MICROCHEM LABORATORY

STUDY REPORT

Study Title

Determination of the Antiviral Effectiveness of Zoono Limited
Test Substance Delivered via Pipette Against Feline Calicivirus

Test Method

ASTM International Standard Test Method E1053
Assessment of the Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces

Study Identification Number

NG7502 + NG7676

Study Sponsor

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Test Facility

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ASTM E1053: General Information

ASTM International, formerly the American Society for Testing and Materials (ASTM), is an internationally recognized organization that develops and publishes product and testing standards. The ASTM E1053 test method is used to determine the virucidal effectiveness of liquid disinfectant products designed for use on hard, nonporous environmental surfaces. In an ASTM E1053 test, a viral inoculum is dried onto carriers, followed by exposure to a test formulation via spray device or pipette (modified use-dilution) for the specified contact time(s). Control carriers are concurrently processed using an equivalent volume of cell culture medium or other suitable buffer. Following neutralization, the carriers are enumerated using standard cell culture (e.g. $TCID_{50}$) or plaque assay techniques. Log_{10} and percent reduction values are calculated to determine the effectiveness of the test product relative to the control carriers. The ASTM E1053 test method for use with spray devices or pipette delivery is recognized by regulatory agencies as an approved method for claim substantiation.

Laboratory Qualifications Specific to the ASTM E1053 Test

Microchem Laboratory has considerable experience in the proper execution of the ASTM E1053 test method. The laboratory has performed many ASTM E1053 tests in order to assess the virucidal efficacy of a broad spectrum of disinfectant products. In addition, the laboratory has experience modifying the method as needed to accommodate customer needs. Each ASTM E1053 test at Microchem Laboratory is performed in a manner appropriate to the test substances submitted by the Study Sponsor, while maintaining the integrity of the study.

Study Timeline For NG7502 (Kill Curve)

Carriers Inoculated and Dried	Test Substance Applied to Carriers	Test Substance Neutralization	Assay Incubation Initiated	Assay Scored/ Calculated	Report Delivered
10 AUG 2016	10 AUG 2016	10 AUG 2016	10 AUG 2016	17 AUG 2016	12 SEP 2016

Study Timeline For NG7676 (24 Hour Contact)

Carriers Inoculated and Dried	Test Substance Applied to Carriers	Test Substance Neutralization	Assay Incubation Initiated	Assay Scored/ Calculated	Report Delivered
13 SEP 2016	13 SEP 2016	14 SEP 2016	14 SEP 2016	20 SEP 2016	20 SEP 2016

Combined report delivered 26 SEP 2016.

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Test Substance Information

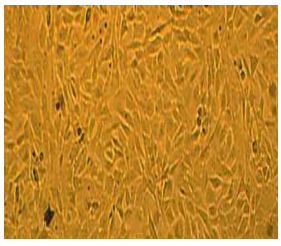
The test substance was received on 01 AUG 2016 and the following picture was taken.

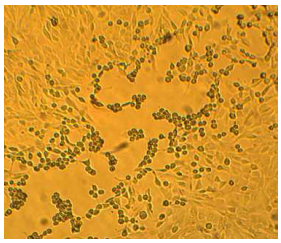
Test Substances Received: Zoono ULTRA germfree24 Hand Sanitizer and Protectant

The test substance arrived as ready to use. The test substance was not diluted prior to use in the study.

Test Microorganism Information

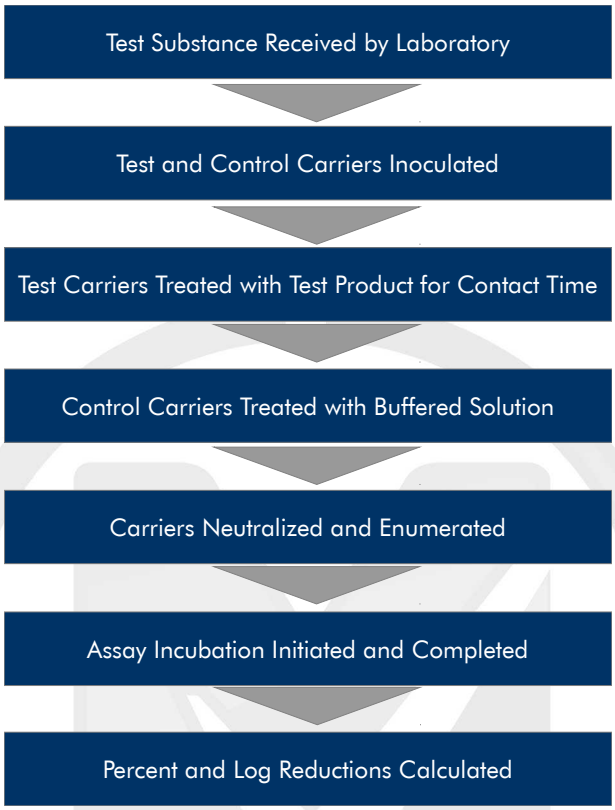
The test microorganism(s) selected for this test:





Feline calicivirus (FCV), ATCC VR-782
This virus is a non-enveloped, positive-stranded RNA member of the genus *Vesivirus*, and a common cause of respiratory infections in cats. Symptoms of infection in felines include nasal discharge and mouth ulcers. As a member of the *Caliciviridae* viral family, FCV is closely related to human noroviruses, which cause acute gastroenteritis marked by nausea, vomiting, and diarrhea. Unlike human norovirus, however, a simple cell culture assay system is available for FCV. Therefore, feline calicivirus is the US EPA-approved surrogate microorganism for human norovirus label claims. Both FCV and human norovirus are able to remain viable on environmental surfaces for extended periods of time and are resistant to a number of disinfectant actives.
Permissive Host Cell Line Selected for FCV: CRFK (Crandell-Rees Feline Kidney Cells), ATCC CCL-94

Diagram of the Procedure



Summary of the Procedure

- Stock virus is thawed and may be supplemented with an organic soil load, if requested.
- Sterile glass petri dish carriers (100 x 15 mm) are inoculated with a volume of virus suspension containing an adequate titer to recover a minimum of 4-$\log_{10}$ infectious viruses per carrier. A sufficient number of test and control carriers are prepared.
- Inoculated carriers are dried at room temperature under laminar flow conditions.
- The test substance is prepared according to the Study Sponsor's instructions as requested, and applied to the test carriers using a spray device or pipette. For spray tests, the distance, angle, and number of sprays applied are recorded. For use-dilution (pipette delivery) tests, the volume applied per carrier is recorded.
- The treated carriers are held for the predetermined contact time(s), and then neutralized in a manner appropriate for the test substance (e.g. dilution and/or gel filtration).
- The control carrier is harvested using an equivalent volume cell culture medium or other suitable buffer.

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Summary of the Procedure (cont.)

- Following neutralization of test and control carriers, the viral suspensions are quantified to determine the levels of infectious virus using standard cell culture (e.g. $TCID_{50}$) or plaque assay techniques.
- Assay trays/plates are incubated for the period most suitable for the virus-host cell system (e.g. 7 days).
- After the incubation period, the assay is scored for the presence/absence of test virus and cytotoxic effects. The appropriate calculations are performed (e.g. Spearman-Karber) to determine viral titers and levels of test substance cytotoxicity, where applicable.
- Log_{10} and percent reductions are computed for viral films exposed to the test product relative to the titer obtained for the study control carrier(s), and reported to the Study Sponsor.

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Criteria for Scientific Defensibility of an ASTM E1053 Study

For Microchem Laboratory to consider a virucidal effectiveness test to be scientifically defensible, the following criteria must be met:

1. A minimum of 4-$\log_{10}$ infectious viruses are recovered from the virus control carrier.
2. Viral cytopathic effects are distinguishable from cytotoxic effects caused by test substance exposure.
3. Neutralization effectiveness is demonstrated by recovery of comparable levels of infectious viruses from control (e.g. PBS), neutralizer (where applicable), and neutralized test substance.
4. Assay wells designated as sterility controls are absent of infectivity, contamination, and cytotoxicity.

Passing Criteria

ASTM International defines passing criteria to be:

1. Complete inactivation of the test virus at all dilutions.
2. If cytotoxicity is observed, a ≥3-log10 reduction in viral titer is observed past the level of cytotoxicity relative to the virus control.

Testing Parameters used for NG7502 (Kill Curve)

Test Substance Diluent:	N/A (Ready To Use)	Carrier Type:	Petri Dishes
Carriers Per Test:	1	Number of Sprays:	N/A
Spray Distance:	N/A	Spray Angle:	N/A
Use-dilution Volume:	2.0 ml of undiluted test substance applied to each test carrier		

Viral Inoculum Volume:	0.200 ml	Carrier Inoculation Area:	10-in^2
Carrier Dry Time:	19 minutes	Carrier Dry Conditions:	Ambient
Contact Time(s):	1 min., 5 min., 10 min.	Contact Conditions:	Ambient
Host Cell Line:	CRFK	Cell Passage Number:	3
Assay Medium:	2% FBS EMEM	Soil Load:	None
Incubation Period:	7 days	Incubation Conditions:	37±2°C, 5% CO_2

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Testing Parameters used for NG7676 (24 Hour Contact)

Test Substance Diluent:	N/A (Ready To Use)	Carrier Type:	Petri Dishes
Carriers Per Test:	1	Number of Sprays:	N/A
Spray Distance:	N/A	Spray Angle:	N/A
Use-dilution Volume:	2.0 ml of undiluted test substance applied to each test carrier		

Viral Inoculum Volume:	0.200 ml	Carrier Inoculation Area:	10-in^2
Carrier Dry Time:	10 minutes	Carrier Dry Conditions:	Ambient
Contact Time(s):	24 hours	Contact Conditions:	Ambient
Host Cell Line:	CRFK	Cell Passage Number:	6
Assay Medium:	2% FBS EMEM	Soil Load:	None
Incubation Period:	6 days	Incubation Conditions:	37±2°C, 5% CO_2

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Study Modifications

No further modifications were made to the method for this study.

Study Notes

In order to ensure the cessation of antiviral activity at the conclusion of the selected contact times for the kill curve testing a chemical neutralizer was used, followed by passage of the test substance-neutralizer mixture through a 10.0 cc prepared Sephacryl S-1000 SF gel filtration column. The chemical neutralizer consisted of 0.1% lecithin suspended in 10% FBS EMEM.

In order to ensure the cessation of antiviral activity at the conclusion of the selected contact time for the 24 hour contact testing a chemical neutralizer was used, followed by passage of the test substance-neutralizer mixture through a 12.5 cc prepared Sephacryl S-1000 SF gel filtration column. The chemical neutralizer consisted of 0.1% lecithin suspended in 10% FBS EMEM.

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Control Results for NG7502(Kill Curve)

Virus Control Titer: See results
Virus Stock Titer: 11.00 $\log_{10}$ $TCID_{50}$/0.1 ml
Neutralization Effectiveness: Confirmed
Cytotoxicity Titer: 2.10 $\log_{10}$/carrier
Sterility Controls: Confirmed

Control Results for NG7676

Virus Control Titer: See results
Virus Stock Titer: 5.50 $\log_{10}$ $TCID_{50}$/1.0 ml
Neutralization Effectiveness: Confirmed
Cytotoxicity Titer: None observed*
Sterility Controls: Confirmed

** While evidence of cytotoxicity was observed, it did not impact the readability of the test assay.*

Calculations

Viral and cytotoxicity titers ($TCID_{50}$/$TCLD_{50}$ and $TCCD_{50}$, respectively) were determined according to the method developed my Spearman-Karber:

$$-\text{Log}_{10} \text{ of 1st Dilution} - \left(\frac{\text{sum of \% mortality at each dilution}}{100}\right) - 0.5$$

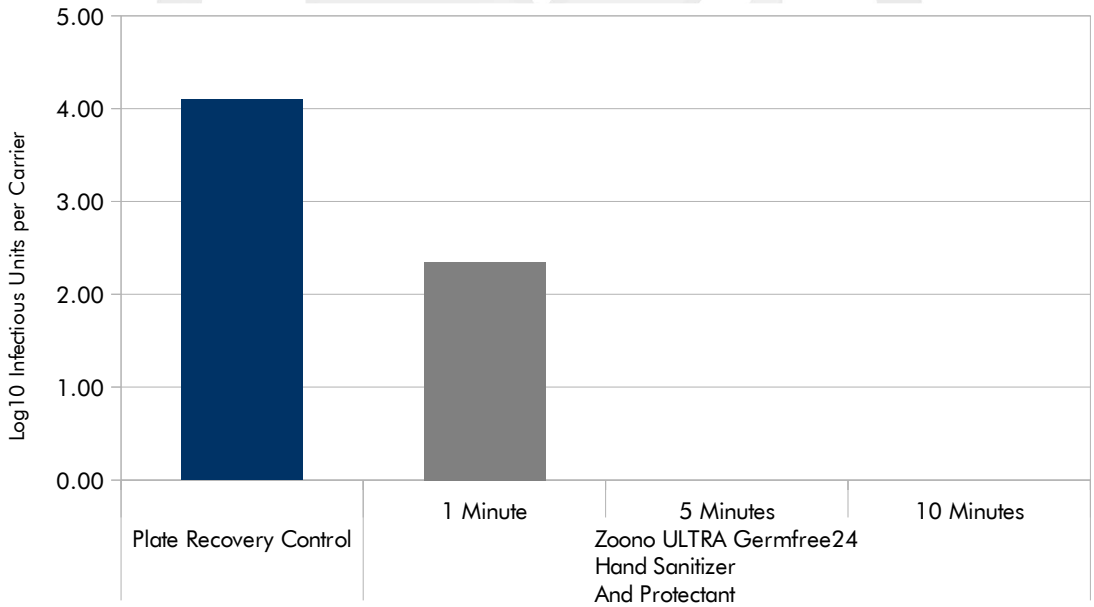
Percent Reduction of Virus is determined according to the following formula:

$$\text{Percent Reduction} = 1 - \left(\frac{C}{B}\right) * 100$$

Where:
B = Log_{10} of Virus Control Carrier
C = Log_{10} of Virus Test Carrier

Results of the Study NG7502 (Kill Curve)

Test Microorganism	Contact Time	Treatment	Log_{10} Infectious Units Per Carrier	Log_{10} Reduction Relative to Controls	Percent Reduction Relative to Controls
Feline calicivirus ATCC VR-782 U.S. EPA-Approved Human Norovirus Surrogate	Plate Recovery Control		4.10	N/A	
	1 Minute	Zoono ULTRA Germfree24 Hand Sanitizer And Protectant	2.35	1.75	98.26%
	5 Minutes		≤2.10	≥2.00	≥99.00%
	10 Minutes		≤2.10	≥2.00	≥99.00%



Note: Values at or below the limit of detection for this assay are depicted as a value of zero in the graph above.

RESULTS

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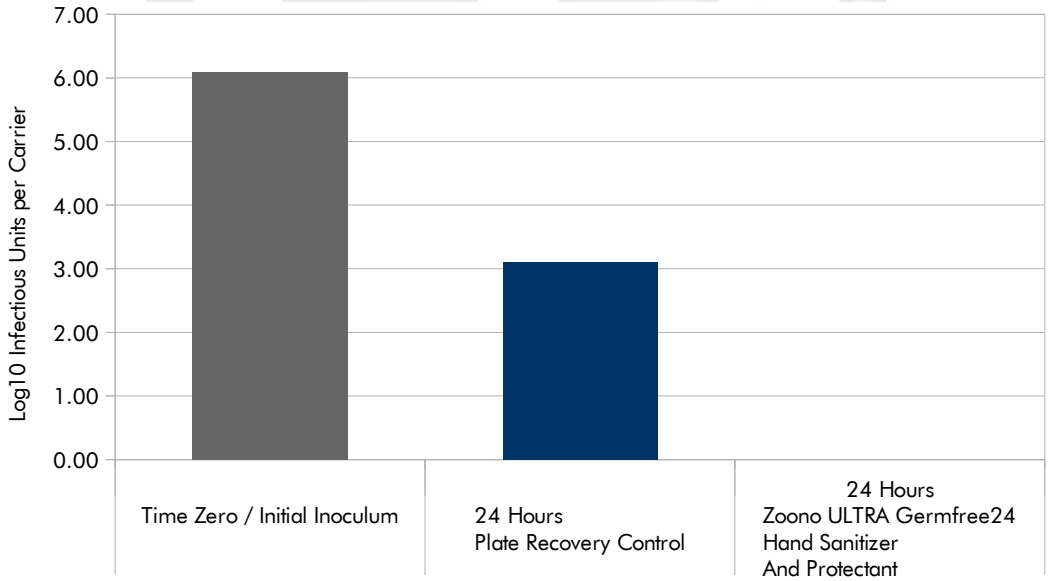
Results of the Study NG7676 (24 Hour Contact)

Test Microorganism	Treatment	Contact Time	Log_{10} Infectious Units Per Carrier	Log_{10} Reduction Relative to Control at Contact Time	Percent Reduction Relative to Control at Contact Time
Feline calicivirus ATCC VR-782 U.S. EPA-Approved Human Norovirus Surrogate	Time Zero / Initial Inoculum		6.10	N/A	
	Plate Recovery Control 24 hours		3.10		
	Zoono ULTRA Germfree24 Hand Sanitizer And Protectant	24 Hours	≤1.10	≥2.00	≥99.00%



Note: Values at or below the limit of detection for this assay are depicted as a value of zero in the graph above. Value provided for initial inoculum in order to demonstrate suitable inoculum titer prior to 24 hour dwell time, which would have unforeseeable impact on the viral plate recovery concentration.

The results of this study apply to the tested substances(s) only. Extrapolation of findings to related materials is the responsibility of the Sponsor.