FINAL STUDY REPORT

STUDY TITLE

Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Utilizing Feline Calicivirus as a Surrogate Virus for Norovirus – Confirmatory Assay

PRODUCT IDENTITY

SNiPER®
Batch # 106-114-1

DATA REQUIREMENT

U.S. EPA 40 CFR Part 158
"Data Requirements for Registration"
Pesticide Assessment Guidelines - Subdivision G, 91-2 (f)

AUTHOR

Mary J. Miller, M.T.
Study Director

STUDY COMPLETION DATE

September 28, 2009

PERFORMING LABORATORY

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

SPONSOR

Global Environmental Restoration, Inc.
108 Maple Ridge Drive
Lafayette, LA 70507

PROJECT NUMBER

A08200

EXACT COPY
INITIALS MP DATE 1/28/09

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: Global Environmental Restoration, Inc.

Company Agent: _____

_____
Title

_____ Date:_____
Signature

GOOD LABORATORY PRACTICE STATEMENT

The study referenced in this report was conducted in compliance with U.S. Environmental Protection Agency Good Laboratory Practice (GLP) regulations set forth in 40 CFR Part 160.

The procedures not performed by or under the direction of ATS Labs are exempt from this Good Laboratory Practice Statement and include: characterization and stability of the test substance(s).

Submitter:_____ Date:_____

Sponsor:_____ Date:_____

Study Director: Mary J. Miller Date: 9-28-09
Mary J. Miller, M.T.

QUALITY ASSURANCE UNIT SUMMARY

Study: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Utilizing Feline Calicivirus as a Surrogate Virus for Norovirus – Confirmatory Assay

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of non-clinical laboratory studies. This study has been performed under Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures and standard protocols. The Quality Assurance Unit maintains copies of study protocols and standard operating procedures and has inspected this study on the dates listed below. This study was inspected at time intervals to assure the integrity of the study.

Phase Inspected	Date	Study Director	Management
Critical Phase	September 10, 2009	September 10, 2009	September 28, 2009
Final Report	September 24, 2009	September 25, 2009	

The findings of these inspections have been reported to management and the Study Director.

Quality Assurance Auditor: _____ Date: 9/28/09

TABLE OF CONTENTS

Title Page 1
Statement of No Data Confidentiality Claims 2
Good Laboratory Practice Statement 3
Quality Assurance Unit Summary 4
Table of Contents 5
Study Personnel 6
General Study Information 7
Test Substance Identity 7
Study Dates 7
Objective 7
Summary of Results 8
Test System 8
Test Method 9
Protocol Changes 11
Data Analysis 11
Study Acceptance Criteria 11
Study Retention 12
References 12
Study Results 13
Study Conclusion 13
Table 1: Virus Controls and Test Substance Assay Results 14
Table 2: Test Substance Cytotoxicity and Neutralization Control Results 15
Test Protocol 16

STUDY PERSONNEL

STUDY DIRECTOR: Mary J. Miller, M.T.

Professional Personnel Involved:

Karen M. Ramm, B.A.*	- Technical Director
Katherine A. Paulson, M.L.T.	- Research Assistant II
Matthew Cantin, B.S.	- Research Assistant II

* Karen Ramm is listed solely as supervisory personnel per 160.185(a)(10) of 40 CFR Part 160. She had no direct participation in the study other than her role as the department supervisor.

STUDY REPORT

GENERAL STUDY INFORMATION

Study Title: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Utilizing Feline Calicivirus as a Surrogate Virus for Norovirus – Confirmatory Assay

Project Number: A08200

Protocol Number: GRS01072909.FCAL

Sponsor: Global Environmental Restoration, Inc.
108 Maple Ridge Drive
Lafayette, LA 70507

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

TEST SUBSTANCE IDENTITY

Test Substance Name: SNiPER®

Lot/Batch(s): Batch # 106-114-1

Test Substance Characterization
Test substance characterization as to content, stability, solubility, storage, etc., (40 CFR, Part 160, Subpart F [160.105]) is the responsibility of the Sponsor.

STUDY DATES

Date Sample Received: May 1, 2009
Study Initiation Date: September 1, 2009
Experimental Start Date: September 10, 2009
Experimental End Date: September 17, 2009
Study Completion Date: September 28, 2009

OBJECTIVE

The purpose of this study was to evaluate the virucidal efficacy of a disinfectant against Feline Calicivirus, used as a surrogate virus for Norovirus, according to test criteria and methods approved by the U.S. Environmental Protection Agency for registration of a product as a virucide.

Norovirus, a member of the *Caliciviridae* family, is a non-enveloped RNA-containing virus and is an important cause of gastroenteritis in humans. Little is known about disinfectant efficacy against this virus due to the inability to propagate the virus in-vitro. Feline Calicivirus, also a member of the *Caliciviridae* family, serves as a valuable model virus for efficacy testing of Norovirus since these viruses share many similar characteristics and Feline Calicivirus can be propagated in cell cultures.

SUMMARY OF RESULTS

Test Substance:	SNiPER®, Batch # 106-114-1
Dilution:	Ready to use (RTU)
Virus:	Feline Calicivirus as a surrogate virus for Norovirus
Exposure Time:	Ten minutes
Exposure Temperature:	Room temperature (20.0°C)
Organic Soil Load:	5% fetal bovine serum
Efficacy Result:	One batch of SNiPER® (Batch # 106-114-1) met the test criteria specified in the study protocol. Under these test conditions, the results indicate **complete inactivation** of Feline Calicivirus as required by the U.S. EPA for claims of virucidal activity.

TEST SYSTEM

1. Virus

The F-9 strain of Feline Calicivirus used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-782). The stock virus was prepared by collecting the supernatant culture fluid from 75-100% infected culture cells. The cells were disrupted and cell debris removed by centrifugation at approximately 2000 RPM for five minutes at approximately 4°C. The supernatant was removed, aliquoted, and the high titer stock virus was stored at ≤-70°C until the day of use. On the day of use, two aliquots of stock virus (ATS Labs Lot FC-48) were removed, thawed, combined and refrigerated until used in the assay. The stock virus culture contained 5% fetal bovine serum as the organic soil load. The stock virus tested demonstrated cytopathic effects (CPE) typical of Feline Calicivirus on Crandel Reese feline kidney cells. The cytopathic effect observed was small, rounding of the cells, with a slight granular look.

2. Indicator Cell Cultures

Cultures of Crandel Reese feline kidney (CRFK) cells were originally obtained from the American Type Culture Collection, Manassas, VA (ATCC CCL-94). The cells were propagated by ATS Labs personnel. The cells were seeded into multiwell cell culture plates and maintained at 36-38°C in a humidified atmosphere of 5-7% CO_2. The confluency of the cells was appropriate for the test virus. This cell line has historically been used as the cell line for propagation and detection of Feline Calicivirus. The cultures were commercially available, were serially propagated, and were capable of showing cytopathic effect in the presence of the virus.

All cell culture documentation was retained for the cell cultures used in this assay with respect to source, passage number, growth characteristics, seeding densities and the general condition of the cells.

3. Test Medium

The test medium used for this assay was Minimum Essential Medium (MEM) supplemented with 5% (v/v) heat inactivated fetal bovine serum. The medium was also supplemented with 10 µg/mL gentamicin, 100 units/mL penicillin and 2.5 µg/mL amphotericin B.

The following table lists the test and control groups, the dilutions assayed, and the number of cultures used. See text for a more detailed explanation.

SAMPLES TESTED FOR THE PRESENCE OF VIRUS			
Test or Control Group	**Dilutions Assayed Per Carrier (log_{10})**	**Cultures per Dilution**	**Total Cultures Inoculated**
Negative Controls	N/A	2-4	2-4/group
Input Virus Control	-4,-5,-6,-7,-8	4	20
Dried Virus Control (performed in duplicate)	-4,-5,-6,-7,-8	4	40
Test Substance – (performed in duplicate)	-1,-2,-3,-4	4	32
Cytotoxicity Control – Test Substance	-1,-2,-3	2	6
Neutralization Control – Test Substance	-1,-2,-3	2	6

TEST METHOD

1. Preparation of Test Substance
   One batch of SNiPER® (Batch # 106-114-1) was used, undiluted, as received from the Sponsor. The test substance was in solution as determined by visual observation. Prior to use, the test substance was pre-equilibrated to the exposure temperature.

2. Preparation of Virus Films
   Films of virus were prepared at staggered intervals by spreading 0.2 mL of virus inoculum uniformly over the bottoms of four separate 100 X 15mm sterile glass petri dishes. The virus films were air-dried at 20.0°C in a relative humidity of 50% until visibly dry (20 minutes).

3. Sephadex Gel Filtration
   To reduce the cytotoxic level of the virus-test substance mixture prior to assay of virus, and/or to reduce the virucidal level of the test substance, virus was separated from test substance by filtration through Sephadex gel. Columns of Sephadex LH-20-100 were equilibrated with phosphate buffered saline containing 1% albumin, centrifuged for three minutes to clear the void volume, loaded with approximately 2.0 mL of virus-test substance mixture and immediately passed through the column utilizing the syringe plunger.

4. Input Virus Control

On the day of testing, the stock virus utilized in the assay was titered by 10-fold serial dilution and assayed for infectivity to determine the starting titer of the virus. The results of this control are for informational purposes only.

5. Treatment of Virus Films with Test Substance

Each of two dried virus films were exposed to a 2.00 mL aliquot of the test substance for the Sponsor specified exposure time of ten minutes at room temperature (20.0°C). Following the exposure time, the plates were individually scraped with a cell scraper to resuspend the contents and the virus-test substance mixtures were immediately passed through individual Sephadex columns utilizing the syringe plunger in order to detoxify the mixture. This dilution was considered the 10^{-1} dilution. A 0.2 mL aliquot of the test virus (the virus film) was resuspended in 2.00 mL of test substance which equals a 1:10 dilution. The filtrates were then titered by 10-fold serial dilution and assayed for infectivity and/or cytotoxicity

6. Treatment of Virus Control Films

Each of two dried virus films were prepared as previously described. The virus control films were individually exposed to 2.00 mL of test medium for ten minutes at room temperature (20.0°C). The virus films were individually scraped with a plastic cell scraper and passed through individual Sephadex columns in the same manner as the test virus. The filtrates (10^{-1} dilution) were then titered by 10-fold serial dilution and assayed for infectivity.

7. Cytotoxicity Control

A 2.00 mL aliquot of the test substance was filtered through a Sephadex column utilizing the syringe plunger. The filtrate (10^{-1} dilution) was then titered by 10-fold serial dilution and assayed for cytotoxicity. Cytotoxicity of the CRFK cell cultures was scored at the same time as the virus-test substance and virus control cultures.

8. Assay of Non-Virucidal Level of Test Substance (Neutralization Control)

Each dilution of the Sephadex-filtered test substance (cytotoxicity control dilutions) was mixed with an aliquot of low titer stock virus. The resulting mixtures of dilutions were assayed for infectivity and/or cytotoxicity in order to determine the dilution(s) of test substance at which virucidal activity, if any, was retained. Dilutions that showed virucidal activity were not considered in determining the reduction in infectivity by the test substance.

9. Infectivity Assays

The CRFK cell line, which exhibits cytopathic effect (CPE) in the presence of Feline Calicivirus, was used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes were inoculated in quadruplicate with 0.1 mL of the dilutions prepared from test and virus control groups. The cytotoxicity and neutralization control dilutions were inoculated in duplicate. Uninfected indicator cell cultures (cell controls) were inoculated with test medium alone. Cultures were incubated at 31-35°C in a humidified atmosphere of 5-7% CO_2 in sterile disposable cell culture labware. The cultures were microscopically scored periodically for seven days for the absence or presence of CPE, cytotoxicity and for viability.

PROTOCOL CHANGES

Protocol Amendments
No protocol amendments were required for this study.

Protocol Deviations:
No protocol deviations occurred during this study.

DATA ANALYSIS

Calculations

Viral and cytotoxicity titers are expressed as $-\log_{10}$ of the 50 percent titration endpoint for infectivity ($TCID_{50}$) or cytotoxicity (TCD_{50}), respectively, as calculated by the method of Spearman Karber.

$$\text{- Log of 1st dilution inoculated} - \left[\left(\left(\frac{\text{Sum of \% mortality at each dilution}}{100}\right) - 0.5\right) \times (\text{logarithm of dilution})\right]$$

Statistical Analysis

The $\log_{10}$ reduction in infectivity was calculated using the revised EPA approved method for calculating the Most Probable Number (MPN) as obtained from the EPA on January 4, 2001.

STUDY ACCEPTANCE CRITERIA

A valid test requires 1) that at least 4 $\log_{10}$ of infectivity be recovered from the dried virus control films; 2) that when cytotoxicity is evident, at least a 3-log reduction in viral titer is demonstrated beyond the cytotoxic level; and 3) that the cell controls be negative for infectivity. **Note:** An efficacious product must demonstrate complete inactivation of the virus at all dilutions.

STUDY RETENTION

Record Retention

All of the original raw data developed exclusively for this study shall be archived at ATS Labs, 1285 Corporate Center Drive, Suite 110, Eagan, MN 55121. 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.

Test Substance Retention

The test substance will be returned following study completion per Sponsor approved protocol. It is the responsibility of the Sponsor to retain a sample of the test material.

REFERENCES

1. Annual Book of ASTM Standards 2000, Section 11 Water and Environmental Technology Volume 11.05 Biological Effects and Environmental Fate: Biotechnology; Pesticides, E1053-97.
2. U.S. Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision G: Product Performance, 91-2(f), November 1982.
3. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, DIS/TSS-7, November 12, 1981.
4. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Schmidt, N.J. and Emmons, R.W. editors. Sixth edition, 1989. p. 18-20.
5. Blackwell, J.H., and J.H.S. Chen. 1970. Effects of various germicidal chemicals on HEP-2 cell culture and Herpes simplex virus. J. AOAC 53:1229-1236.
6. Environmental Protection Agency Federal Register: August 25, 2000 (Volume 65, Number 166).
7. Statistical Analysis of Hepatitis B Carrier Test Data Revised (1-01) Template for Calculating the Log Reduction (LR) and Associated Standard Error (SE). M. Hamilton, Center for Biofilm Engineering, Montana State University, January 9, 2001, Published January 4, 2001.
8. Inactivation of feline Calicivirus, a Norwalk virus surrogate, Journal of Hospital Infection (1999) 41: 51-57.
9. Virucidal Efficacy of Four New Disinfectants, Journal of the American Animal Hospital Association, Vol. 38 No. 3, May/June 2002, Pages 231-234.
10. Efficacy of Commonly Used Disinfectants for the Inactivation of Calicivirus on Strawberry, Lettuce, and Food-Contact Surface, Journal of Food Protection, Vol. 64, No. 9, 2001, Pages 1430-1434.
11. Concentration and Detection of Caliciviruses from Food Contact Surfaces, Journal of Food Protection, June 2002; 65 (6).

STUDY RESULTS

Results of tests with one batch of SNiPER® (Batch # 106-114-1), ready to use, exposed to Feline Calicivirus in the presence of a 5% fetal bovine serum soil load at room temperature (20.0°C) for ten minutes are shown in Tables 1-2. The input titer (not dried) of the virus was 7.75 $\log_{10}$. The titer of the dried virus control was 6.75 $\log_{10}$ for Replicate #1 and 6.75 $\log_{10}$ for Replicate #2. The MPN for the two dried virus control replicates is 3849323 and 2184004, respectively. Following exposure, test virus infectivity was not detected in either replicate of the virus-test substance mixture at any dilution tested ($\leq$0.5 $\log_{10}$). Based on the cytotoxicity and neutralization control results, the MPN for both test replicates is $\leq$23.979. Test substance cytotoxicity was observed in the cytotoxicity control at 1.5 $\log_{10}$. The neutralization control (non-virucidal level of the test substance) indicates that the test substance was neutralized at $\leq$1.5 $\log_{10}$. Utilizing the statistical program provided by the EPA and based on the cytotoxicity and neutralization control results, the log reduction in viral titer is $\geq$5.08 and the standard error of the log reduction is 0.12.

STUDY CONCLUSION

Under the conditions of this investigation and in the presence of a 5% fetal bovine serum soil load, one batch of SNiPER® (Batch # 106-114-1), ready to use, demonstrated complete inactivation of Feline Calicivirus following a ten minute exposure time at room temperature (20.0°C) as required by the U.S. EPA for virucidal label claims.

In the opinion of the Study Director, there were no circumstances that may have adversely affected the quality or integrity of the data.

TABLE 1: Virus Controls and Test Substance Assay Results

Effects of SNiPER® (Batch # 106-114-1) Following a Ten Minute Exposure to Feline Calicivirus Dried on an Inanimate Surface

Dilution	Input Virus Control	Dried Virus Control		Feline Calicivirus + Batch # 106-114-1	
		Replicate #1	Replicate #2	Replicate #1	Replicate #2
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	NT	NT	NT	0 0 0 0	0 0 0 0
10^{-2}	NT	NT	NT	0 0 0 0	0 0 0 0
10^{-3}	NT	NT	NT	0 0 0 0	0 0 0 0
10^{-4}	+ + + +	+ + + +	+ + + +	0 0 0 0	0 0 0 0
10^{-5}	+ + + +	+ + + +	+ + + +	NT	NT
10^{-6}	+ + + +	+ + + +	+ + + 0	NT	NT
10^{-7}	+ + + +	0 + 0 0	0 0 + +	NT	NT
10^{-8}	0 + 0 0	0 0 0 0	0 0 0 0	NT	NT
$TCID_{50}$/0.1 mL	$10^{7.75}$	$10^{6.75}$	$10^{6.75}$	$\leq 10^{0.5}$	$\leq 10^{0.5}$
MPN	NA	3849323	2184004	≤23.979*	≤23.979*
Log_{10} MPN	NA	6.58538	6.33925	≤1.37983*	≤1.37983*
Log Reduction	NA	NA		≥5.08*	

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present
(NT) = Not tested
(NA) = Not applicable
(MPN) = Most probable number
(*) = Based on the cytotoxicity and neutralization control results

TABLE 2: Test Substance Cytotoxicity and Neutralization Control Results

Dilution	Cytotoxicity Control Batch # 106-114-1	Neutralization Control Batch # 106-114-1
Cell Control	0 0	0 0
10^{-1}	T T	T T
10^{-2}	0 0	+ +
10^{-3}	0 0	+ +
TCD_{50}/0.1 mL	$10^{1.5}$	See below

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present
(T) = Test substance cytotoxicity present

The results of the neutralization control indicate the test substance was neutralized at the dilution equivalent to ≤ 1.5 $\log_{10}$ $TCID_{50}$/0.1 mL as compared to the treated test samples.

(For Laboratory Use Only)
ATS Labs Project # A08200
On 9/2/09

ATS LABS

PROTOCOL

Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Utilizing Feline Calicivirus as a Surrogate Virus for Norovirus - Confirmatory Assay

PROTOCOL NUMBER

GRS01072909.FCAL

PREPARED FOR

Global Environmental Restoration, Inc.
108 Maple Ridge Drive
Lafayette, LA 70507

PERFORMING LABORATORY

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

PREPARED BY

Mary J. Miller, M.T.
Research Scientist II

DATE

July 29, 2009

EXACT COPY
INITIALS MM DATE 9-28-09

1285 Corporate Center Drive, Suite 110 • Eagan, MN 55121 • 877.287.8378 • 651.379.5510 • Fax: 651.379.5549

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Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Utilizing Feline Calicivirus as a Surrogate Virus for Norovirus – Confirmatory Assay

SPONSOR: Global Environmental Restoration, Inc.
108 Maple Ridge Drive
Lafayette, LA 70507

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

PURPOSE AND SCOPE

The purpose of this study is to evaluate the virucidal efficacy of a disinfectant against Feline Calicivirus, to be used as a surrogate virus for Norovirus, according to test criteria and methods approved by the U.S. Environmental Protection Agency for registration of a product as a virucide.

Norovirus, a member of the *Caliciviridae* family, is a non-enveloped RNA-containing virus and is an important cause of gastroenteritis in humans. Little is known about disinfectant efficacy against this virus due to the inability to propagate the virus in-vitro. Feline Calicivirus, also a member of the *Caliciviridae* family, serves as a valuable model virus for efficacy testing of Norovirus since these viruses share many similar characteristics and Feline Calicivirus can be propagated in cell cultures.

TEST SUBSTANCE CHARACTERIZATION

Test substance characterization as to content, stability, solubility, storage, etc., is the responsibility of the Sponsor. The test substance shall be characterized before the experimental start date of 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.

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 August 18, 2009. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of September 8, 2009. 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, because of 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 United States FDA or EPA of its submission 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.

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JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM

The U.S. Environmental Protection Agency requires that a specific virucidal claim for a disinfectant intended for use on hard surfaces be supported by appropriate scientific data demonstrating the efficacy of the disinfectant against the claimed virus. The agency will accept adequate data generated by any appropriate technique in support of a virucidal efficacy claim. This is accomplished by treating the target virus with the disinfectant (test substance) under conditions, which simulate as closely as possible, in the laboratory, the actual conditions under which the disinfectant is designed to be used. For disinfectant products intended for use on hard surfaces (dry, inanimate environmental surfaces), a carrier method is used in the generation of the supporting virological data. The Crandel Reese feline kidney cell line, which supports the growth of the Feline Calicivirus, will be used in this study. The experimental design in this protocol meets these requirements.

TEST PRINCIPLE

A film of virus, dried on a glass surface, is exposed to the test substance for a specified contact time. Following exposure, the virucidal and cytotoxic activities are removed from the virus-test substance mixture, and the mixture is assayed for viral infectivity by an accepted assay method. Appropriate virus, test substance cytotoxicity, and neutralization controls are run concurrently.

STUDY DESIGN

Four dried virus films are prepared in parallel and used as follows:

Two films: for one batch of test substance.
Two films for virus control titration (titer of virus after drying).

Following the specified exposure time, resuspended virus-test substance mixtures will be detoxified and made non-virucidal by immediately adding the contents to a Sephadex gel filtration column followed by 10-fold serial dilutions in test medium. Each dilution is assayed for viral infectivity by inoculation into Crandel Reese feline kidney cell cultures. The resuspended virus control films and the test substance alone (for toxicity determinations) will be treated in exactly the same manner. For analysis of infectivity, the cell cultures will be held for the appropriate incubation period and microscopically observed for the presence of the test virus or cytotoxicity. Uninfected indicator cell cultures will be maintained to serve as a negative control. In addition to the above titrations for infectivity and toxicity, the residual virucidal activity of the test substance following neutralization will be determined by adding a low titer of stock virus to each dilution of the detoxified test substance (toxicity control dilutions). The resulting mixtures of dilutions are assayed for infectivity in order to determine the dilution(s) of test substance at which virucidal activity, if any, is retained.

The following table outlines the specific parameters that will be tested:

SAMPLES TO BE TESTED FOR THE PRESENCE OF VIRUS			
Test or Control Group	**Dilutions Assayed Per Carrier (log_{10})**	**Cultures per Dilution**	**Total Cultures Inoculated**
Negative Controls	N/A	2-4	2-4/group
Input Virus Control (not dried)	-4,-5,-6,-7*	4	16
Dried Virus Control (performed in duplicate)	-4,-5,-6,-7*	4	32
Test Sample - Batch #1 (performed in duplicate)	-1,-2,-3,-4	4	32
Cytotoxicity Control - Batch #1	-1,-2,-3	2	6
Neutralization Control - Batch #1	-1,-2,-3	2	6

* The ending dilution assayed may change depending on the titer of the virus.

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VIRUS

The F-9 strain of Feline Calicivirus to be used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-782). Stock virus is prepared by collecting the supernatant culture fluid from 75-100% infected culture cells. The cytopathic effect can be described as small, rounding of the cells, with a slight granular look. The CPE starts to develop in 1-2 days following inoculation and advances to involve the entire cell sheet following an incubation period of approximately seven days. The cells are disrupted and cell debris removed by centrifugation. The supernatant is removed, aliquoted, and the high titer stock virus may be stored at ≤-70°C until the day of use. On the day of use an aliquot is removed, thawed and refrigerated until use in the assay. **Note:** The percent FBS contained in the stock virus aliquot will be adjusted to yield a minimum of a 5% organic soil load (if necessary). If the Sponsor chooses a soil load greater than 5%, the percent FBS contained in the stock virus aliquot will be adjusted to yield the percent soil load requested.

INDICATOR CELL CULTURES

Cultures of Crandel Reese feline kidney (CRFK) cells were originally obtained from the American Type Culture Collection, Manassas, VA (ATCC CCL-94). The cells are propagated by ATS Labs personnel. The cells are seeded into multiwell cell culture plates and maintained at 36-38°C in a humidified atmosphere of 5-7% CO_2. The confluency of the cells will be appropriate for the test virus. This cell line has historically been used as the cell line for propagation and detection of Feline Calicivirus. The cultures are commercially available, can be serially propagated, and are capable of showing cytopathic effect in the presence of the virus.

All cell culture documentation is retained for the cell cultures used in this assay with respect to source, passage number, growth characteristics, seeding densities and the general condition of the cells.

TEST MEDIUM

The test medium used for this assay is Minimum Essential Medium (MEM) supplemented with 1-10% (v/v) heat inactivated fetal bovine serum. The medium may also be supplemented with one or more of the following: 10 µg/mL gentamicin, 100 units/mL penicillin and 2.5 µg/mL amphotericin B.

PREPARATION OF THE TEST SUBSTANCE

The dilution of the test substance will be used as recommended by the Sponsor. The product will be pre-equilibrated to the desired test temperature if applicable. One batch of test substance will be assayed.

PREPARATION OF THE VIRUS FILMS

Films of virus will be prepared by spreading 0.2 mL of virus inoculum uniformly over the bottoms of four 100 X 15mm sterile glass petri dishes. The virus will be air-dried at 10°C-30°C until visibly dry (≥20 minutes). The drying conditions (temperature and humidity) will be appropriate for the test virus for the purpose of obtaining maximum survival following drying. The actual drying conditions will be clearly documented.

TEST METHOD

Sephadex Gel Filtration

To reduce the cytotoxic level of the virus-test substance mixture prior to assay of virus, and/or to reduce the virucidal level of the test substance, virus is separated from the test substance by filtration through Sephadex gel. Columns of Sephadex LH-20-100 are equilibrated with phosphate buffered saline containing 1% albumin, centrifuged for three minutes to clear the void volume, loaded with approximately 2.0 mL of virus-test substance mixture (or the amount recovered from the plate for spray products) and immediately passed through the column utilizing the syringe plunger.

Input Virus Control

On the day of test, the stock virus utilized in the assay will be titered by 10-fold serial dilution and assayed for infectivity to determine the starting titer of the virus. The results of this control are for informational purposes only.

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Treatment of Virus Films with the Test Substance
Two dried virus films are exposed to 2.0 mL of the use dilution of the test substance (liquid products), or to the amount of spray released under use conditions (spray products) for the specified exposure time and temperature. The actual temperature will be recorded. Following the exposure time, each plate is individually scraped with a cell scraper to resuspend the contents and the virus-test substance mixture is immediately passed through individual Sephadex columns utilizing the syringe plunger in order to detoxify the mixture. This dilution is considered the 10^{-1} dilution. A 0.2 mL aliquot of the test virus (the virus film) is resuspended in approximately 2.0 mL of test substance which equals a 1:10 dilution. The filtrates are then titered by serial dilution and assayed for infectivity and/or cytotoxicity. To further aid in removing the cytotoxic effects of the test substance to the indicator cell cultures, individual dilutions may be passed through individual Sephadex columns.

Treatment of Virus Control Films
Two virus films are prepared as described above. The virus control films are run in parallel to the test virus but 2.0 mL of test medium is added in lieu of the test substance. The control films are exposed to the test medium for the same exposure time and at the same exposure temperature as the test films are exposed to the test substance. The virus control films are scraped as previously described and the mixtures are immediately passed through individual Sephadex columns. The filtrates are then titered by serial dilution and assayed for infectivity. If additional Sephadex columns were used to further reduce the cytotoxic effects in the test substance assay, the same dilutions of the virus control that are assayed for infectivity will be passed through individual Sephadex columns.

Cytotoxicity Controls
A 2.0 mL aliquot of the test substance (liquid products) or the amount of the test substance recovered when sprayed onto a sterile petri dish (spray products) is filtered through individual Sephadex columns and the filtrates are diluted serially in medium and inoculated into cell cultures for assay of cytotoxicity concurrently with the virus control and test substance-treated virus samples. If additional Sephadex columns were used to further reduce the cytotoxic effects in the test substance assay, the same dilutions of the cytotoxicity control will be passed through individual Sephadex columns.

Neutralization Control
Serial dilutions of the diluted neutralized test substance (cytotoxicity control dilutions) will be mixed with low titer stock virus and the resulting mixtures of dilutions will be assayed for infectivity and/or cytotoxicity in order to determine the dilution(s) of the test substance at which virucidal activity, if any, is retained. Dilutions that show virucidal activity will not be considered in determining reduction in infectivity by the test substance.

Infectivity Assays
The CRFK cell line, which exhibits cytopathic effect (CPE) in the presence of Feline Calicivirus, will be used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes will be inoculated in quadruplicate with 0.1 mL of the dilutions prepared from test and virus control groups. The cytotoxicity and neutralization control dilutions will be inoculated in duplicate. Uninfected indicator cell cultures (cell controls) will be inoculated with test medium alone. Cultures are incubated at 31-35°C in a humidified atmosphere of 5-7% CO_2 in sterile disposable cell culture labware. The cultures will be microscopically scored periodically for approximately seven days for the absence or presence of CPE, cytotoxicity and for viability.

CALCULATION OF TITERS

Viral and cytotoxicity titers will be expressed as $-\log_{10}$ of the 50 percent titration endpoint for infectivity ($TCID_{50}$) or cytotoxicity (TCD_{50}), respectively, as calculated by the method of Spearman Karber.

$$-\text{Log of 1st dilution inoculated} - \left[\left(\left(\frac{\text{Sum of \% mortality at each dilution}}{100}\right) - 0.5\right) \times (\text{logarithm of dilution})\right]$$

Statistical Methods
The $\log_{10}$ reduction in infectivity will be calculated using the revised EPA approved method for calculating the Most Probable Number (MPN) as obtained from the EPA on January 4, 2001.

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PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM
The specialized virucidal testing section of ATS Labs maintains Standard Operating Procedures (SOPs) relative to virucidal efficacy testing studies. Virucidal 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 virus and cell stocks for purposes of identification, receipt and use of chemical reagents including cell culture medium components, etc. These procedures are designed to document each step of virucidal 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 virucidal efficacy test is assigned a unique Project Number when the Study Director initiates the protocol for the study. This number is used for identification of the test culture plates, etc. during the course of the test. Test culture plates are also labeled with reference to the test virus, experimental start date, and test product. These measures are designed to document the identity of the test system.

METHOD FOR CONTROL OF BIAS: N/A

TEST CRITERIA
A valid test requires 1) that at least 4 $\log_{10}$ of infectivity be recovered from the dried virus control films; 2) that when cytotoxicity is evident, at least a 3-log reduction in titer is demonstrated beyond the cytotoxic level; 3) that the cell controls be negative for infectivity. If any of the previous requirements are not met, the test may be repeated under the current protocol number. **Note:** An efficacious product must demonstrate complete inactivation of the virus at all dilutions.

FINAL REPORT
The report will include, but not be limited to, identification of the sample and date received, dates on which the test was initiated and completed, identification of the virus strain used and composition of the inoculum, description of cells, medium and reagents, description of the methods employed, tabulated results, calculated titers for infectivity and cytotoxicity, and a conclusion as it relates to the purpose of the test.

PROTOCOL CHANGES
If it becomes necessary to make changes in the approved protocol, the revision and reasons for change 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.

PRODUCT DISPOSITION
Test substance retention shall be the responsibility of the Sponsor. Unused test material will be **discarded** following study completion unless otherwise requested.

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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 the 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. Annual Book of ASTM Standards 2000, Section 11 Water and Environmental Technology Volume 11.05 Biological Effects and Environmental Fate: Biotechnology; Pesticides, E1053-97.
2. U.S. Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision G: Product Performance, 91-2(f), November 1982.
3. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, DIS/TSS-7, November 12, 1981.
4. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Schmidt, N.J. and Emmons, R.W. editors. Sixth edition, 1989. p. 18-20.
5. Blackwell, J.H., and J.H.S. Chen. 1970. Effects of various germicidal chemicals on HEP-2 cell culture and Herpes simplex virus. J. AOAC 53:1229-1236.
6. Environmental Protection Agency Federal Register: August 25, 2000 (Volume 65, Number 166).
7. Statistical Analysis of Hepatitis B Carrier Test Data Revised (1-01) Template for Calculating the Log Reduction (LR) and Associated Standard Error (SE). M. Hamilton, Center for Biofilm Engineering, Montana State University, January 9, 2001, Published January 4, 2001.
8. Inactivation of feline Calicivirus, a Norwalk virus surrogate, Journal of Hospital Infection (1999) 41: 51-57.
9. Virucidal Efficacy of Four New Disinfectants, Journal of the American Animal Hospital Association, Vol. 38 No. 3, May/June 2002, Pages 231-234.
10. Efficacy of Commonly Used Disinfectants for the Inactivation of Calicivirus on Strawberry, Lettuce, and Food-Contact Surface, Journal of Food Protection, Vol. 64, No. 9, 2001, Pages 1430-1434.
11. Concentration and Detection of Caliciviruses from Food Contact Surfaces, Journal of Food Protection, June 2002; 65 (6).

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STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Sponsor (Date/Initial): AC 8/13/09

Test Substance (Name and Batch Number - exactly as it should appear on final report):
SNiPER® ~~#1: 106-114-1~~ *mm 9-1-09, ~~#2: 106-114-2~~ *mm 9-1-09

Expiration Date: 5/2011

Product Description:

- ☐ Quaternary ammonia
- ☐ Iodophor
- ☐ Sodium hypochlorite
- ☐ Peracetic acid
- ☐ Peroxide
- ■ Other Chlorine Dioxide 2000 ppm

Test Substance Active Concentration (upon submission to ATS Labs): 2000 ppm

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)
- ☐ *Dilutions/Concentrations to be tested _____
  - ☐ Deionized Water (Filter Sterilized)
  - ☐ Tap Water (Filter Sterilized)
  - ☐ AOAC Synthetic Hard Water: _____PPM
  - ☐ Other_____

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

Test Virus: Feline Calicivirus as a surrogate virus for Norovirus

Exposure Time: 10 Min

Exposure Temperature: ☑ Room temperature (18-22°C)
☐ Other:_____°C (please specify range)

Directions for application of aerosol/spray products (if applicable): ~~Shake bottle + spray~~ *mm 9-1-09

☑ Check here if spray instructions are not applicable. *mm 9-1-09

Organic Soil Load

- ☑ 5% fetal bovine serum (minimum amount accepted by the EPA)
- ☐ Other _____

* Per 9-1-09 email, only 1 batch of test substance to be tested. Batch# 106-114-1 will be tested. It will be applied as a liquid, not as a spray. The test substance bottle will be shaken prior to application. mm 9-1-09

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:_____
☑ Sender (if other than Sponsor): Shore Chemical, 2917 Spruce Way Pittsburg, PA 15201

COMPLIANCE

This study will be conducted in compliance with the EPA Good Laboratory Practices Regulations of 40 CFR Part 160 (Federal Register Notice [August 17, 1989]) and in accordance to standard operating procedures.
☑ Yes
☐ No (Non-GLP Study)

PROTOCOL MODIFICATIONS

☑ Approved without modification
☐ Approved with modification - Supplemental Information Form Attached - ☐ Yes ☐ No

APPROVAL SIGNATURES

SPONSOR:

NAME: Alan Campbell TITLE: General Mgr

SIGNATURE: Alan Campbell DATE: 8/13/09

PHONE: 337-235-4710;337-319-4983 (cell) FAX:337-235-7153 EMAIL:alanbud@environmentrestoration.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: Mary J. Miller
Study Director

SIGNATURE: Mary J. Miller DATE: 9-1-09
Study Director