ATS LABS

FINAL STUDY REPORT

STUDY TITLE

Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Virus: Human Coronavirus

PRODUCT IDENTITY

TMXPAE
Lot # TMXPAE-101117-1 and Lot # TMXPAE-101117-2

DATA REQUIREMENTS

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

AUTHOR

Shanen Conway, B.S.
Study Director

STUDY COMPLETION DATE

March 4, 2011

PERFORMING LABORATORY

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

PROJECT NUMBER

A10859

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).

Signature

Date: 2011-03-08

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 studies 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).

Study Director: Shanen K. Conway
Shanen Conway, B.S.

Date: 3/4/11

QUALITY ASSURANCE UNIT SUMMARY

Study: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of non-clinical laboratory studies. These studies have 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. Studies are inspected at time intervals to assure the integrity of the study.

Phase Inspected	Date of Phase Inspection	Date Reported to Study Director	Date Reported to Management
Critical Phase Audit	February 2, 2011	February 2, 2011	February 2, 2011
Draft Report	February 15, 2011	February 16, 2011	February 16, 2011
Final Report	March 3, 2011	March 3, 2011	March 4, 2011

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

Quality Assurance Auditor: Joy Saarela Date: 3/4/11

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STUDY PERSONNEL

STUDY DIRECTOR: Shanen Conway, B.S.

Professional Personnel Involved:

Kelleen Gutzmann, M.S.	- Manager, Virology Operations
Matthew Cantin, B.S.	- Research Assistant II
Katherine A. Paulson, M.L.T.	- Research Assistant II
Andrea Molina, B.S.	- Research Assistant I

STUDY REPORT

GENERAL STUDY INFORMATION

Study Title: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Project Number: A10859

Protocol Number: GRP01102910.COR

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

TEST SUBSTANCE IDENTITY

Test Substance Name: TMXPAE

Lot/Batch(s): Lot # TMXPAE-101117-1 and Lot # TMXPAE-101117-2

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. The Sponsor Test Material Characterization Reports may be found in Attachments I and II.

STUDY DATES

Date Sample Received: December 9, 2010
Study Initiation Date: January 25, 2011
Experimental Start Date: February 2, 2011
Experimental End Date: February 11, 2011
Study Completion Date: March 4, 2011

OBJECTIVE

The objective of this study was to evaluate the virucidal efficacy of a test substance against Human Coronavirus according to test criteria and methods approved by the U.S. Environmental Protection Agency for registration of a product as a virucide.

SUMMARY OF RESULTS

Test Substance:	TMXPAE, Lot # TMXPAE-101117-1 and Lot # TMXPAE-101117-2
Dilution:	Ready to use (RTU), trigger spray
Virus:	Human Coronavirus, ATCC VR-740, Strain 229E
Exposure Time:	1 minute
Exposure Temperature:	Room temperature (20.0°C)
Organic Soil Load:	5% fetal bovine serum
Efficacy Result:	Two lots of TMXPAE met the test criteria specified in the study protocol. The results indicate **complete inactivation** of Human Coronavirus under these test conditions as required by the U.S. EPA for claims of virucidal activity.

TEST SYSTEM

1. Virus
The 229E strain of Human Coronavirus used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-740). 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, an aliquot of stock virus (ATS Labs Lot HCV-63) was removed, thawed and maintained at a refrigerated temperature until used in the assay. The stock virus culture was adjusted to contain 5% fetal bovine serum as the organic soil load. The stock virus tested demonstrated cytopathic effects (CPE) typical of Human Coronavirus on WI-38 (human lung) cells.

2. Indicator Cell Cultures
Cultures of WI-38 (human lung) cells were originally obtained from the American Type Culture Collection, Manassas, VA (ATCC CCL-75). 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. On the day of testing, the cells were observed as having proper cell integrity and confluency, and therefore, were acceptable for use in this study.

   All cell culture documentation was retained for the cell cultures used in the 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 in this study was Minimum Essential Medium (MEM) supplemented with 2% (v/v) heat-inactivated fetal bovine serum (FBS), 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 the report text for a more detailed explanation.

PARAMETERS TESTED FOR VIRUCIDAL EFFICACY ASSAY			
Test or Control Group	**Dilutions Assayed (log_{10})**	**Cultures per dilution**	**Total Cultures**
Cell Control	N/A	2-4	2-4/group
Input Virus Control	-1,-2,-3,-4,-5,-6,-7	2	14
Dried Virus Control (Group A)	-1,-2,-3,-4,-5,-6	4	24
Sample lot #1 + virus (Group B)	-1,-2,-3,-4,-5,-6	4	24
Sample lot #2 + virus (Group B)	-1,-2,-3,-4,-5,-6	4	24
Cytotoxicity of lot #1 (Group C)	-1,-2,-3,-4,-5,-6	4	24
Cytotoxicity of lot #2 (Group C)	-1,-2,-3,-4,-5,-6	4	24
Non-Virucidal level - lot #1 (Group D)	-1,-2,-3,-4,-5,-6	4	24
Non-Virucidal level - lot #2 (Group D)	-1,-2,-3,-4,-5,-6	4	24

TEST METHOD

1. Preparation of Test Substance
Two lots of TMXPAE (Lot # TMXPAE-101117-1 and Lot # TMXPAE-101117-2), ready to use trigger spray, were used as received from the Sponsor. The test substance was at room temperature (20.0°C) prior to use. The test substance was applied according to the use directions provided by the Sponsor. (See report section on the Treatment of Virus Films with the Test Substance).

2. Preparation of Virus Films
Films of virus were prepared by spreading 0.2 mL of virus inoculum uniformly over the bottoms of three separate 100 x 15 mm 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. Preparation of Sephadex Gel Filtration Columns
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 the test substance by filtration through Sephadex gel. The Sephadex LH-20 gel was prepared by equilibration with phosphate buffered saline containing 1% albumin. On the day of testing, Sephadex columns were prepared by centrifuging the prepared Sephadex gel in sterile syringes for three minutes to clear the void volume. The columns were then ready to be used in the assay.

4. Input Virus Control (TABLE 1)
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 the Test Substance (GROUP B, TABLE 1)
For each lot of test substance, one dried virus film was individually exposed for 1 minute at room temperature (20.0°C) to the amount of spray released under use conditions. The carriers were sprayed until thoroughly wet (3 sprays at a distance of 6 to 8 inches) and held covered for the remainder of the exposure time. The virus films were completely covered with the test substance. 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 plungers in order to detoxify the mixtures. The filtrates (10^{-1} dilution) were then titered by 10-fold serial dilution and assayed for infectivity and/or cytotoxicity.

6. Treatment of Dried Virus Control Film (GROUP A, TABLE 1)
A virus film was prepared as previously described (paragraph 2). The virus control film was exposed to 2.00 mL of test medium in lieu of the test substance and held covered for 1 minute at room temperature (20.0°C). Following the exposure time, the virus control was scraped with a cell scraper and the virus mixture was immediately passed through a Sephadex column in the same manner as the test virus (paragraph 5). The filtrate (10^{-1} dilution) was then titered by 10-fold serial dilution and assayed for infectivity.

7. Cytotoxicity Controls (GROUP C, TABLE 2)
Each lot of the test substance was sprayed as previously described onto separate sterile petri dishes and held covered for the 1 minute exposure time at room temperature (20.0°C). Following exposure, the plates were individually scraped with a cell scraper, the contents were transferred to a Sephadex column and immediately passed through the 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 WI-38 (human lung) 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)
(GROUP D, TABLE 3)
Each dilution of the neutralized test substance (cytotoxicity control dilutions) was challenged with an aliquot of low titer stock virus 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 reduction of the virus by the test substance.

Using the cytotoxicity control dilutions prepared above, an additional set of indicator cell cultures was inoculated with a 0.1 mL aliquot of each dilution in quadruplicate. A 0.1 mL aliquot of low titer stock virus was inoculated into each cell culture well and the indicator cell cultures were incubated along with the test and virus control plates.

9. Infectivity Assays
The WI-38 cell line, which exhibits cytopathic effect (CPE) in the presence of Human Coronavirus, 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 control groups. The input virus control was inoculated in duplicate. Uninfected indicator cell cultures (cell controls) were inoculated with test medium alone. The 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 scored periodically for nine days for the absence or presence of CPE, cytotoxicity, and for viability.

10. Statistical Methods: Not applicable

PROTOCOL CHANGES

Protocol Amendments:
No protocol amendments were required for this study.

Protocol Deviations:
No protocol deviations occurred during this study.

CALCULATION OF TITERS

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.

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

Calculation of Log Reduction

Dried Virus Control $TCID_{50}$ – Test Substance $TCID_{50}$ = Log Reduction

TEST CRITERIA

A valid test requires 1) that at least 4 $\log_{10}$ of infectivity be recovered from the dried virus control film; 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. **Note:** An efficacious product must demonstrate complete inactivation of the virus at all dilutions.

RECORD RETENTION

Study Specific Documents
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 the final study report.
7. Study-specific SOP deviations made during the study.

Test Substance Retention
The test substance will be discarded following study completion per Sponsor approved protocol. It is the responsibility of the Sponsor to retain a sample of the test substance.

REFERENCES

1. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E 1053-97 (Reapproved 2002).
2. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E 1482-04.
3. U.S. Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision G: Product Performance, 91-2(f), November, 1982.
4. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, DIS/TSS-7, November 12, 1981.
5. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette, E.H., Lennette, D.A., and Lennette, E.T., editors. Seventh edition, 1995.
6. 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.

STUDY RESULTS

Results of tests with two lots of TMXPAE (Lot # TMXPAE-101117-1 and Lot # TMXPAE-101117-2), ready to use trigger spray, exposed to Human Coronavirus in the presence of a 5% fetal bovine serum organic soil load at room temperature (20.0°C) for 1 minute are shown in Tables 1-3. All cell controls were negative for test virus infectivity. The titer of the input virus control was 5.5 $\log_{10}$. The titer of the dried virus control was 4.75 $\log_{10}$. Following exposure, test virus infectivity was not detected in the virus-test substance mixture for either lot at any dilution tested ($\leq$1.5 $\log_{10}$). Test substance cytotoxicity was observed in both lots 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 $\log_{10}$ for both lots. Taking the cytotoxicity and neutralization control results into consideration, the reduction in viral titer was $\geq$3.25 $\log_{10}$ for both lots.

STUDY CONCLUSION

Under the conditions of this investigation and in the presence of a 5% fetal bovine serum organic soil load, TMXPAE (Lot # TMXPAE-101117-1 and Lot # TMXPAE-101117-2), ready to use trigger spray, demonstrated complete inactivation of Human Coronavirus following a 1 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 Results

Effects of TMXPAE (Lot # TMXPAE-101117-1 and Lot # TMXPAE-101117-2) Following a 1 Minute Exposure to Human Coronavirus Dried on an Inanimate Surface

Dilution	Input Virus Control	Dried Virus Control (GROUP A)	Human Coronavirus + Lot # TMXPAE-101117-1 (GROUP B)	Human Coronavirus + Lot # TMXPAE-101117-2 (GROUP B)
Cell Control	0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	+ +	+ + + +	T T T T	T T T T
10^{-2}	+ +	+ + + +	0 0 0 0	0 0 0 0
10^{-3}	+ +	+ + + +	0 0 0 0	0 0 0 0
10^{-4}	+ +	+ + + +	0 0 0 0	0 0 0 0
10^{-5}	+ +	0 0 0 0	0 0 0 0	0 0 0 0
10^{-6}	0 0	+ 0 0 0	0 0 0 0	0 0 0 0
10^{-7}	0 0	NT	NT	NT
$TCID_{50}$/0.1 mL	$10^{5.5}$	$10^{4.75}$	$\leq 10^{1.5}$	$\leq 10^{1.5}$

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present
(T) = Cytotoxicity present
(NT) = Not tested

TABLE 2: Cytotoxicity Control Results

Cytotoxicity of TMXPAE on WI-38 (Human Lung) Cell Cultures

Dilution	Cytotoxicity Control Lot # TMXPAE-101117-1 (GROUP C)	Cytotoxicity Control Lot # TMXPAE-101117-2 (GROUP C)
Cell Control	0 0 0 0	0 0 0 0
10^{-1}	T T T T	T T T T
10^{-2}	0 0 0 0	0 0 0 0
10^{-3}	0 0 0 0	0 0 0 0
10^{-4}	0 0 0 0	0 0 0 0
10^{-5}	0 0 0 0	0 0 0 0
10^{-6}	0 0 0 0	0 0 0 0
TCD_{50}/0.1 mL	$10^{1.5}$	$10^{1.5}$

(0) = No test virus recovered and/or no cytotoxicity present
(T) = Cytotoxicity present

TABLE 3: Neutralization Control Results

Non-Virucidal Level of the Test Substance (Neutralization Control)

Dilution	Test Virus + Cytotoxicity Control Lot # TMXPAE-101117-1 (GROUP D)	Test Virus + Cytotoxicity Control Lot # TMXPAE-101117-2 (GROUP D)
Cell Control	0 0 0 0	0 0 0 0
10^{-1}	T T T T	T T T T
10^{-2}	+ + + +	+ + + +
10^{-3}	+ + + +	+ + + +
10^{-4}	+ + + +	+ + + +
10^{-5}	+ + + +	+ + + +
10^{-6}	+ + + +	+ + + +

(+) = Positive for the presence of test virus after low titer stock virus added (neutralization control)
(0) = No test virus recovered and/or no cytotoxicity present
(T) = Cytotoxicity present

Results of the non-virucidal level control indicate that the test substance was neutralized at a $TCID_{50}$/0.1 mL of ≤1.5 log_{10} for both lots.

Attachment I: Sponsor Test Material Characterization - TMXPAE, Lot # TMXPAE-101117-1

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APPENDIX B: CERTIFICATE OF ANALYSIS

Product Safety Labs

CERTIFICATE OF ANALYSIS

Product: TMXPAE

Lot No.: TMXPAE-101117-1

PSL Reference No.: 101201-14D

Date of Analysis: Dec. 9, 2010

	Result:
Thymol	0.203%

This product was analyzed in compliance with Good Laboratory Practice standards. Data are reported in EPSL GLP Study No. 31310.

Approval: Catherine Wo Dec. 13, 2010
Catherine Wo, Ph.D. — Date
Analytical Services
Product Safety Labs

QA Release: Rhonda S. Krick Dec 20, 2010
Rhonda Krick, B.S. — Date
Quality Assurance
Product Safety Labs

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Attachment II: Sponsor Test Material Characterization - TMXPAE, Lot # TMXPAE-101117-2

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APPENDIX B: CERTIFICATE OF ANALYSIS

Product Safety Labs

CERTIFICATE OF ANALYSIS

Product: TMXPAE

Lot No.: TMXPAE-101117-2

PSL Reference No.: 101201-15D

Date of Analysis: Dec. 9, 2010

	Result:
Thymol	0.205%

This product was analyzed in compliance with Good Laboratory Practice standards. Data are reported in EPSL GLP Study No. 31311.

Approval: Catherine Wu — Dec. 13, 2010
Catherine Wu, Ph.D.
Analytical Services
Product Safety Labs
Date

QA Release: Rhonda S. Krick — Dec 20, 2010
Rhonda Krick, B.S.
Quality Assurance
Product Safety Labs
Date

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(For Laboratory Use Only)
ATS Labs Project # A10859

LC 3/24/11

ATS LABS

PROTOCOL

Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Virus: Human Coronavirus

PROTOCOL NUMBER

GRP01102910.COR

PERFORMING LABORATORY

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

PREPARED BY

Shanen Conway, B.S.
Research Scientist I

DATE

October 29, 2010

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Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

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

PURPOSE
The purpose of this study is to evaluate the virucidal efficacy of a test substance against Human Coronavirus according to test criteria and methods approved by the U.S. Environmental Protection Agency for registration of a product as a virucide.

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. 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 November 11, 2010. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of Decmeber 22, 2010. 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.

JUSTIFICATION FOR SELECTION OF THE TEST SYSTEM
The U.S. Environmental Protection Agency (EPA) 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 test substance 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 WI-38 cell line, which supports the growth of the Human Coronavirus, 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 exposure 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
The appropriate number of dried virus films will be prepared in parallel and used as follows:

One film for each batch of test substance assayed per exposure time requested.

One film for virus control titration (titer of virus after drying) per exposure time requested.

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 inoculated into indicator cell cultures. The resuspended virus control film and each batch of test substance alone will be treated in exactly the same manner. For analysis of infectivity, cultures will be held for the appropriate incubation period at the end of which time cultures will be scored for the presence of the test virus. Cultures will be monitored at that time for cell viability. Uninfected indicator cell cultures will be carried in parallel and similarly monitored. For analysis of cytotoxicity, the viability of cultures inoculated with dilutions of each test and control will be determined. In addition to the above titrations for infectivity and cytotoxicity, the residual virucidal activity of the test substance after neutralization will be determined by adding a low titer of stock virus to each dilution of the test substance (cytotoxicity 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.

VIRUS
The 229E strain of Human Coronavirus to be used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-740). Stock virus is prepared by collecting the supernatant culture fluid from 75-100% infected culture cells. 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 maintained at a refrigerated temperature until used in the assay. **Note:** If the Sponsor requests an organic soil load challenge, fetal bovine serum (FBS) or the requested organic soil will be incorporated into the stock virus aliquot. The stock virus aliquot will be adjusted to yield the percent organic soil load requested.

INDICATOR CELL CULTURES
Cultures of WI-38 (human lung) cells were originally obtained from the American Type Culture Collection, Manassas, VA (ATCC CCL-75). 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. WI-38 cells obtained from an alternate, reputable source may be used. The source of the cells will be specified in the final report.

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. The composition of the test medium may be altered based on the virus and/or cells. The composition of the medium will be specified in the final report.

PREPARATION OF TEST SUBSTANCE
The dilution of test substance(s) will be used as recommended by the Sponsor. The product will be pre-equilibrated to the desired test temperature if applicable. Two batches of test substance must be assayed for registration of a test substance as a virucide with the EPA.

PREPARATION OF VIRUS FILMS

Films of virus will be prepared by spreading 0.2 mL of virus inoculum uniformly over the bottom of the appropriate number of 100 X 15 mm 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

Preparation of Sephadex Gel Filtration Columns

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. The Sephadex gel is prepared by equilibration with phosphate buffered saline containing 1% albumin. The type of Sephadex used will be specified in the final report. On the day of testing, Sephadex columns are prepared by centrifuging the prepared Sephadex gel in sterile syringes for three minutes to clear the void volume. The columns are now ready to be used in the assay.

Input Virus Control

On the day of testing, 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.

Treatment of Virus Films with the Test Substance

A dried virus film is 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) and held covered for the specified exposure time(s) and temperature. The actual temperature will be recorded. Following the exposure time, the plate is scraped with a cell scraper to resuspend the contents of the plate and the virus-test substance mixture is immediately passed through a Sephadex column utilizing the syringe plunger in order to detoxify the mixture. The filtrate (10^{-1} dilution) is then titered by serial dilution and assayed for infectivity and/or cytotoxicity. To further aid in the removing of the cytotoxic effects of the test substance to the indicator cell cultures, individual dilutions may be passed through a second individual Sephadex column.

Treatment of Dried Virus Control Film

A virus film is prepared as described above for each exposure time assayed. The virus control film is run in parallel to the test virus but 2.0 mL of test medium is added in lieu of the test substance. The virus control is held covered for the same exposure time and at the same exposure temperature as the test substance. Following the exposure time, the virus film is scraped as previously described and the mixture is immediately passed through a Sephadex column utilizing the syringe plunger. The filtrate (10^{-1} dilution) is 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 will be passed through individual Sephadex columns.

Cytotoxicity Controls

A 2.0 mL aliquot of test substance (liquid products) or the amount of the test substance recovered when sprayed onto a sterile petri dish (spray products), is filtered through a Sephadex column utilizing the syringe plunger and the filtrate is diluted serially in medium and inoculated into cell cultures for assay of cytotoxicity concurrently with the virus control and test substance-treated virus samples. For spray products, the cytotoxicity control will be held covered for the longest requested exposure time at the requested exposure temperature. 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.

Assay of Non-Virucidal Level of Test Substance (Neutralization Control)
Each dilution of the neutralized test substance (cytotoxicity control dilutions) will be challenged with an aliquot of low titer stock virus to determine the dilution(s) of test substance at which virucidal activity, if any, is retained. Dilutions that show virucidal activity will not be considered in determining reduction of the virus by the test substance.

Using the cytotoxicity control dilutions prepared above, an additional set of indicator cell cultures will be inoculated with a 0.1 mL aliquot of each dilution in quadruplicate. A 0.1 mL aliquot of low titer stock virus will be inoculated into each cell culture well and the indicator cell cultures will be incubated along with the test and virus control plates.

Infectivity Assays
The WI-38 cell line, which exhibits cytopathic effect (CPE) in the presence of Human Coronavirus, 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 control groups. The input virus control 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 scored periodically for approximately ten 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.

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

Calculation of Log Reduction
Dried Virus Control $TCID_{50}$ – Test Substance $TCID_{50}$ = Log Reduction

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 film; 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.

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.

PROPOSED STATISTICAL METHODS: N/A

REFERENCES

1. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E1053-97 (Reapproved 2002).
2. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E 1482-04.
3. U.S. Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision G: Product Performance, 91-2(f), November 1982.
4. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, DIS/TSS-7, November 12, 1981.
5. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette, E.H., Lennette, D.A. and Lennette, E.T. editors. Seventh edition, 1995.
6. 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.

STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Sponsor (Date/Initial): 2011-01-13/UDD

Test Substance (Name and Batch Number - exactly as it should appear on final report):

~~TMXPAE Lot #TMXPAE-1071117-1 & Lot # TMXPAE-1071117-2~~ TMXPAE-101117-1 & Lot # TMXPAE-101117-2 UDD 2011-01-13

Expiration Date: NA

Product Description

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

Test Substance Active Concentration (upon submission to ATS Labs): ~0,21%

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:

_____ defined as _____ + _____
(example: 1 oz/gallon) (amount of test substance) (amount of diluent)

- ☐ Deionized Water (Filter or Autoclave Sterilized)
- ☐ Tap Water (Filter or Autoclave Sterilized)
- ☐ AOAC Synthetic Hard Water: _____PPM
- ☐ Other _____

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

Test Virus: Human Coronavirus

Exposure Time: 1 minute

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

Directions for application of aerosol/spray products: Spray until thoroughly wet
☐ Check here if spray instructions are not applicable.

Organic Soil Load

- ☐ 1% fetal bovine serum (minimum level that can be tested)
- ☑ 5% fetal bovine serum
- ☐ 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:_____

☐ Sender (if other than Sponsor):_____

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
1. draft report verbiage as on others

2.ATS-labs supplied spray bottles will be used

APPROVAL SIGNATURES

ATS Labs:

NAME: Sharen Conway
Study Director

SIGNATURE: Sharen J. Conway
Study Director

DATE: 1/25/11