(For Laboratory Use Only)
ATS Labs Project # A16709

ATS LABS

EXACT COPY
INITIALS MW DATE 7-15-14

PROTOCOL

Standard Test Method for Efficacy of Sanitizers Recommended for Inanimate Non-Food Contact Surfaces (Dilutable)

Test Organisms:

Cronobacter sakazakii (ATCC 12868)
Salmonella enterica subsp. *enterica* Typhimurium (ATCC 13311)

PROTOCOL NUMBER

ECO01050114.NFS.2

PREPARED FOR

Ecolab
Ecolab Schuman Campus
655 Lone Oak Drive
Eagan, MN 55121-1560

PERFORMING LABORATORY

ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

DATE

May 1, 2014

Revised May 6, 2014

PROPRIETARY INFORMATION

THIS DOCUMENT IS THE PROPERTY OF AND CONTAINS PROPRIETARY INFORMATION OF ATS LABS. NEITHER THIS DOCUMENT, NOR INFORMATION CONTAINED HEREIN IS TO BE REPRODUCED OR DISCLOSED TO OTHERS, IN WHOLE OR IN PART, NOR USED FOR ANY PURPOSE OTHER THAN THE PERFORMANCE OF THIS WORK ON BEHALF OF THE SPONSOR, WITHOUT PRIOR WRITTEN PERMISSION OF ATS LABS.

Standard Test Method for Efficacy of Sanitizers Recommended for Inanimate Non-Food Contact Surfaces (Dilutable)

SPONSOR: Ecolab
Ecolab Schuman Campus
655 Lone Oak Drive
Eagan, MN 55121-1560

TEST FACILITY: ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

PURPOSE

The purpose of this study is to determine the antimicrobial efficacy of sanitizers on hard, inanimate, non-porous, non-food contact surfaces. This method is in compliance with the requirements of the U.S. Environmental Protection Agency (EPA) and Health Canada.

TEST SUBSTANCE CHARACTERIZATION

According to (40 CFR, Part 160, Subpart F [160.105]) test substance characterization as to identity, strength, purity, solubility and composition, as applicable, shall be documented before its use in this study. The stability of the test substance shall be determined prior to or concurrently with this study. Pertinent information, which may affect the outcome of this study, shall be communicated in writing to the Study Director upon sample submission to ATS Labs. ATS Labs will append Sponsor-provided Certificates of Analysis (C of A) to this study report, if requested and supplied. Characterization and stability studies not performed by or under the direction of ATS Labs will be noted in the Good Laboratory Practice compliance statement.

SCHEDULING AND DISCLAIMER OF WARRANTY

Experimental start dates are generally scheduled on a first-come/first-serve basis once ATS Labs receives the Sponsor approved/completed protocol, signed fee schedule and corresponding test substance(s). Based on all required materials being received at this time, the proposed experimental start date is May 19, 2014. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of June 16, 2014. To expedite scheduling, please be sure all required paperwork and test substance documentation is complete/accurate upon arrival at ATS Labs.

If a test must be repeated, or a portion of it, due to failure by ATS Labs to adhere to specified procedures, it will be repeated free of charge. If a test must be repeated, or a portion of it, due to failure of internal controls, it will be repeated free of charge. "Methods Development" fees shall be assessed, however, if the test substance and/or test system require modifications due to complexity and difficulty of testing.

If the Sponsor requests a repeat test, they will be charged for an additional test. Neither the name of ATS Labs nor any of its employees are to be used in advertising or other promotion without written consent from ATS Labs.

The Sponsor is responsible for any rejection of the final report by the regulating agencies concerning report format, pagination, etc. To prevent rejection, Sponsor should carefully review the ATS Labs final report and notify ATS Labs of any perceived deficiencies in these areas before submission of the report to the regulatory agency. ATS Labs will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM

The U.S. Environmental Protection Agency requires that a specific claim for a sanitizer be supported by appropriate scientific data demonstrating the efficacy of the sanitizer against the claimed organism. In addition, Health Canada requires that the product be recognized as a disinfectant prior to accepting sanitizer claims. This is accomplished in the laboratory by treating the target organism with the test substance under conditions which simulate as closely as possible, the actual conditions under which the test substance is designed to be used. For products intended for use on non-food contact surfaces, a carrier method is used in the generation of the supporting data. The test system to be used in this study follows the ASTM approved method for the evaluation of the antimicrobial efficacy of sanitizers on inanimate, nonporous, non-food contact surfaces.

TEST PRINCIPLE

A film of organism cells dried on a surface of appropriate carriers is exposed to the test substance for a specified exposure time. After exposure, the carriers are neutralized and assayed for survivors. Appropriate sterility, culture purity, carrier population, neutralization confirmation and inoculum count controls are performed. The current version of Standard Operating Procedure CGT-4150 reflects the methods which shall be used in this study.

TEST METHOD

Test Organism	ATCC #	Growth Medium	Incubation Parameters
Cronobacter sakazakii	12868	Nutrient Broth	25-30°C, aerobic
Salmonella enterica subsp. *enterica* Typhimurium	13311	Nutrient broth	35-37°C, aerobic

The test organisms to be used in this study were obtained from the American Type Culture Collection (ATCC), Manassas, VA.

Subculture Agar: An appropriate subculture agar medium such as Tryptic Soy Agar+5% Sheep's blood will be used in testing. The agar used in the test will be the same as that which is used in the control procedures which substantiates test organism recovery.

Carriers

Glass 1" x 1" carriers shall be dipped in 95% alcohol, rinsed with deionized water, and air dried before sterilization. The carriers will be placed into a vessel and sterilized in hot air oven for ≥2 hours at ≥180°C. After sterilization, each carrier will be placed into a sterile Petri dish.

Preparation of Test Organism

From a stock slant no more than 5 transfers from original stock and ≤1 month old, an initial tube (10 mL) of culture broth will be inoculated. This culture is termed the "initial broth suspension." From this initial broth suspension, at least three consecutive daily transfers using 1 loopful (10 µL) of culture into 10 mL of culture media will be performed prior to use as an inoculum. Incubate each daily transfer for 24±2 hours using the appropriate growth medium. The final test culture will be incubated for 48-54 hours.

A 48-54 hour culture will be vortex-mixed and allowed to settle for ≥15 minutes. The upper 2/3rds of the culture will be removed and transferred to a sterile vessel for use in testing. The culture may be adjusted by dilution in growth medium or by centrifuge concentration, if necessary. An organic soil load may be added to the test culture per Sponsor request. The test culture will be thoroughly mixed prior to use.

Preparation of Test Substance

The test substance will be prepared according to the directions for intended use of the product. The test substance shall be used within three hours of preparation if additional preparation is required by ATS Labs.

Contamination of Carriers

Inoculate each sterile carrier with 0.02 mL (20 µL) of culture using a calibrated pipettor spreading the inoculum to within approximately 3 mm of the edges of the carrier. Dry the inoculated carriers for 20-40 minutes at 35-37°C ~~or~~ ① ① ~~25-30°C~~ until visibly dry. A drying humidity should be selected to encourage maximum survival of the test organism (targeting approximately 40% humidity, for example). The lids may be left slightly ajar or intact during drying if die-off is a concern. *The drying conditions for organisms not defined in the ASTM method have been modified to ensure adequate recovery of the test organism.* A constant humidity chamber will be used in place of a desiccating chamber to ensure uniform humidification conditions and to overcome slow re-equilibration of a desiccator after opening.

① removed per email. KWS 5-16-14

Exposure Conditions
Following the completion of drying, transfer each carrier to individual sterile 2 oz. (60 mL) polypropylene jars using sterile forceps with the inoculum facing up. Using staggered intervals, transfer 5.0 mL of prepared test substance to each jar. The liquid should completely cover the carrier during exposure. Continue treating the test carriers using staggered intervals. Allow the carriers to expose at the Sponsor specified exposure temperature for the Sponsor specified exposure time. Following exposure, transfer 20 mL of neutralizer to the jars using identical staggered intervals. Rotate the jar vigorously on an even plane for approximately 50 rotations to suspend the surviving organisms or vortex mix the jars for 10-15 seconds.

Test System Recovery
Within 30 minutes of neutralization, plate 1.0 mL and 0.1 mL aliquots of the neutralized subcultures (10^0) in duplicate onto appropriate agar.

If neutralization of the test substance cannot be achieved chemically, filter-neutralization may be performed. Within 30 minutes of neutralization, transfer duplicate 1.0 mL and 0.1 mL of the neutralized solution, to individual filter units pre-wetted with 10 mL of sterile diluent. Evacuate the contents and rinse each filter with a minimum of 50 mL of sterile diluent. Transfer each filter to an appropriate agar using sterile forceps.

Incubation and Observation
Incubate *S. enterica* plates at 35-37°C for 48±4 hours. Incubate *C. sakazakii* plates for 48±4 hours at 25-30°C. Following incubation, the subcultures will be visually enumerated. If necessary, the subcultures may be placed at 2-8°C for up to three days prior to examination. Representative test plates showing growth may be subcultured, stained and/or biochemically assayed to confirm or rule out the presence of the test organism. If possible, subcultures containing 30-300 colonies will be used for calculations. When membrane filtration is used, the upper limit used for counting/calculations should be 200 CFU.

STUDY CONTROLS

Carrier Population Control
Inoculated, dried control carriers will be treated as in the test procedure utilizing sterile deionized water in place of test substance. If multiple exposure times were followed in testing, the carriers will be exposed for the shortest exposure time followed in the test procedure. Following exposure, the carriers will be neutralized as in the test. The carriers will be mixed as in the test. Ten-fold serial dilutions will be prepared and 0.1 mL aliquots of the 10^{-1} to 10^{-4} dilutions will be plated in duplicate. The plates will be incubated as in the test procedure and enumerated. The acceptance criterion for this study control is a minimum geometric mean value of 2.5×10^4 CFU/carrier which is required to show a 99.9% reduction and has been modified for test organisms not defined in the ASTM method.

Carrier Sterility Control
A representative, uninoculated carrier will be added to the neutralizer. The vessel will be mixed and 1.0 mL will be plated onto appropriate agar and incubated. The acceptance criterion is a lack of growth following incubation.

Neutralizer Sterility
A 1.0 mL aliquot of neutralizer will be plated onto appropriate agar and incubated. The acceptance criterion is a lack of growth following incubation.

Culture Purity
A "streak plate for isolation" will be performed on the organism culture and following incubation examined in order to confirm the presence of a pure culture. The acceptance criterion for this study control is a pure culture demonstrating colony morphology typical of the test organism.

Organic Soil Load Sterility
If applicable, a 1.0 mL aliquot of the serum used in testing will be added to a tube of Fluid Thioglycollate Medium. This control will be incubated and examined. The acceptance criterion is a lack of growth following incubation.

Neutralization Confirmation Control
In a manner consistent with the AOAC 960.09 method, the following neutralization confirmation control will be performed prior to testing or concurrent with testing. To represent worst-case conditions, only the most concentrated test substance and/or shortest exposure time needs to be utilized in this control when multiple test substance concentrations or multiple exposure times are being evaluated in the study.

Serially dilute the prepared test culture to target $2x10^4$ – $2x10^5$ CFU/mL (to target a result of 10-100 CFU plated in each control run). Multiple organism dilutions may be prepared. *(Typically the 10^{-3}, 10^{-4} or 10^{-5} dilutions will provide a culture in range depending on expected titer. Alternate or partial dilutions may be used where appropriate.)* If all the organism dilution(s) used in this control fail to provide adequate numbers (10-100 CFU) which coincides in a failure to meet the acceptance criterion for this study control, the control may be repeated in its entirety.
Test Culture Titer (TCT)
Add 0.1 mL of diluted test organism to 25 mL of sterile diluent and vortex mix. Hold the mixture for a minimum of 30 minutes and spread plate or filter plate duplicate 0.1 mL aliquots using the same method used in the test. The acceptance criterion for this study control is growth. If the test culture titer fails to yield countable numbers or if the culture titer is too low resulting in failing results, the entire neutralization confirmation control may be repeated in its entirety, as necessary, to properly validate neutralization.

Neutralization Confirmation Control Treatment (NCT)
Immerse a sterile carrier (one per test organism dilution to be used, per test substance to be evaluated) in 5.0 mL of test substance as in the test. Expose for the exposure time and neutralize each carrier with 20 mL of neutralizer. Rotate the jar vigorously on an even plane for approximately 50 rotations or vortex mix the jars for 10-15 seconds. Within 5 minutes, add 0.1 mL of diluted test organism to the neutralized contents and vortex mix. Hold the mixture for a minimum of 30 minutes and spread plate or filter plate duplicate 0.1 mL aliquots using the same method used in the test. The acceptance criterion for this study control is growth within 1 $\log_{10}$ of the test culture titer (TCT).

Neutralizer Toxicity Treatment (NTT)
Add 0.1 mL of diluted test organism to 25 mL of sterile neutralizer and vortex mix. Hold the mixture for a minimum of 30 minutes and spread plate or filter plate duplicate 0.1 mL aliquots using the same method used in the test. The acceptance criterion for this study control is growth within 1 $\log_{10}$ of the test culture titer (TCT).

Inoculum Count
Serially dilute and plate the test organism in duplicate using 0.1 mL aliquots and appropriate dilutions and incubate as in the test. This control is for informational purposes and therefore has no acceptance criterion.

PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM

ATS Labs maintains Standard Operating Procedures (SOPs) relative to efficacy testing studies. Efficacy testing is performed in strict adherence to these SOPs which have been constructed to cover all aspects of the work including, but not limited to, receipt, log-in, and tracking of biological reagents including test organism strains for purposes of identification, receipt and use of chemical reagents. These procedures are designed to document each step of efficacy testing studies. Appropriate references to medium, batch number, etc. are documented in the raw data collected during the course of each study.

Additionally, each efficacy test is assigned a unique Project Number when the protocol for the study is initiated by the Study Director. This number is used for identification of the test subcultures, etc. during the course of the test. Test subcultures are also labeled with reference to the test organism, experimental start date, and test product. Microscopic and/or macroscopic evaluations of positive subcultures are performed in order to confirm the identity of the test organism. These measures are designed to document the identity of the test system.

METHOD FOR CONTROL OF BIAS: **N/A**

STUDY ACCEPTANCE CRITERIA

Test Substance Performance Criteria
The efficacy performance requirements for label claims state that the test substance must demonstrate a minimum 99.9% reduction of test survivors as compared to the population control to be considered an effective non-food contact sanitizer.

Control Acceptance Criteria
The study controls must perform according to the criteria detailed in the study controls description section. If any control acceptance criteria are not met, the test may be repeated under the current protocol number.

REPORT
The report will include, but not be limited to, identification of the sample, date received, initiation and completion dates, identification of the organism strains used, description of media and reagents, description of the methods employed, tabulated results and conclusion as it relates to the purpose of the test, and all other items required by 40 CFR Part 160.185.

PROTOCOL CHANGES
If it becomes necessary to make changes in the approved protocol, the revision and reasons for changes will be documented, reported to the Sponsor and will become a part of the permanent file for that study. Similarly, the Sponsor will be notified as soon as possible whenever an event occurs that may have an effect on the validity of the study.

Standard operating procedures used in this study will be the correct effective revision at the time of the work. Any minor changes to SOPs (for this study) or methods used will be documented in the raw data and approved by the Study Director.

TEST SUBSTANCE RETENTION
It is the responsibility of the Sponsor to retain a sample of the test substance. All unused test substance will be discarded following study completion unless otherwise indicated by Sponsor.

RECORD RETENTION

Study Specific Documents

All of the original raw data developed exclusively for this study shall be archived at ATS Labs. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of final study report.
7. Study-specific SOP deviations made during the study.

Facility Specific Documents

The following records shall also be archived at ATS Labs. These documents include, but are not limited to, the following:

1. SOPs which pertain to the study conducted.
2. Non study-specific SOP deviations made during the course of this study which may affect the results obtained during this study.
3. Methods which were used or referenced in the study conducted.
4. QA reports for each QA inspection with comments.
5. Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
6. Current curriculum vitae, training records, and job descriptions for all personnel involved in the study.

REFERENCES

1. U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines, OCSPP 810.2000: General Considerations for Uses of Antimicrobial Agents, September 4, 2012.
2. 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 4, 2012.
3. American Society for Testing and Materials (ASTM). Standard Test Method for Efficacy of Sanitizers Recommended for Inanimate Non-Food Contact Surfaces, E1153-14.
4. Association of Official Analytical Chemists (AOAC) Official Method 960.09, Germicidal and Detergent Sanitizing Action of Disinfectants Method. In Official Methods of Analysis of the AOAC, 2013 Edition.
5. Health Canada, January, 2014. Guidance Document – Safety and Efficacy Requirements for Hard Surface Disinfectant Drugs.
6. Health Canada, January, 2014. Guidance Document - Disinfectant Drugs.

DATA ANALYSIS

Calculations

$$\text{CFU/mL} = \frac{\text{(average CFU) x (dilution factor)}}{\text{(volume plated in mL)}}$$

Number of Organisms Surviving per Carrier

$$\text{CFU/carrier} = \frac{\text{(average CFU) x (dilution factor) x (volume neutralized solution in mL)}}{\text{(volume plated or filtered in mL)}}$$

Geometric Mean of Number of Organisms Surviving on Test or Control Carriers

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

Where: X equals CFU/carrier
N equals number of carriers

Percent Reduction

% reduction = [(a - b) / a] x 100

where:
a = geometric mean of the number of organisms surviving on the population control carriers.
b = geometric mean of the number of organisms surviving on the test carriers.

Recovery Log_{10} Difference = (Log_{10} Numbers Control) – (Log_{10} Neutralization Results)
Used for the neutralization confirmation control

Statistical Methods
None Used.

STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Test Substance (Name & Batch Numbers) exactly as it should appear on final report:
HX-16228 Batches P111431-2 and P120331-2

Product Description:

- ☐ Quaternary ammonia
- ☐ Iodophor
- ☐ Sodium hypochlorite
- ☑ Peracetic acid
- ☑ Peroxide
- ☐ Other _____

Test Substance Active Concentration (upon submission to ATS Labs): ~10.70 % hydrogen peroxide, ~0.63% peroxyoctanoic acid, ~2.38% peroxyacetic acid

Neutralization/Subculture Broth:
- ☑ DE Broth
- ☐ ATS Labs' Discretion. By checking, the Sponsor authorizes ATS Labs, at their discretion, to perform neutralization confirmation assays at the Sponsor's expense prior to testing to determine the most appropriate neutralizer. (See Fee Schedule).

Storage Conditions:
- ☑ Room Temperature
- ☐ 2-8°C
- ☐ Other: _____

Hazards:
- ☐ None known: Use Standard Precautions
- ☑ Material Safety Data Sheet, Attached for each product
- ☐ As Follows: _____

Product Preparation
- ☐ No dilution required, Use as received (RTU)
- ☑ *Dilution(s) to be tested: 1oz/8gallons defined as 1.56 grams + 1498.44 grams
  (example: 1 oz/gallon) (amount of test substance) (amount of diluent)
- ☐ Sterile Deionized Water
- ☐ Sterile Tap Water (Soft)
- ☑ AOAC Synthetic Hard Water: 500 PPM
- ☐ Other _____

The test substance will be diluted at 1oz/8gallons to result in the actives at or below the lower limits of 114ppm hydrogen peroxide, 23.9ppm peroxyacetic acid and 5.74ppm peroxyoctanoic acid as shown below. The test substance and diluent weights may vary by +/-0.03g.

Note: An equivalent dilution may be made unless otherwise requested by the Sponsor.

Test Organisms:
- ☑ *Cronobacter sakazakii* (ATCC 12868)
- ☑ *Salmonella enterica* subsp. *enterica* Typhimurium (ATCC 13311)

Carrier Number: 5 test carriers per batch and 3 population control carriers

Exposure Time: 5 Minutes

Exposure Temperature: 18-25°C

Organic Soil Load:
- ☑ Minimum 5% Organic Soil Load (Fetal Bovine Serum)
- ☐ No Organic Soil Load Required
- ☐ Other: _____

TEST SUBSTANCE SHIPMENT STATUS

- ☐ Has been used in one or more previous studies at ATS Labs .
- ☐ Has been shipped to ATS Labs (but has not been used in a previous study).
  Date shipped to ATS Labs:_____ Sent via *overnight* delivery? ☐ Yes ☐ No
- ☑ Will be shipped to ATS Labs.
  Date of expected receipt at ATS Labs: 5/9/14
- ☐ Sender (if other than Sponsor):_____

COMPLIANCE

Study to be performed under EPA Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures.

- ☑ Yes
- ☐ No (Non-GLP or Development Study) - Test Substance Characterization is not required.

TEST SUBSTANCE CHARACTERIZATION

[Verification required per 40 CFR Part 160 Subpart B (160.31(d))]

- ☐ A Certificate of Analysis (C of A) will be provided for each lot of test substance.
- ☑ If C of A is not available, other documentation will be provided to verify test substance characterization has been performed (written or verbal confirmation).

  Sponsor's study #(s) for characterization and/or stability testing (if applicable): The chemical characterization of the test substance was conducted under Ecolab GLP study # 1300150. The chemical characterization of the test substance use-solution was conducted under Ecolab GLP study # 1400009.
- ☐ No test substance characterization information is available. If no characterization verification is provided, it will be noted in the compliance statement in the final report.

PROTOCOL MODIFICATIONS

- ☑ Approved without modification
- ☑ Approved with modification

wrong box pto 5/7/14

Please include Ecolab GLP study # 1300150CLS in the final report. Please use Brain Heart Infusion Broth as the growth medium for S. enterica subsp. enterica Typhimurium. Please use stainless steel, non-corrosive, 25x25mm (1x1") carriers. Following exposure and addition of the neutralizer, please vortex jars vigorously on an even plane for approximately 50 revolutions to suspend the surviving organisms.

PROTOCOL ATTACHMENTS

Supplemental Information Form Attached - ☐ Yes ☑ No

① Box checked per email. KW 5-16-14

APPROVAL SIGNATURES

SPONSOR:

NAME: Ms. Laurinda Holen TITLE: Senior Microbiologist

SIGNATURE: Laurinda Holen DATE: 5/7/14

PHONE: (651) 795 - 5974 FAX: (651) 204 - 7501 EMAIL: laurinda.holen@ecolab.com

For confidentiality purposes, study information will be released only to the sponsor/representative signing the protocol (above) unless other individuals are specifically authorized in writing to receive study information.
Other individuals authorized to receive information regarding this study: ☐ See Attached

ATS Labs:

NAME: Kristen Niehaus
Study Director

SIGNATURE: Kristen Niehaus DATE: 5-19-14
Study Director