Final Report for ProKure Solutions, LLC (PKS) and Pantheon Enterprises, Inc. (PEI)

Jan Biotech Project ID: PKSPEI-2014-01

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Testing Performed: 7th-13th October 2014

Study:	**Virucidal Efficacy of 100ppm ProKure against Murine Norovirus 1 in Suspension and on a Nonporous Surface**
Sponsor:	ProKure Solutions, LLC, and Pantheon Enterprises, Inc.
Testing Facility:	Jan Biotech, Inc., at Cornell University Nanobiotechnology Center (NBTC)
Test Substance:	ProKure at 100 ppm
Test Format:	Suspension, Surface
Contact Times:	10, 30 secs
Temperature:	22^{o}C

OBJECTIVE

The objective of this study was to evaluate the virucidal efficacy of the test substance ProKure at 100 ppm against Murine Norovirus (MVN) according to test criteria based on US EPA Guidelines DIS/TSS-7, Initial and Confirmatory testing, Hoelzer et al., 2013, and Nims and Plavsic, 2013. The tests followed exactly the ASTM E1052-11 protocol for viruses in suspension and ASTM E1053-11 for viruses on a nonporous inanimate environmental surface.

STATEMENT OF WORK

ProKure at 100 ppm was tested for virucidal efficacy at 10 and 30 second exposure to Murine Norovirus (MVN) in suspension and on a surface (nonporous glass), with an organic soil load simulated by 5% FBS. The completed tests were assayed for virus survival through serial dilution to 10^{-5}, with four cultures tested per dilution. An indicator mouse macrophage cell line (RAW264.7) was used for viral survival counts (infectivity assays), with an incubation period of 48 hours. Cytotoxicity of residual ProKure on treated virus samples was tested.

Starting and remaining virus titers after treatment of test substance and positive and negative controls are expressed as -$\log_{10}$ of the 50% endpoint for infectivity ($TCID_{50}$), calculated using the Spearman-Karber method. Log reduction was calculated by the starting virus titer - endpoint virus titer.

LABORATORY AND SCIENTIST QUALIFICATION STATEMENT

The reported testing was performed at Cornell University's Nanobiotechnology Center (NBTC) BSL-2 mammalian tissue culture facility. Jan Biotech Inc. scientists are microbiologists trained to perform research and testing work in accordance with standard operating procedures (SOPs), including ASTM, ISO, and other organization and custom study procedures. Dr. Janet Huie holds a Ph.D. in Molecular Biology from Princeton University, with postdoctoral work completed at New Jersey Medical School and Princeton University, and attained tenure as a Microbiology Associate Professor. Her work resulted in a change in EPA regulations for disinfection of airline water systems, with commercial sales to 12 airlines in the U.S. and internationally. More recently, she was Director of R&D for the use of supercritical Carbon dioxide for tissue and medical device sterilization.

TEST PARAMETERS USED IN THIS STUDY

Testing Substance Con.:	100 ppm	Test Substance Volume:	1.35 mL
Test Substance Diluent:	N/A	Replicates:	1
Control Substance:	DMEM	Control Substance Volume:	1.35 mL
Neutralization Method:	Addition of neutralizer at 100 ppm		

Virus: Murine Norovirus 1 (MVN-1), ATCC PTA-5935

Viral Inoculum Volume:	0.15 mL	Target Inoculum:	5.68 $\log_{10}$
Contact Times:	10 sec, 30 sec	Contact Temperature:	22 ^{o}C
Host Cell Line:	**RAW264.7**	Cell Passage Number:	5
Assay Medium:	5% FBS DMEM	Soil Load:	5% FBS
Incubation Period:	48 hours	Incubation Conditions:	37 ^{o}C, 5% CO_2

Table 1. Testing Matrix Performed

Parameter	Summary	Replicates
Cell culture	medium alone	4/group
Virus control	1 part virus + 9 parts medium	4/dilution
Virucidal test	1 part virus + 9 parts test substance	4/dilution
Cytotoxicity control	1 part medium + 9 parts test substance	4/dilution
Neutralization control	neutralized test substance + virus	4/dilution

CONTROL RESULTS:

Sterility: Validated Virus Control Titer: 6.50 $\log_{10}$
Cytotoxicity Titer: ≤ 0.50 $\log_{10}$; no toxicity observed.
Neutralization: Validated. No toxicity observed. Viral CPE observed in all wells.

CRITERIA FOR SCIENTIFIC DEFENSIBILITY OF ASTM E1052 AND E1053 STUDIES

Virucidal Suspension and Surface Time Kill tests must meet the following criteria:

(a) Minimum 4Log_{10} infectious virus recovered from virus controls.
(b) Viral cytopathic effects (CPE) distinguishable from cytotoxic effects due to test substance.
(c) Effectiveness of neutralization method established.
(d) Assay wells designated as sterility controls absent of viral CPE, contamination and cytotoxicity.

PASSING CRITERIA

Federal regulatory agency, including the U.S. EPA, require:

(a) Complete inactivation of test virus at all dilutions.
(b) If cytotoxicity observed, ≥3Log_{10} viral titer reduction past cytotoxicity level relative to virus control.

CALCULATIONS:

The 50% tissue culture infectious dose per mL (TCID_{50}/mL) was determined using the Spearman-Karber method using the following formula:

$$m = x_k + (d/2) - d\Sigma p_i$$

where:

m = the logarithm of the titer relative to the test volume
x_k = the logarithm of the smallest dosage which induces infection in all cultures
d = the logarithm of the dilution factor
p_i = the proportion of positive results at dilution i

The values were converted to TCID_{50}/mL using a sample inoculum of 1.0 mL.

The Log_{10} Reduction Factor (LRF) was calculated in the following manner:
Log_{10} Reduction = Log_{10} TCID_{50} (Virus Recovery Control) — Log_{10} TCID_{50} (Test)

The Load (Log_{10} TCID_{50}) per carrier was calculated in the following manner:
Load (Log_{10} TCID_{50}) = Titer (Log_{10} TCID_{50}/mL) + Log_{10} [volume per carrier (mL)]

Key (for all tables):
X/y = X wells out of y wells inoculated exhibited viral cytopathic effects (CPE).
0/y = 0 wells out of y wells inoculated exhibited viral CPE, no cytotoxicity or bacterial contamination was observed in any of the wells inoculated.

RESULTS

Summary: The test substance, ProKure, at 100ppm achieved ≥6Log_{10} reduction at both 10 and 30 seconds in suspension against Murine Norovirus. The same concentration achieved ≥6Log_{10} reduction at 30 seconds, with a ≥2.25Log_{10} reduction at 10 seconds, on a nonporous surface (glass) against Murine Norovirus. The results were validated by cell culture, viral titer and neutralization controls.

Table 1: Test Agent Results for 100 ppm ProKure with Virus Suspension

Dilution	10 sec	30 sec
10^{-2}	0/4	0/4
10^{-3}	0/4	0/4
10^{-4}	0/4	0/4
10^{-5}	0/4	0/4
Titer (Log_{10} $TCID_{50}$/mL)	≤0.5	≤0.5
Load (Log_{10} $TCID_{50}$) per carrier (0.15 mL challenge)	≤-0.32	≤-0.32
Log_{10} Reduction	≥6.00	≥6.00

Table 2: Test Agent Results for 100 ppm ProKure with Virus Dried on Surface

Dilution	10 sec	30 sec
10^{-2}	4/4	0/4
10^{-3}	3/4	0/4
10^{-4}	0/4	0/4
10^{-5}	0/4	0/4
Titer (Log_{10} $TCID_{50}$/mL)	4.25	≤0.5
Load (Log_{10} $TCID_{50}$) per carrier (0.15 mL challenge)	3.43	≤-0.32
Log_{10} Reduction	2.25	≥6.00

Table 3: Neutralizer Effectiveness and Cytotoxicity Controls

Dilution	Neutralizer Effectiveness	Cytotoxicity
10^{-2}	4/4	0/4
10^{-3}	4/4	0/4
10^{-4}	4/4	0/4
10^{-5}	4/4	0/4
Titer (Log_{10} $TCID_{50}$/mL)	≥6.50	N/A
Load (Log_{10} $TCID_{50}$) per carrier (0.15 mL challenge)	≥5.68	N/A

Table 4: Viability Control Results

Cell Viability Control
0/4 (Cells were viable; media was sterile)

Table 5: Virus Recovery Controls

Dilution	Virus Recovery Control
10^{-2}	4/4
10^{-3}	3/4
10^{-4}	4/4
10^{-5}	4/4
Titer (Log_{10} $TCID_{50}$/mL)	≥6.50
Load (Log_{10} $TCID_{50}$) per carrier (0.15 mL challenge)	≥5.68

REFERENCES

ASTM E1052-11. Standard Test Method to Assess the Activity of Microbicides against Viruses in Suspension.

ASTM E1053-11. Standard Test Method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces.

EPA DIS/TSS-7 / Nov. 12, 1981. EFFICACY DATA REQUIREMENTS: VIRUCIDES. http://www.epa.gov/oppad001/dis_tss_docs/dis-07.htm

Initial Virucidal Effectiveness Test — Efficacy of pre-saturated or impregnated towelettes for hard, non-porous surface disinfection against Feline Calicivirus (FCV), a surrogate virus for Norovirus - Initial (8/19/05).

Confirmatory Virucidal Effectiveness Test — Efficacy of pre-saturated or impregnated towelettes for hard, non-porous surface disinfection against Feline Calicivirus (FCV), a surrogate virus for Norovirus - Confirmatory (8/19/05).

Hoelzer, K., Fanaselle, W., Pouillot, R., Van Doren, J.M., Dennis, S. 2013. Virus inactivation on hard surfaces or in suspension by chemical disinfectants: systematic review and meta-analysis of norovirus surrogates. *J. Food Prot.* **76:**1006–16.

Nims, R., Plavsic, M. 2013. Inactivation of caliciviruses. *Pharmaceuticals (Basel)* **6:**358-92.