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ATS LABS

FINAL STUDY REPORT

STUDY TITLE

Test for Antiviral Activity and Efficacy – Modification of JIS Z 2801

Virus: Coxsackievirus type A16

PRODUCT IDENTITY

VirusGuard and VV-0 (control)

AUTHOR

Mary J. Miller, M.T.
Study Director

STUDY COMPLETION DATE

October 30, 2013

PERFORMING LABORATORY

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

SPONSOR

Nippon Paint (Singapore) Co. Pte Ltd
1 First Lok Yang Road
Jurong, Singapore 629728

PROJECT NUMBER

A15681

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).

Submitter:_____ Date:_____

Sponsor:_____ Date:_____

Study Director: Mary J. Miller Date: 10-30-13
Mary J. Miller, M.T.

QUALITY ASSURANCE UNIT SUMMARY

Study: Test for Antiviral Activity and Efficacy – Modification of JIS Z 2801

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of nonclinical 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	October 11, 2013	October 11, 2013	October 11, 2013
Final Report	October 29, 2013	October 29, 2013	October 30, 2013

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

Quality Assurance Auditor: Judy Heidemann Date: 10-30-13

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STUDY PERSONNEL

STUDY DIRECTOR:	Mary J. Miller, M.T.
Professional Personnel Involved:	
Kelleen Gutzmann, M.S.	- Director, Virology & Microbial ID Operations
Shanen Conway, B.S.	- Senior Virologist
Katherine A. Paulson, M.L.T.	- Senior Virologist
Matthew Cantin, B.S.	- Lead Virologist
Erica Flinn, B.A.	- Associate Virologist

STUDY REPORT

GENERAL STUDY INFORMATION

Study Title: Test for Antiviral Activity and Efficacy – Modification of JIS Z 2801

Project Number: A15681

Protocol Number: NPC01092513.COX

Sponsor: Nippon Paint (Singapore) Co. Pte Ltd
1 First Lok Yang Road
Jurong, Singapore 629728

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

TEST SUBSTANCE IDENTITY

Test Substance: VirusGuard

Control Substance: VV-0

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

STUDY DATES

Date Sample Received: October 2, 2013

Study Initiation Date: September 30, 2013

Experimental Start Date: October 10, 2013

Experimental End Date: October 18, 2013

Study Completion Date: October 30, 2013

OBJECTIVE

The objective of this study was to determine the antiviral efficacy of the Sponsor's product as compared to an untreated control following modifications of the JIS Z 2801 method.

SUMMARY OF RESULTS

Test Substance:	VirusGuard
Control Substance:	VV-0
Product Preparation:	Ready to use (RTU)
Virus:	Coxsackievirus type A16, Strain G10, ATCC VR-174
Exposure Time:	24 hours
Exposure Temperature:	Room temperature (20.0°C) in a relative humidity of 50%
Organic Soil Load:	5% fetal bovine serum
Efficacy Result:	Under these test conditions, VirusGuard demonstrated a 99.3% mean reduction in viral titer following a 24 hour exposure time, as compared to the mean titer of the zero time virus control. The mean log reduction in viral titer was 2.13 $\log_{10}$, as compared to the mean titer of the zero time virus control. A 92.6% mean reduction in viral titer was demonstrated following a 24 hour exposure time, as compared to the mean titer of the 24 hour virus control. The mean log reduction in viral titer was 1.13 $\log_{10}$, as compared to the mean titer of the 24 hour virus control.

TEST SYSTEM

1. Virus

The G10 strain of Coxsackievirus type A16 used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-174). 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, one aliquot of stock virus (ATS Labs Lot CX16-66) 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 Coxsackievirus on LLC-MK_2 cells.

2. Indicator Cell Cultures

Cultures of LLC-MK_2 (Rhesus monkey kidney) cells were obtained from the American Type Culture Collection, Manassas, VA (ATCC CCL-7.1). 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 this assay with respect to source, passage number, growth characteristics, seeding densities and the general condition of the cells.

3. Test Medium

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.

TEST METHOD

1. Test and Control Material (Carrier) Preparation

The Sponsor provided the VirusGuard test material, dry paint film coated on a glass panel, pre-cut to approximately 50 mm x 50 mm (2 inch x 2 inch). A control material (VV-0) cut to the same size was supplied as well. Prior to use in testing, the test and control materials were wiped slightly with ethanol and allowed to dry prior to use in testing. The test and control materials were equilibrated to the exposure temperature prior to use.

2. Carrier Film Preparation

A carrier film was prepared to fit over the test and control materials. The film was approximately 40 mm x 40 mm and was prepared from a sterile stomacher bag. A separate carrier film was prepared for each test and control carrier.

3. Input Virus Control (TABLE 1)

On the initial day of testing (October 10, 2013), 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.

The stock virus utilized in the assay following the 24 hour exposure time on October 11, 2013, was titered by 10-fold serial dilution and assayed for infectivity to determine the starting titer of the virus. This virus was used for the non-virucidal level control (neutralization control). The results of this control are for informational purposes only.

4. Inoculation and Recovery of the Test and Control Materials (TABLES 1-2)
Two test carriers and two control carriers, contained in individual sterile petri dishes, were inoculated with a 100 µL aliquot of the test virus. The inoculum was covered with the carrier film and the film was pressed down so the test virus spread over the film, but did not spread past the edge of the film. The exposure time began when each sample was inoculated. The samples were transferred to a controlled chamber set to room temperature (20.0°C) in a relative humidity of 50% for the duration of the Sponsor specified 24 hour exposure time.

Following the 24 hour exposure time, using sterile forceps the film was lifted off and a 1.00 mL aliquot of test medium was pipetted individually onto each test and control carrier as well as the underside of the film used to cover each sample (side exposed to the test sample or control). The surface of each carrier was individually scraped with a sterile plastic cell scraper. The test medium was collected (10^{-1} dilution), mixed using a vortex type mixer, and serial 10-fold dilutions were prepared.

5. Inoculation of Zero Time Virus Control (TABLE 1)
Two control carriers, contained in individual sterile petri dishes, were inoculated with a 100 µL aliquot of the test virus. Immediately following inoculation, a 1.00 mL aliquot of test medium was pipetted individually onto each control carrier. The surface of each carrier was individually scraped with a sterile plastic cell scraper. The test medium was collected, mixed using a vortex type mixer and serial 10-fold dilutions were prepared.

6. Cytotoxicity Control (TABLE 3)
One test carrier, contained in an individual sterile petri dish, was inoculated with a 100 µL aliquot of the test medium containing the Sponsor requested organic soil load (5% fetal bovine serum), in lieu of virus. The inoculum was covered with the carrier film and the film was pressed so that the test medium spread over the film, but did not spread past the edge of the film. The exposure time began when the sample was inoculated. The sample was transferred to a controlled chamber set to room temperature (20.0°C) in a relative humidity of 50% for the duration of the 24 hour exposure time.

Following the 24 hour exposure time, using sterile forceps the film was lifted off and a 1.00 mL aliquot of test medium was pipetted onto the cytotoxicity control carrier as well as the underside of the film used to cover the sample (side exposed to the carrier). The surface of the carrier was scraped with a sterile plastic cell scraper. The test medium was collected (10^{-1} dilution), mixed using a vortex type mixer, and serial 10-fold dilutions were prepared.

7. Assay of Non-Virucidal Level of Test Substance (Neutralization Control) (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 100 µL aliquot of each dilution in duplicate. A 100 µL 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.

8. Infectivity Assay
The LLC-MK_2 cell line, which exhibits CPE in the presence of Coxsackievirus type A16, was used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes were inoculated in quadruplicate with 100 µL of the dilutions prepared from test and virus control groups. The cytotoxicity, neutralization, and input virus controls were inoculated in duplicate. Uninfected indicator cell cultures (cell controls) were inoculated with test medium alone. The cultures were incubated at 36-38°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.

9. Statistical Methods: Not applicable

PROTOCOL CHANGES

Protocol Amendments:
No protocol amendments occurred during 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.

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

Geometric Mean = Antilog of: $\frac{\text{Log}_{10}X_1 + \text{Log}_{10}X_2}{2}$

(X equals $TCID_{50}$/volume inoculated for each of the two test or control replicates.)

Calculation of the Mean Log Reduction

Mean Zero Time Virus Control Log_{10} $TCID_{50}$ – Mean Test Substance Log_{10} $TCID_{50}$ = Mean Log Reduction

Mean Virus Control Log_{10} $TCID_{50}$ – Mean Test Substance Log_{10} $TCID_{50}$ = Mean Log Reduction

Calculation of the Mean Percent Reduction

$$\text{Mean \% Reduction} = 1 - \left[\frac{\text{Mean } TCID_{50} \text{ test}}{\text{Mean } TCID_{50} \text{ zero time virus control}}\right] \times 100$$

$$\text{Mean \% Reduction} = 1 - \left[\frac{\text{Mean } TCID_{50} \text{ test}}{\text{Mean } TCID_{50} \text{ virus control}}\right] \times 100$$

STUDY ACCEPTANCE CRITERIA

A valid test requires 1) that infectivity be recovered from the virus control; 2) that the cell controls be negative for infectivity. **Note:** Minimum percent and log reduction values do not exist to specify "passing" or "failing" test material.

STUDY 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. Antimicrobial Products – Test for Antimicrobial Activity and Efficacy Japanese Industrial Standard (JIS) Method JIS Z 2801:2000. Copyright 2000 Japanese Standards Association.

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 1053 (current version).

3. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette, E.H., Lennette, D.A., and Lennette, E.T. editors. Seventh edition, 1995.

4. 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.

5. Techniques in HIV Research, A. Aldovini and B. Walker, 1990.

STUDY RESULTS

Results of tests with VirusGuard, dry paint film coated on a glass panel, exposed to Coxsackievirus type A16 in the presence of a 5% fetal bovine serum organic soil load at room temperature (20.0°C) in a relative humidity of 50% for the Sponsor requested 24 hour exposure times are shown in Tables 1-3. All cell controls were negative for test virus infectivity.

The titer of the initial input virus control (October 10, 2013) was 6.00 $\log_{10}$ and the titer of the input virus control following the 24 hour exposure time (October 11, 2013) was 5.50 $\log_{10}$. The titer of the zero time virus control (VV-0) was 5.75 $\log_{10}$ for Replicate #1 and 6.00 $\log_{10}$ for Replicate #2. The mean titer of the zero time virus control replicates was 5.88 $\log_{10}$. The titer of the 24 hour virus control (VV-0) was 4.50 $\log_{10}$ for Replicate #1 and 5.25 $\log_{10}$ for Replicate #2. The mean titer of the 24 hour virus control replicates was 4.88 $\log_{10}$.

Following the 24 hour exposure time, test virus infectivity was detected in the virus-test substance sample for VirusGuard at 3.50 $\log_{10}$ for Replicate #1 and 4.00 $\log_{10}$ for Replicate #2. The mean titer on the two test replicates was 3.75 $\log_{10}$. Test substance cytotoxicity was not observed at any dilution assayed (≤0.50 $\log_{10}$). The neutralization control (non-virucidal level of the test substance) indicates that the test substance was neutralized at ≤0.50 $\log_{10}$.

STUDY CONCLUSIONS

Under the conditions of this investigation and in the presence of a 5% fetal bovine serum organic soil load, VirusGuard, a dry paint film coated on a glass panel, demonstrated a 99.3% mean reduction in viral titer following a 24 hour exposure time at room temperature (20.0°C) in a relative humidity of 50% to Coxsackievirus type A16, as compared to the mean titer of the zero time virus control. The mean log reduction in viral titer was 2.13 $\log_{10}$, as compared to the mean titer of the zero time virus control.

A 92.6% mean reduction in viral titer was demonstrated following a 24 hour exposure time, as compared to the mean titer of the 24 hour virus control. The mean log reduction in viral titer was 1.13 $\log_{10}$, as compared to the mean titer of the 24 hour virus control.

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 Control Results

Dilution	Input Virus Control		Zero Time Virus Control		24 Hour Virus Control	
	October 10, 2013	October 11, 2013	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}	+ +	+ +	+ + + +	+ + + +	+ + + +	+ + + +
10^{-2}	+ +	+ +	+ + + +	+ + + +	+ + + +	+ + + +
10^{-3}	+ +	+ +	+ + + +	+ + + +	+ + + +	+ + + +
10^{-4}	+ +	+ +	+ + + +	+ + + +	+ + + +	+ + + +
10^{-5}	+ +	+ +	+ + + +	+ + + +	0 0 0 0	0 + + +
10^{-6}	+ 0	0 0	+ 0 0 0	0 + + 0	0 0 0 0	0 0 0 0
10^{-7}	0 0	0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-8}	0 0	0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
$TCID_{50}$/100 µL	$10^{6.00}$	$10^{5.50}$	$10^{5.75}$	$10^{6.00}$	$10^{4.50}$	$10^{5.25}$
Mean $TCID_{50}$/100 µL	NA	NA	$10^{5.88}$		$10^{4.88}$	

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present
(NA) = Not applicable

TABLE 2: Test Results

Results of VirusGuard Exposed to Coxsackievirus Type A16 Following a 24 Hour Exposure Time

Dilution	Test Coxsackievirus type A16 + VirusGuard	
	Replicate #1	Replicate #2
Cell Control	0 0 0 0	0 0 0 0
10^{-1}	+ + + +	+ + + +
10^{-2}	+ + + +	+ + + +
10^{-3}	+ + + +	+ + + +
10^{-4}	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
10^{-7}	0 0 0 0	0 0 0 0
10^{-8}	0 0 0 0	0 0 0 0
$TCID_{50}$/100 µL	$10^{3.50}$	$10^{4.00}$
Mean $TCID_{50}$/100 µL	$10^{3.75}$	
Mean Percent Reduction (Based on the zero time virus control)	99.3%	
Mean Log_{10} Reduction (Based on the zero time virus control)	2.13 Log_{10}	
Mean Percent Reduction (Based on the 24 hour virus control)	92.6%	
Mean Log_{10} Reduction (Based on the 24 hour virus control)	1.13 Log_{10}	

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

TABLE 3: Cytotoxicity and Neutralization Control Results

Dilution	Cytotoxicity Control	Neutralization Control
	VirusGuard	VirusGuard
Cell Control	0 0	0 0
10^{-1}	0 0	+ +
10^{-2}	0 0	+ +
10^{-3}	0 0	+ +
10^{-4}	0 0	+ +
10^{-5}	0 0	+ +
10^{-6}	0 0	+ +
10^{-7}	0 0	+ +
10^{-8}	0 0	+ +
TCD_{50}/100 µL	$\leq 10^{0.50}$	Neutralized at a $TCID_{50}$/100 µL of $\leq 10^{0.50}$

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

(For Laboratory Use Only)
ATS Labs Project # A15681

300 10-7-13

ATS LABS

PROTOCOL

Test for Antiviral Activity and Efficacy – Modification of JIS Z 2801

Virus: Coxsackievirus type A16

PROTOCOL NUMBER

NPC01092513.COX

PREPARED FOR

Nippon Paint (Singapore) Co. Pte Ltd
1 First Lok Yang Road
Jurong, Singapore 629728

PERFORMING LABORATORY

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

PREPARED BY

Mary J. Miller, M.T.
Senior Virologist

DATE

September 25, 2013

EXACT COPY
INITIALS MM DATE 10-30-13

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Test for Antiviral Activity and Efficacy – Modification of JIS Z 2801

SPONSOR: Nippon Paint (Singapore) Co. Pte Ltd
1 First Lok Yang Road
Jurong, Singapore 629728

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

PURPOSE
The purpose of this study is to determine the antiviral efficacy of the Sponsor's product as compared to an untreated control following modifications of the JIS Z 2801 method.

TEST SUBSTANCE CHARACTERIZATION
Test substance characterization as to content, stability, etc., (40 CFR, Part 160, Subpart F [160.105]) is the responsibility of the Sponsor. The test substance shall be characterized by the Sponsor prior to 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 September 30, 2013. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of October 27, 2013. 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 or 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 any regulatory agency 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

A specific antiviral claim for a test substance must be supported by appropriate scientific data demonstrating the efficacy of the test substance against the claimed virus. This is accomplished in the laboratory by inoculating the treated material (test substance) under conditions which simulate as closely as possible the actual conditions under which the test substance is designed to be used. The LLC-MK_2 cell line, which supports the growth of the Coxsackievirus type A16, will be used in this study. The experimental design in this protocol meets these requirements. **This protocol has not been reviewed by regulatory agencies for registration compliance. Acceptance of this protocol by a regulatory agency is the responsibility of the Sponsor.**

TEST PRINCIPLE

An aliquot of test virus is placed on the surface of each test and control material and the materials are covered with a carrier film. Following the Sponsor requested exposure time, neutralization medium (test medium) will be added to the test and control materials; the surface of each material will be scraped and the test medium collected. Serial 10-fold dilutions will be performed and the dilutions will be assayed for viral infectivity by an accepted method. Appropriate virus, test substance cytotoxicity, and neutralization controls are run concurrently.

VIRUS

The G10 strain of Coxsackievirus type A16 to be used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-174). 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 LLC- MK_2 (Rhesus monkey kidney) cells were originally obtained from the American Type Culture Collection, Manassas, VA (ATCC CCL 7.1). 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. LLC- MK_2 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 the virucidal assays is Minimum Essential Medium (MEM) supplemented with 1-10% (v/v) heat inactivated FBS. 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.

TEST METHOD

Test and Control Material (Carrier) Preparation

The Sponsor will provide the test material, pre-cut to approximately 50 mm x 50 mm (2 inch x 2 inch), 25 mm x 25 mm (1 inch x 1 inch) or an alternate size as provided by the Sponsor. A control substance cut to the same size will be supplied as well. The size of the test and control material will be documented in the raw data and reported. If a control material is not provided, ATS Labs may provide a control material that is acceptable with the Sponsor. The type of control material provided by ATS Labs will be documented and reported. If the test or control material is not provided pre-cut, ATS Labs may cut the materials to the Sponsor requested size.

The test and control materials will be wiped with ethanol and will be allowed to air dry prior to use in testing. If the test and/or control material could become compromised by this procedure, alternate methods of cleaning / sterilizing may be employed. Alternately, this procedure may be omitted entirely by Sponsor request. (Refer to the study information page.)

Carrier Film Preparation
A carrier film will be prepared to fit over the test and control material. The film will be approximately 40 mm x 40 mm for the 50 mm x 50 mm samples or approximately 20 mm x 20 mm for the 25 mm x 25 mm samples and will consist of an appropriate sterile material such as a glass slide, stomacher bag, or other appropriate material. The size of the carrier film may be adjusted based on the size of the test and control material. The size of the carrier film will be documented in the raw data and reported. If the carrier film does not adhere to the test and/or control material or carrier due to shape or hydrophobic interactions, the film may be omitted completely or by request.

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.

Inoculation and Recovery of the Test and Control Materials
Inoculate the appropriate number of test and control carriers, contained in a sterile petri dish, with an aliquot of the test virus. The volume of virus utilized will be dependent on the size of the test and control materials. The volume of virus utilized will be recorded in the raw data and reported. Cover the inoculum with the film; pressing down on the film so that the test virus spreads over the film, paying attention so that the inoculum does not spread past the edge of the film. The exposure time begins when each sample is inoculated. Transfer the samples to a controlled chamber set to the Sponsor requested exposure conditions (temperature and appropriate humidity) for the duration of the Sponsor specified exposure time(s). A desiccator containing water, or other appropriate method, may be used and placed inside of an incubator or controlled chamber set at the requested exposure temperature.

Following the exposure time, using sterile forceps the film will be lifted off and an aliquot of test medium will be pipetted individually onto each test and control carrier as well as the underside of the film used to cover the sample (side exposed to the test sample or control). The surface of the carrier will be scraped with a sterile plastic cell scraper. The test medium will be collected, mixed using a vortex type mixer, and serial 10-fold dilutions will be prepared.

If excess cytotoxicity to the indicator cell cultures is caused by the test substance, the affected dilution(s) may be passed through individual Sephadex gel columns to reduce the toxicity. If this procedure is performed, the same dilutions of the zero time virus control and cytotoxicity control must also be passed through individual columns.

Inoculation of Zero Time Virus Control
Inoculate the control carriers, contained in a petri dish, with an aliquot of the test virus. Immediately following inoculation, an aliquot of test medium will be pipetted individually onto each control carrier. The surface of the carrier will be scraped with a sterile plastic cell scraper. The test medium will be collected, mixed using a vortex type mixer, and serial 10-fold dilutions will be prepared.

Cytotoxicity Control
Inoculate one test carrier, contained in a petri dish, with an aliquot of test medium containing the Sponsor requested organic soil load in lieu of virus. The volume utilized will be the same volume utilized for the test and control carriers. Cover the inoculum with the film; pressing down on the film so that the test medium spreads over the film, paying attention so that the inoculum does not spread past the edge of the film. The exposure time begins when the sample is inoculated. Transfer the sample to a controlled chamber set to the Sponsor requested exposure conditions (temperature and appropriate humidity) for the longest Sponsor specified exposure time. If necessary, depending on the requested exposure times, additional cytotoxicity controls may be performed at the discretion of the Study Director. A desiccator containing water, or other appropriate method, may be used and placed inside of an incubator or controlled chamber set at the requested exposure temperature.

Following the exposure time, using sterile forceps the film will be lifted off and an aliquot of test medium will be pipetted individually onto the carrier as well as the underside of the film used to cover the sample (side exposed to the carrier). The surface of the carrier will be scraped with a sterile plastic cell scraper. The test medium will be collected, mixed using a vortex type mixer, and serial 10-fold dilutions will be prepared.

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 100-500 µL aliquot of each dilution in duplicate. A 100 µL 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 Assay
The LLC- MK_2 cell line, which exhibits cytopathic effect (CPE) in the presence of Coxsackievirus type A16, will be used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes will be inoculated in quadruplicate with 100-500 µL of the dilutions prepared from test and control groups. The cytotoxicity, neutralization, and input virus controls will be inoculated in duplicate. Uninfected indicator cell cultures (cell controls) will be inoculated with test medium alone. Cultures are incubated at 36-38°C in a humidified atmosphere of 5-7% CO_2 in sterile disposable cell culture labware. The cultures will be scored periodically for approximately seven days for the absence or presence of CPE, cytotoxicity and for viability.

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: NA

STUDY ACCEPTANCE CRITERIA

A valid test requires 1) that infectivity be recovered from the virus control; 2) 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:** Minimum percent and log reduction values do not exist to specify a "passing" or "failing" test material.

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, 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. Antimicrobial Products – Test for Antimicrobial Activity and Efficacy Japanese Industrial Standard (JIS) Method JIS Z 2801:2000. Copyright 2000 Japanese Standards Association.
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 1053 (current version).
3. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette, E.H., Lennette, D.A. and Lennette, E.T. editors. Seventh edition, 1995.
4. 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.
5. Techniques in HIV Research, A. Aldovini and B. Walker, 1990.

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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.

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

Geometric Mean = Antilog of: $\frac{Log_{10}X_1 + Log_{10}X_2 + Log_{10}X_3}{3^*}$

(X equals $TCID_{50}$/volume inoculated for each test or control replicate)

**This value (or number of values for X) may be adjusted depending on the number of replicates requested.*

Calculation of Log Reduction

Zero Time Virus Control Log_{10} $TCID_{50}$ – Test Substance Log_{10} $TCID_{50}$ = Log Reduction **and/or**

Virus Control Log_{10} $TCID_{50}$ – Test Substance Log_{10} $TCID_{50}$ = Log Reduction

Calculation of Percent Reduction

$$\% \text{ Reduction} = 1 - \left[\frac{TCID_{50}\text{ test}}{TCID_{50}\text{ zero time virus control}}\right] \times 100 \textbf{ and/or}$$

$$\% \text{ Reduction} = 1 - \left[\frac{TCID_{50}\text{ test}}{TCID_{50}\text{ virus control}}\right] \times 100$$

Statistical Analysis
None used.

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STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

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

VirusGuard and VV-0 (control)

Expiration Date: NA

Product Description

- ☐ Quaternary ammonia
- ☐ Iodophor
- ☐ Sodium hypochlorite
- ☐ Peracetic acid
- ☐ Peroxide
- ☑ Other Silver ion containing paint film

Test Substance Active Concentration (upon submission to ATS Labs): 10-1000 ppm per 9-25-13 email. MM 9-30-13

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 preparation required, use as received (RTU)
- ☐ Preparation required: _____

Product Cleaning

- ☐ No cleaning required, Use as received (RTU)
- ☑ Wipe test and control carriers with alcohol
- ☐ Wipe test and control carriers with: _____

Test Virus: Coxsackievirus type A16

Exposure Times: 24 hours

Number of test and control carriers: ☐ One ☑ Two ☐ Three ☐ Other:_____

Exposure Temperature: ☑ Room temperature (18-24°C) at appropriate humidity
☐ Other:_____°C in a humidified atmosphere of _____% relative humidity
(please state the temperature and humidity range)

Organic Soil Load

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

TEST SUBSTANCE SHIPMENT STATUS

* mm 9-30-13
☑ 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):_____
* = Per 9-29-13 email, use new samples being sent by DHL via Worldwide express. mm 9-30-13

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 Study)

PROTOCOL MODIFICATIONS

☑ Approved without modification
☐ Approved with modification

_____

_____

PROTOCOL ATTACHMENTS

Supplemental Information Form Attached - ☑ Yes ☐ No

APPROVAL SIGNATURES

SPONSOR:

NAME: Anne Lim TITLE: Asst. General Manager, Head of Technical/QA

SIGNATURE:_____ DATE: 26/9/2013

PHONE: (65) 6265 5355 FAX: (65) 6264 1603 EMAIL: annelim@nipponpaint.com.sg
Corrected per 9-28-13 email. mm 9-30-13

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: 9-30-13
Study Director

ATS LABS

Attachment I: Other Individuals Authorized to Receive Information Regarding This Study

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MM 9-30-13

EXACT COPY
INITIALS MM DATE 10-30-13