subculture media will be included in the test and incubated with the other tubes.
b. Negative Carrier: uninoculated carrier in 10 – 20 mL of TSB or neutralizer broth incubated at 36 ± 1 °C for 3 - 10 days.

7.5.4.2. Inoculated Controls

a. Neutralizer-primary positive control: four tubes of fresh neutralizer, unexposed to sanitizer, one tube for each inoculum dilution.
b. Neutralizer-secondary positive control: four tubes of secondary subculture media, unexposed to sanitizer, one tube for each inoculum dilution.

7.5.5. Treatment Inoculation

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7.5.5.1. After step 7.4.3.7, inoculate all tubes concurrently using Table 1 below.

Table 1

	Dilutions Added			
Treatment	**10^{-4}**	**10^{-5}**	**10^{-6}**	**10^{-7}**
Product Test: Neutralizer Primary Subculture Treatment				
Primary Subculture Treatment	0.1mL	0.1mL	0.1mL	0.1mL
Product Test: Neutralizer Secondary Subculture Treatment				
Primary Subculture Treatment	0.1mL	0.1mL	0.1mL	0.1mL
Secondary Subculture Treatment	0.1mL	0.1mL	0.1mL	0.1mL
Inoculated Control: Neutralizer Primary Subculture Treatment				
Primary Subculture Treatment	0.1mL	0.1mL	0.1mL	0.1mL
Inoculated Control: Neutralizer Secondary Subculture Treatment				
Primary Subculture Treatment	0.1mL	0.1mL	0.1mL	0.1mL
Secondary Subculture Treatment	0.1mL	0.1mL	0.1mL	0.1mL
Negative Control: Neutralizer Primary Subculture Treatment				
Primary Subculture Treatment	N/A	N/A	N/A	N/A
Negative Control: Neutralizer Secondary Subculture Treatment				
Primary Subculture Treatment	N/A	N/A	N/A	N/A
Secondary Subculture Treatment	N/A	N/A	N/A	N/A

7.5.5.2. Shake tubes thoroughly.
7.5.5.3. Incubate all tubes at 36 ± 1 °C for 48 ± 2 hours.

7.5.6. Results and Confirmation

7.5.6.1. Record results as "+" for turbidity and "-" for no growth.
7.5.6.2. For each treatment and control group, confirm a minimum of one positive tube per treatment as stated below. Select the tube with the highest dilution showing growth to confirm.

a. *S. aureus*: gram stain and culture on Baird Parker Agar. Should be gram positive cocci under microscope. Selective agar should show black colonies with hemolytic zones.

7.5.7. Interpretation

7.5.7.1. Numbers Control

a. One of the four dilutions should provide counts within 50-100 cfu/mL range.

7.5.7.2. Controls

a. Negative controls should show no growth. Growth in this case indicates sterility issues and the test will need to be repeated. Positive controls should show consistent growth among primary and secondary neutralizing broths. If growth rates differ greatly, and/or if no growth is observed in tubes with high level of

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inoculum, this indicates poor media performance and alternative media may be necessary.

7.5.7.3. Treatments

a. If growth in primary treatment tubes, only one neutralization step is necessary. If no growth is observed in primary treatment, but growth is observed in secondary treatment, primary neutralization is not sufficient and secondary transfer is necessary for ASTM E1153-14 method. If no growth is observed in either primary or secondary tubes, an alternative neutralizing broth is necessary, and test should be repeated.

7.6. **ASTM E1153-14**

This procedure describes the efficacy determination of liquid-based sanitizer against *S. aureus* and *E. aerogenes* on hard nonporous, non-food contact surfaces. Borosilicate glass slide carriers are inoculated and exposed to test product.

Prior to conducting ASTM E1153-14 method, perform all procedures above in section 7 for each product. All tests must provide acceptable results before continuing with ASTM E1153-14 procedures.

A minimum of 3 different product lots are to be evaluated using 5 test slide carriers each lot. One lot of product must be a minimum of 60 days old.

Inoculum Preparation

7.6.1.1. Prepare inoculum according to section 7.3.3.

7.6.1.2. Vortex 48-54 hour culture of test organism.

7.6.1.3. Allow to settle $\geq$15 minutes.

7.6.1.4. Remove upper two thirds of this suspension by aspiration for use in this method.

7.6.1.5. If testing for product intended for lightly soiled surfaces, add this suspension to 1mL bovine serum for a 5% concentration.

7.6.1.6. Target Dilutions: Average surviving organism present on control slide carriers must be $\geq 7.5x10^5$.

7.6.1.7. Use inoculum within 30 minutes or refrigerate at 4 ± 1 °C for up to two hours.

7.6.2. Inoculating Carriers

7.6.2.1. Before inoculating carriers, remove negative control slide carriers.

a. Aseptically and sequentially transfer one uninoculated slide carrier into sterile centrifuge tubes containing 5mL of test product.
b. Repeat twice, transferring slide carriers every 30 or 60 seconds.
c. After 5 minutes, sequentially add 20mL neutralizer to slide carriers in 30 or 60 second intervals.
d. Rotate centrifuge tube vigorously for approximately 50 rotations, or vortex for 10-15s.
e. Transfer within $\pm$5 seconds of specified time.

7.6.2.2. Inoculate each sterile glass carrier with 0.01-0.03mL of inoculum.

a. Spread inoculum within approximately 3 mm of the edges of the slide carrier.
b. Keep slide carriers in the order in which they were inoculated.

7.6.2.3. Incubate in humidity-controlled chamber set to 37$\pm$2ºC, 40-41%RH, for 20-40 minutes.

a. Make sure slide carriers are visibly dry prior to use.

7.6.2.4. Numbers Control: Plate inoculum onto 3M APC plates (or equivalent) using appropriate dilutions to obtain a countable range.

a. Incubate *S. aureus* for 48$\pm$4 hours at 37$\pm$2ºC.

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b. Incubate *E. aerogenes* for 48+4 hours at 25-32ºC.

7.6.3. Selecting Control Carriers

7.6.3.1. Select three positive control carrier slides per test organism.

a. Aseptically and sequentially transfer one inoculated slide carriers into sterile centrifuge tubes containing 5mL of sterile water each.
b. Repeat twice, transferring slide carriers every 30 or 60 seconds.
c. After 5 minutes, sequentially add 20mL neutralizer to slide carriers in 30 or 60 second intervals.
d. Rotate centrifuge tube vigorously for approximately 50 rotations, or vortex for 10-15s.
e. Transfer within +5 seconds of specified time.
f. Repeat steps 7.6.3.1 a-e for each test organism.

7.6.3.2. Select one viability control carrier slide per test organism.

a. Aseptically transfer one inoculated slide carrier per test organism into sterile centrifuge tube containing 25mL neutralizer.
b. Rotate centrifuge tube vigorously for approximately 50 rotations, or vortex for 10-15s.
c. Transfer within +5 seconds of specified time.
d. Repeat steps 7.6.3.2 a-c for each test organism.

7.6.3.3. Plate all control slide carriers in duplicate with appropriate dilutions to obtain a countable range within 30 minutes of neutralizer addition.

a. Incubate *S. aureus* for 48+4 hours at 37+2ºC.
b. Incubate *E. aerogenes* for 48+4 hours at 25-32ºC.

7.6.4. Sanitizer Test Procedure

7.6.4.1. Prepare sanitizer according to manufacturer instructions and section 7.4.

a. Dispense 5 mL product into sterile centrifuge tubes, 1 tube per carrier.
b. Allow tubes to equilibrate at least 10 minutes to 20 ± 1 °C.

7.6.4.2. Aseptically and sequentially transfer sterile slide carriers from petri dish to sterile centrifuge tubes containing 5mL sanitizer product at 30 second or 60 second intervals.

a. Add one carrier to a product tube and swirl 2-3 times, ensuring slide carrier is completely submerged in sanitizer.
b. Add carrier within +5 seconds of specified time.
c. Use alternating sterile forceps or equivalent to transfer slide carriers. Flame sterilize forceps between slide carriers.

7.6.4.3. Following 5-minute exposure time, sequentially add 20mL neutralizer media to each test carrier slide.

a. Rotate centrifuge tube vigorously for approximately 50 rotations, or vortex for 10-15s.
b. Transfer within +5 seconds of specified time.

7.6.4.4. Plate all slide carriers in duplicate 1.0 and 0.1mL dilutions within 30 minutes of neutralizer addition.

a. Incubate *S. aureus* for 48+4 hours at 37+2ºC.
b. Incubate *E. aerogenes* for 48+4 hours at 25-32ºC.

8. Records and Results

8.1. Calculations

8.1.1. Colony Forming Units per Milliliter in Neutralizer Solution

8.1.1.1. Determine average CFU on each of duplicate countable plates.

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8.1.1.2. Divide by average volume plated (in mL).

a. This provides average CFU/mL, surviving organism per milliliter neutralizer.

8.1.2. Number of Organisms Surviving per Slide Carrier

8.1.2.1. Multiply CFU/mL by total volume added to centrifuge tube (5mL sanitizer + 20mL neutralizer= 25mL total volume).

a. This provides average CFU/slide carrier, surviving organism per slide carrier.
b. Do this for both test slide carriers and control slide carriers.

8.1.3. Geometric Mean of Number of Organisms Surviving on Slide Carrier

8.1.3.1. Calculate average Log_{10} CFU/slide carrier.

8.1.3.2. Calculate antilog of average Log_{10}(CFU/slide carrier).

This provides the geometric mean per test or control.

8.1.4. Percent Reduction

8.1.4.1. Calculate (geometric mean of control slide carriers) – (geometric mean test slide carriers).

8.1.4.2. Multiply this number by 100.

8.1.4.3. Divide by geometric mean of control slide carriers.

a. This provides % reduction of sanitizer.
b. Repeat for each organism tested.

8.1.4.4. See Appendix for Equations and example calculation.

8.2. Results and Interpretation

8.2.1. Record all colony counts in cfu/mL.

8.2.2. Viability Controls: Growth should be observed. If no growth is observed, repeat the test.

8.2.3. Sterility Controls: If negative carrier has growth, issues in sterility is indicated. The test must be repeated.

8.2.4. Test Carriers: Record results for test carriers. Acceptance criteria is as follows:

8.2.4.1. Sanitizer must prove at least 99.9% reduction for each organism tested.

8.3. A final, signed report will be issued to the client. A copy of all record sheets and final report will be retained in accordance with ESS policy 1.6.8 Records and Data Management. All reagents and medias shall be traceable. All timing, temperatures, and procedures shall be documented. All quality control measures shall be documented.

9. Quality Control

Studies must include the following controls (as specified in each section above).

9.1. Slide Carrier Controls

9.1.1. Sterility Control:

9.1.1.1. For each test lot, three uninoculated slide carriers were collected and exposed to 5mL test solution, where 20mL neutralizing broth was then added alongside all test slide carriers.

9.1.2. Viability Control

9.1.2.1. For each test lot, an inoculated, untreated carrier was collected and incubated in 25 mL of neutralizer alongside all test carriers.

9.1.3. Neutralization Subculture Sterility Controls

9.1.3.1. Four primary neutralizing subculture media tubes and four secondary neutralizing subculture media tubes were incubated alongside test carriers.

9.1.4. Positive Control

9.1.4.1. For each organism, three inoculated slid carriers were collected and exposed to 5mL sterile DI water, where 20mL neutralizing broth was then added alongside all test slide carriers.

9.1.5. Media Sterility Controls

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9.1.5.1. All media lots went through quality control testing including inoculation with test organisms to ensure growth promotion, sterility evaluation, and pH monitoring.

9.1.6. Test Microorganism Purity Control

9.1.6.1. Test cultures used in this study were subcultured onto blood agar and visually examined for purity prior to evaluation.

9.1.7. Confirmation of Positive

9.1.7.1. Positive tubes with turbidity/growth were confirmed via microscopic exam and/or colony morphology on selective agar plates.

10. Definitions

10.1. Carrier- A surrogate surface or matrix that facilitates the interaction of test microorganisms and treatments

10.2. CFU- Colony forming unit

10.3. Efficacy- the proven performance of a product established under defined conditions of testing

10.4. Hard water- water which contains a standardized concentration of calcium and magnesium ions

10.5. Inoculum- the viable microorganisms used to contaminate a sample, device, or surface, often expressed as to number and type

10.6. Mean Log Density (LD)- average $\log_{10}$ converted carrier count cfu/mL of data per test day

10.7. Geometric Mean (GM): Antilog (Average Log_{10} cfu/mL) of sample set

10.8. Neutralization- the process for inactivating or quenching the activity of a microbiocide, often achieved through physical or chemical means

10.9. Sanitizer- chemical or physical agent(s) used to reduce the number of microorganisms to a level judged to be appropriate for a defined purpose and/or claim

10.10. Test substance- an antimicrobial formulation used in testing

11. Safety

11.1. Use appropriate PPE for all testing. Lab coat, safety goggles, and gloves are required for all methods listed above due to the unknown formulation and safety hazards of test substances.

11.2. Perform test product preparation (section 7.3) in a hood due to product off gassing when hydrated.

12. Attachments

12.1. None

13. Appendix

13.1. Calculations Equations:

- $Antilog = log\ base^{Value}$
- $Geometric\ Mean = Antilog\ x\ Average\ Log10\left(\frac{cfu}{carrier slide}\right)$
- $\%\ Reduction = \frac{(GM\ control - GM\ test)x100}{GM\ control}$

13.2. Example Calculations:

Test Data set:

Dilution	Test 1	Test 2	Test 3	Test 4	Test 5
1mL	5 colonies	0 colonies	3 colonies	1 colony	2 colonies
Dilution	Control 1	Control 2	Control 3		
0.000001	32	18	22		

Test Calculation:

- $Avg.\frac{CFU}{mL} = \frac{5+0+3+1+2}{5} = 2.2$

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- $Avg.\frac{CFU}{carrier slide} = 2.2\frac{cfu}{mL} x\ 25mL\ volume = 55.2\frac{CFU}{carrier slide}$
- $GM = \frac{Antilog}{} \left(\frac{\text{Log10}(25)+\text{Log10}(1)+\text{Log10}(75)+\text{Log10}(25)+\text{Log10}(50)}{5}\right)$

$= 10^{(1.4138)}$

$= 25.9284$

Control Calculation:

- $Avg.\frac{CFU}{mL} = \frac{32+18+22}{3} = 24\ x\ 100{,}000 = 2{,}400{,}000$
- $Avg.\frac{CFU}{carrier slide} = 2{,}400{,}000\frac{cfu}{mL} x\ 25mL\ volume = 60{,}000{,}000\frac{CFU}{carrier\ slide}$
- $GM = \frac{Antilog}{} \left(\frac{\text{Log10}(8000000)+\text{Log10}(45000000)+\text{Log10}(55000000)}{3}\right)$

$= 10^{(2.7656)}$

$= 582.8477$

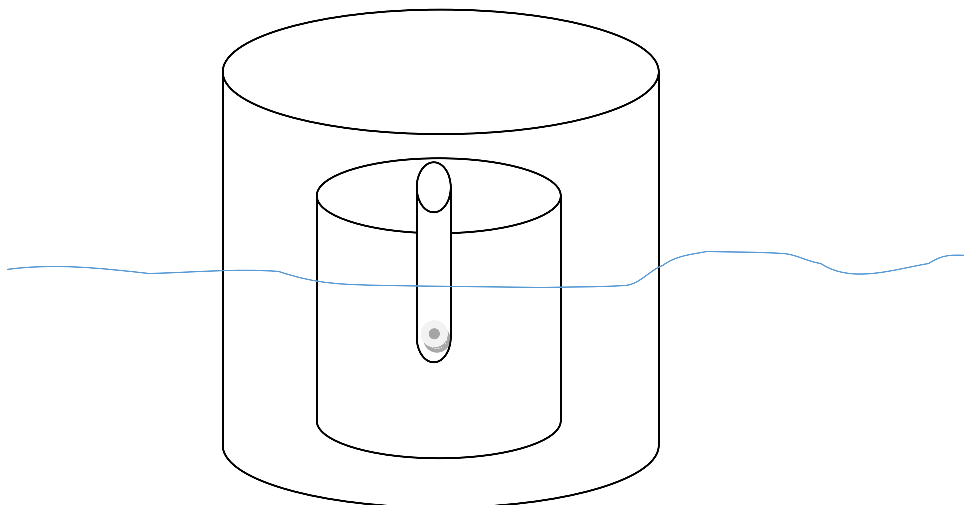
Percent Reduction Calculation:

- $\%\ Reduction = \frac{(582.8477 - 25.9284) x\ 100}{582.8477}$

$= 95.5514$

13.3 Diagram 1: Sonication Process

Ensure water/media levels are even and that both test tube and inner beaker do not touch the bottom of the sonicator nor the sides of outer containers.



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Certificate of Analysis

Client:	Synergy Americas, Inc 127 Stream Road Winterport, ME 04496	**Invoice Number:**	21-16912
		PO Number:	EPA-GLP3
		Received Date:	10/6/2021
Phone:	207.944.3495	**Approved By:**	
		Approved Date:	11/3/2021
Email:	michael@synergy-americas.com	**Project Name:**	ASTM Testing- Envirotab

Lab #: 68744 **Sample: Synergy Envirotab 4g- Lots 2020/12-05, 2020/12-06, 2020/12-07**

Analyte	Results	Units	Detection Limit	Method	Analyst	Date Analyzed
Administration						
Katherine	**See Word Doc**	PSU		ESS_1.1.27	KS	11/3/2021

ISO 17025 ANAB Accredited Laboratory Cert. #: AT-1754
I = ISO 17025 accredited method
ND = Not detected above the listed detection limit
MM = Modified Method
RR = Revised Result
SS = Analysis was run on a separate submission

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