STUDY TITLE
ISO 22196 Method

Study Identification Number
GLP1454
L-A-B ISO 17025 Accredited – Certificate Number L2450 - Testing

Protocol Number
P1570

Product Identity
DBC/CC504/18124 Black Anti-Microbial Compound
Lot#: 71088

Test Microorganism(s)
Feline calicivirus, Strain F-9, ATCC VR-782
(U.S. EPA-Approved Human Norovirus Surrogate)

Study Director
Erika Guin, B.S.

Data Requirements
ISO/IEC 17025:2005
ISO 22196:2011 (E)

Study Completion Date
27SEP2016

Testing Facility
Microchem Laboratory
1304 W. Industrial Blvd.
Round Rock, Texas 78681

Study Sponsor
Paul Cook
Titan Healthcare (Antibacterial) Products LTD
Titan House, 375 Babbacombe Road
Torquay, Devon TQ1 3TB, United Kingdom

GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study meets International Organization for Standardization (ISO/IEC) 17025:2005 with the following exception:

- Records concerning treated test surface characteristics (i.e. composition, purity, stability, strength, solubility) are maintained by the Study Sponsor.

Study Director

Company: Microchem Laboratory

Name: Erika Guin, B.S.

Title: Study Director

Signature: _____ Date: 27 SEP 2016

Study Sponsor

Company: Titan Healthcare (Antibacterial) Products LTD

Name: Paul Cook

Title: Study Sponsor

Signature: _____ Date: _____

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QUALITY ASSURANCE STATEMENT

Study Title: ISO 22196 Method

Study ID#: GLP1454

The following quality assurance audits were conducted in accordance with Good Laboratory Practice Standards outlined in ISO 17025 Sec. 4.14 and reported to management and the Study Director:

Phase Inspected	Date Inspected	Date Reported to Study Director	Date Reported to Management
In Phase	28 JUN 2016	28 JUN 2016	28 JUN 2016
Final Report	27 SEP 2016	27 SEP 2016	27 SEP 2016

Quality Assurance Unit:

Signature: Travis Chesser Date: 27SEP2016

Name: Travis Chesser, B.S.

Title: Quality Assurance Specialist

LABORATORY ACCREDITATION BUREAU a division of A-S-B ACCREDITED ISO/IEC 17025 Certificate # L2450 Testing

PERSONNEL INVOLVED IN THE STUDY

Study Director
Name: Erika Guin, B.S.
Company: Microchem Laboratory
Title: Study Director

Scientific Director
Name: Ben Tanner, Ph.D.
Company: Microchem Laboratory
Title: Scientific Director

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FINAL STUDY REPORT SUMMARY

Study Title
ISO 22196 Method

Study Identification Number
GLP1454

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Test Microorganism(s)
Feline calicivirus, Strain F-9, ATCC VR-782
(U.S. EPA-Approved Human Norovirus Surrogate)

Study Sponsor
Paul Cook
Titan Healthcare (Antibacterial) Products LTD
Titan House, 375 Babbacombe Road
Torquay, Devon TQ1 3TB, United Kingdom

Testing Facility
Microchem Laboratory
1304 W. Industrial Blvd.
Round Rock, Texas 78681

Study Director
Erika Guin, B.S

Study Completion Date
TBD

Study Objective
To determine to antimicrobial efficacy of the submitted test surface against the test microorganisms listed in this report using an ISO 22196 assessment of antimicrobial efficacy on non-porous surface(s) with a contact time of 8 hours and 12 hours.

Study Conclusion
After a contact time of 8 ± 1 hours the DBC/CC504/18124 Black Anti-Microbial Compound (Lot#: 71088) test surface showed a 99.17% or 2.08 $\log_{10}$ reduction against Feline calicivirus, Strain F-9, ATCC VR-782. After a contact time of 12 ± 1 hours the DBC/CC504/18124 Black Anti-Microbial Compound (Lot#: 71088) test surface showed a 99.74% or 2.59 $\log_{10}$ reduction against Feline calicivirus, Strain F-9, ATCC VR-782.

FINAL STUDY REPORT

Important Dates

Study Initiation Date:	21JUN2016
Experimental Start Date:	28JUN2016/0813
Experimental End Date:	06JUL2016/1708

Test Substance Information

Name:	DBC/CC504/18124 Black Anti-Microbial Compound
Manufacture Date:	08JUN2016
Date Received:	15JUN2016
Lot Number:	71088
Active Ingredients:	Not provided at time of Study
Expiration Dates:	Not provided at time of Study
Storage Conditions:	Ambient "Room" Temperature under Fluorescent Lighting or in a cabinet

FINAL STUDY REPORT (CONT.)

Test Parameters

Microorganism:	Feline calicivirus, Strain F-9, ATCC VR-782
Stock Lot Number:	FCV_16FEB2016
Culture Manipulation:	Viral stocks were manipulated by dilution in phosphate buffered saline prior to use in testing.
Host Cell Line:	CRFK (ATCC CCL-94) Subculture Passage Number: 240
Test Assay Medium:	Eagle's Minimum Essential Medium (EMEM) supplemented with 2% fetal bovine serum plus antibiotics [100 µg/ml kanamycin sulfate solution and antibiotic-antimycotic solution (100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B)].
Number of Test Carriers:	2 (1 dried virus film per test substance lot per contact time)
Organic Soil Load:	None
Test Contact Time:	8 hours ± 1 hour, 12 hours ± 1 hour
Test Temperature:	23.0 – 25.5 °C
Test Humidity:	44 – 47 % relative humidity
Primary Neutralizer:	0.1% sodium thiosulfate in 2% FBS EMEM
Neutralization Hold Time:	15 minutes
Hold Time Temperature:	25.0 °C
Hold Time Humidity:	45 % relative humidity
Assay Conditions:	37 ± 2.0 °C; 5 ± 1% CO_2
Assay Period:	7 days

Test Method

The test was conducted according to the attached protocol except as noted on page 9.

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PROTOCOL CHANGES

Protocol Amendments

No amendments were generated for the approved protocol.

Protocol Deviations

1. A deviation from the approved protocol occurred wherein certain individual plate recovery control titers from both dates of testing were determined to be below the protocol specified minimum of 4.00 $\log_{10}$ per carrier. It is the determination of the Study Director that, because the average plate recovery control titer on each date of testing was above this lower limit, the test product was sufficiently challenged and the study outcome was not adversely impacted.

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STUDY CONTROLS

Cytotoxicity Control

A cytotoxicity control was run to determine if any active contained within the neutralization and recovery media was toxic to the selected host cell line. One test surface was processed exactly as in the test procedure, but instead of viral inoculum cell culture media was applied and spread using a plastic film. Following neutralization, two aliquots of the neutralization and recovery media were prepared- one for use in the cytotoxicity control, one for use in the neutralization effectiveness control. The aliquot reserved for the cytotoxicty control was subject to serial 10-fold dilutions in 0% FBS EMEM through to the appropriate dilution and applied in quadruplicate per dilution to the appropriate host cell culture monolayers prepared to suitable confluency in multi-well trays.

Neutralization Effectiveness Control

An aliquot of the Cytotoxicity Control filtrate (neutralized test substance) generated from the submitted test substance was used to determine the effectiveness of the prescribed neutralization method. An aliquot of PBS was prepared as a control substance to determine if comparable levels of infectious viral units were recovered from the control and the neutralized test substance filtrates. Each viral stock was diluted in order to add a low number (e.g. 1000 to 5000) of infective units of the respective test system into each neutralized test substance filtrate and PBS control substance preparation. The PBS control and neutralized test substance filtrate preparations were each inoculated with 0.100 ml of the low virus titer suspension and allowed to sit undisturbed for 10 to 20 minutes at room temperature. The two mixtures were then diluted and enumerated to determine the comparative levels of infectious viruses by plating to the appropriate test culture monolayers (quadruplicate per dilution).

Cell Culture Control

To ensure that the host cell monolayers were not contaminated with bacteria, fungi, or any cytopathogenic viruses other than those used in the test, and to confirm the viability of the cells during the incubation period of the assay, at least four host cell monolayers were left untreated on the day of testing. These wells received an aliquot of test culture/assay medium only.

STUDY ACCEPTANCE CRITERIA

Study Success Criteria

The experimental success (controls) criteria follow:

1. A minimum of 4.00 $\log_{10}$ infectious viruses/carrier ($TCID_{50}$) is recovered from each plate recovery control film.
2. Quantification of the plate recovery control titer, the virus titer following test substance exposure, cytotoxicity levels, and test substance neutralization controls is conducted at a minimum of four determinations per dilution for each assay system.
3. The test results are reported as the reduction of the virus titer due to the activity of the test substance [$TCID_{50}$ of the plate recovery control less the $TCLD_{50}$ of the virus test carrier(s)] expressed as the logarithm to the base of 10 ($\log_{10}$) and calculated by a statistical method (e.g. Spearman-Karber).
4. Assay wells designated as cell viability (sterility) controls be absent of infectivity, contamination, and cytotoxicity.

Test substance performance criteria:

1. To be determined by the Study Sponsor.

CALCULATIONS AND STATISTICAL ANALYSIS

The Spearman-Karber Method was used to calculate the Plate Recovery Control titer ($TCID_{50}$), the viral titer following test substance exposure ($TCLD_{50}$), and the titer of host cell cultures exhibiting cytotoxicity following test substance exposure ($TCCD_{50}$).

The $TCID_{50}$ (Tissue Culture Infectivity Dose) represented the endpoint dilution where 50% of the cell cultures exhibited cytopathic effects due to infection by the test virus. The dose required to kill 50% of the test viruses after the given exposure time was referred to as the Tissue Culture Lethal Dose ($TCLD_{50}$), and the endpoint dilution at which 50% of the host cell monolayers exhibited cytotoxicity was termed the Tissue Culture Cytotoxic Dose ($TCCD_{50}$). The $TCID_{50}$, $TCLD_{50}$, and $TCCD_{50}$ were determined according to the method of Spearman-Karber as follows:

[-Log of 1st dilution inoculated – [(Sum of % mortality at each dilution / 100) – 0.5 x (logarithm of dilution)]

Calculation of Virus Inactivation Due to Test Substance Exposure

Mean Plate Recovery Control Log_{10} $TCID_{50}$ – Mean Virus-Test Substance Film Log_{10} $TCLD_{50}$ = Log_{10} Reduction of Virus Due to Inactivation by Test Substance

STUDY RECORD AND TEST SUBSTANCE RETENTION

Study Record Retention

The study report and corresponding data will be held in the archives of Microchem Laboratory for at least 2 years after the date of the final report. After 2 years, documentation may be returned to the Study Sponsor for archiving.

Test Substance Retention

The test substance may be returned to the Study Sponsor at Sponsor's request and expense within 30 days of study completion. If the Study Sponsor does not request return of the sample, it will be destroyed >30 days after study completion. Archiving of test substances is the responsibility of the Sponsor.

RESULTS

Key: + = Virus recovered; O = Virus not recovered and/or no cytotoxicity observed;
T = Toxicity observed

Table 1.
The following was the result of initial recovery controls from testing performed on 28JUN2016 for evaluating the DBC/CC504/18124 Black Anti-Microbial Compound (Lot# 71088) test substance evaluated against Feline calicivirus, Strain F-9, ATCC VR-782 at a contact time of 8 hours.

Dilution	Initial Control Replicate 1				Initial Control Replicate 2				Initial Control Replicate 3			
10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-3}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-4}	O	O	+	O	O	O	O	O	O	O	O	+
10^{-5}												
10^{-6}												
Per 1.00 ml	3.75 $\log_{10}$ $TCID_{50}$				3.50 $\log_{10}$ $TCID_{50}$				3.75 $\log_{10}$ $TCID_{50}$			
Per Carrier	4.23 $\log_{10}$ $TCID_{50}$				3.98 $\log_{10}$ $TCID_{50}$				4.23 $\log_{10}$ $TCID_{50}$			
Mean Per Carrier Value	4.15 $\log_{10}$ $TCID_{50}$											

Table 2.
The following was the result of plate recovery controls from testing performed on 28JUN2016 for evaluating the DBC/CC504/18124 Black Anti-Microbial Compound (Lot#: 71088) test substance when evaluated against Feline calicivirus, Strain F-9, ATCC VR-782 at a contact time of 8 hours.

Dilution	Plate Recovery Control Replicate 1				Plate Recovery Control Replicate 2				Plate Recovery Control Replicate 3			
10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-3}	+	O	+	+	+	+	+	+	+	+	+	+
10^{-4}	O	O	O	+	+	O	O	O	O	+	+	+
10^{-5}												
10^{-6}												
Per 1.00 ml	3.50 $\log_{10}$ $TCID_{50}$				3.75 $\log_{10}$ $TCID_{50}$				4.25 $\log_{10}$ $TCID_{50}$			
Per Carrier	3.98 $\log_{10}$ $TCID_{50}$				4.23 $\log_{10}$ $TCID_{50}$				4.73 $\log_{10}$ $TCID_{50}$			
Mean Per Carrier Value	4.31 $\log_{10}$ $TCID_{50}$											

RESULTS (CONT.)

Key: + = Virus recovered; O = Virus not recovered and/or no cytotoxicity observed;
T = Toxicity observed

Table 3.
The following was the result of DBC/CC504/18124 Black Anti-Microbial Compound (Lot#: 71088) when tested against Feline calicivirus, Strain F-9, ATCC VR-782 at a contact time of 8 hours.

Dilution	Virus Test Film (Lot: 71088) Replicate 1				Virus Test Film (Lot: 71088) Replicate 2				Virus Test Film (Lot: 71088) Replicate 3			
10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	O	+	O	O	O	+	+	O	O	O	O	O
10^{-3}	O	O	O	O	O	O	O	O	O	O	O	O
10^{-4}	O	O	O	O	O	O	O	O	O	O	O	O
10^{-5}	O	O	O	O	O	O	O	O	O	O	O	O
10^{-6}	O	O	O	O	O	O	O	O	O	O	O	O
Per 1.00 ml	1.75 $\log_{10}$ $TCLD_{50}$				2.00 $\log_{10}$ $TCLD_{50}$				1.50 $\log_{10}$ $TCLD_{50}$			
Per Carrier	2.23 $\log_{10}$ $TCLD_{50}$				2.48 $\log_{10}$ $TCLD_{50}$				1.98 $\log_{10}$ $TCLD_{50}$			
Mean Per Carrier Value	2.23 $\log_{10}$ $TCID_{50}$											

Table 4.
The following was the result of initial recovery controls from testing performed on 29JUN2016 for evaluating the DBC/CC504/18124 Black Anti-Microbial Compound (Lot# 71088) test substance evaluated against Feline calicivirus, Strain F-9, ATCC VR-782 at a contact time of 12 hours.

Dilution	Initial Control Replicate 1				Initial Control Replicate 2				Initial Control Replicate 3			
10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-3}	+	+	+	+	+	O	+	O	+	+	+	+
10^{-4}	O	+	+	O	+	O	+	O	O	+	O	O
10^{-5}												
10^{-6}												
Per 1.00 ml	4.00 $\log_{10}$ $TCID_{50}$				3.50 $\log_{10}$ $TCID_{50}$				3.75 $\log_{10}$ $TCID_{50}$			
Per Carrier	4.48 $\log_{10}$ $TCID_{50}$				3.98 $\log_{10}$ $TCID_{50}$				4.23 $\log_{10}$ $TCID_{50}$			
Mean Per Carrier Value	4.23 $\log_{10}$ $TCID_{50}$											

RESULTS (CONT.)

Key: + = Virus recovered; O = Virus not recovered and/or no cytotoxicity observed;
T = Toxicity observed

Table 5.
The following was the result of plate recovery controls from testing performed on 29JUN2016 for evaluating the DBC/CC504/18124 Black Anti-Microbial Compound (Lot#: 71088) test substance when evaluated against Feline calicivirus, Strain F-9, ATCC VR-782 at a contact time of 12 hours.

Dilution	Plate Recovery Control Replicate 1				Plate Recovery Control Replicate 2				Plate Recovery Control Replicate 3			
10^{-1}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	+	+	+	+	+	+	+	+	+	+	+	+
10^{-3}	+	O	+	O	+	+	+	+	O	+	+	+
10^{-4}	O	O	O	O	+	+	+	+	O	O	+	O
10^{-5}	O	O	O	O	O	O	O	O	O	O	O	O
10^{-6}	O	O	O	O	O	O	O	O	O	O	O	O
Per 1.00 ml	3.00 $\log_{10}$ $TCID_{50}$				4.50 $\log_{10}$ $TCID_{50}$				3.50 $\log_{10}$ $TCID_{50}$			
Per Carrier	3.48 $\log_{10}$ $TCID_{50}$				4.98 $\log_{10}$ $TCID_{50}$				3.98 $\log_{10}$ $TCID_{50}$			
Mean Per Carrier Value	4.15 $\log_{10}$ $TCID_{50}$											

Table 6.
The following was the result of DBC/CC504/18124 Black Anti-Microbial Compound (Lot#: 71088) when tested against Feline calicivirus, Strain F-9, ATCC VR-782 at a contact time of 12 hours.

Dilution	Virus Test Film (Lot: 71088) Replicate 1				Virus Test Film (Lot: 71088) Replicate 2				Virus Test Film (Lot: 71088) Replicate 3			
10^{-1}	O	+	O	+	O	O	+	O	+	+	O	+
10^{-2}	O	O	O	O	O	O	O	O	O	+	O	O
10^{-3}	O	O	O	O	O	O	O	O	O	O	O	O
10^{-4}	O	O	O	O	O	O	O	O	O	O	O	O
10^{-5}	O	O	O	O	O	O	O	O	O	O	O	O
10^{-6}	O	O	O	O	O	O	O	O	O	O	O	O
Per 1.00 ml	1.00 $\log_{10}$ $TCLD_{50}$				0.75 $\log_{10}$ $TCLD_{50}$				1.50 $\log_{10}$ $TCLD_{50}$			
Per Carrier	1.48 $\log_{10}$ $TCLD_{50}$				1.23 $\log_{10}$ $TCLD_{50}$				1.98 $\log_{10}$ $TCLD_{50}$			
Mean Per Carrier Value	1.56 $\log_{10}$ $TCID_{50}$											

RESULTS (CONT.)

Key: + = Virus recovered; O = Virus not recovered and/or no cytotoxicity observed;
T = Toxicity observed

Table 7.
The following was the result of viral stock titer determination performed on 28JUN2016.

Dilution	Viral Stock Titer Determination			
10^{-5}	+	+	+	+
10^{-6}	O	O	+	O
Per 1.00 ml	5.75 $\log_{10}$ $TCID_{50}$			

Table 8.
The following was the result of viral stock titer determination performed on 29JUN2016.

Dilution	Viral Stock Titer Determination			
10^{-5}	+	+	+	+
10^{-6}	O	O	O	O
Per 1.00 ml	5.50 $\log_{10}$ $TCID_{50}$			

Table 9.
The following were the neutralization effectiveness control results.

Dilution	Neutralization Inoculum Titer				Neutralization PBS Control Titer				Neutralization Test Control Titer			
10^{-1}									+	+	+	+
10^{-2}	+	+	+	+	+	+	+	+	+	O	+	+
10^{-3}	O	O	+	+	O	O	O	O	O	O	O	O
10^{-4}												
10^{-5}												
10^{-6}												
Per 1.00 ml	3.00 $\log_{10}$ $TCID_{50}$				2.50 $\log_{10}$ $TCID_{50}$				2.25 $\log_{10}$ $TCID_{50}$			

RESULTS (CONT.)

Key: + = Virus recovered; O = Virus not recovered and/or no cytotoxicity observed;
T = Toxicity observed

Table 10.
The following were the cytotoxicity control results for this study.

Dilution	Cytotoxicity Control (Lot: 71088) 28 JUN 2016				Cytotoxicity Control (Lot: 71088) 29 JUN 2016			
10^{-1}	O	O	O	O	O	O	O	O
10^{-2}	O	O	O	O	O	O	O	O
10^{-3}	O	O	O	O	O	O	O	O
10^{-4}								
10^{-5}								
10^{-6}								
Per 0.100 ml	≤ 0.50 $\log_{10}$ $TCCD_{50}$				≤ 0.50 $\log_{10}$ $TCCD_{50}$			
Per Carrier	≤ 0.98 $\log_{10}$ $TCCD_{50}$				≤ 0.98 $\log_{10}$ $TCCD_{50}$			

STUDY CONCLUSION

For Study Identification Number GLP1454, one microorganism was tested to determine the antimicrobial efficacy of the submitted test surface, DBC/CC504/18124 Black Anti-Microbial Compound (Lot#: 71088), at the contact times of 8 and 12 hours under the ISO 22196 method specifications, modified for viruses.

After incubation for the first selected contact time (8 hours ± 1 hour) at ambient conditions, the DBC/CC504/18124 Black Anti-Microbial Compound (Lot#: 71088) test surface demonstrated an average 2.08 $\log_{10}$ (99.17%) reduction against Feline calicivirus, Strain F-9, ATCC VR-782 when compared to the control surface.

After incubation for the second selected contact time (12 hours ± 1 hour) at ambient conditions, the DBC/CC504/18124 Black Anti-Microbial Compound (Lot#: 71088) test surface demonstrated an average 2.59 $\log_{10}$ (99.74%) reduction against Feline calicivirus, Strain F-9, ATCC VR-782 when compared to the control surface.

The neutralization effectiveness control results demonstrated that the selected neutralization method was adequate for use in this study.

This study was carried out in compliance with the approved Protocol, number P1570.

REFERENCES

1. International Organization for Standardization. ISO 22196 "Measurement of Antibacterial Activity on Plastics and Other Non-Porous Surfaces". 2011. ISO 22196:2011 (E)

2. International Organization for Standardization. ISO/IEC17025:2005 "General Requirements for the Competence of Testing and Calibration Laboratories."

PROTOCOL

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Test Microrganism
Feline calicivirus, Strain F-9, ATCC VR-782
U.S. EPA-Approved Human Norovirus Surrogate

Product Identity
DBC/CC504/18124 Black Anti-Microbial Compound
Lot#: 71088

Study Sponsor
Paul Cook
Titan Healthcare (Antibacterial) Products LTD
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Performing Laboratory
Microchem Laboratory
1304 W. Industrial Blvd.
Round Rock, Texas 78681

Protocol Number
P1570

Study Director
Erika Guin, B.S.

Date
20JUN2016

PROTOCOL (CONT.)

I. Introduction

This document describes the materials and procedure for evaluating the antimicrobial effectiveness of non-porous surface(s) using the International Organization for Standardization (ISO) 22196 test method. This document also explains the terms and conditions of testing.

II. Purpose

The purpose of this study is to document the efficacy of the test substance against the Sponsor requested test system (microorganism) under the test parameters specified in this protocol.

III. Justification for the Selection of Test System (Microorganism)

The microorganism listed on page 1 of this protocol was selected by the Sponsor.

IV. Terms and Conditions

Studies by Microchem Laboratory are conducted in accordance with general terms and conditions posted on www.MicrochemLab.com/terms

Prior to study initiation, Microchem Laboratory must receive the approved and signed protocol, test substance and payment. Changes to the signed, approved protocol will require amendment and may incur additional fees. Cancellation of the study any time after the protocol has been signed will result in a cancellation fee of up to 100% of the total study cost, to be determined by laboratory management at its sole discretion.

Microchem Laboratory may repeat studies, free of charge, in the event of unintended protocol non-conformance, if the non-conformance is determined by the Study Director to have affected the study outcome. If the neutralization system specified for a study is not adequate, the study will be deemed "inconclusive" and the Study Sponsor will be responsible for the cost of the study. In addition, the Study Sponsor is responsible for the cost of all studies performed to confirm the outcome of a previous study and for ensuring that the study will meet their regulatory objectives.

The Study Sponsor must obtain written consent from Microchem Laboratory to use or publish its protocols, study reports (or parts thereof), logo or employee names for marketing purposes.

Test substance characterization as to content, stability, etc., is the responsibility of the Study Sponsor.

PROTOCOL (CONT.)

V. Test Substance Identification, Characterization, and Handling

Test Substance Name – DBC/CC504/18124 Black Anti-Microbial Compound
Lot Number(s) – 71088
Manufacture Date – 08JUN2016

Special Handling Requirements — None

Test substance characterization as to content, stability, etc., is the responsibility of the Study Sponsor.

Test substances and devices are handled as follows:

- The test substance is stored at ambient (room) temperature under fluorescent lighting or in a cabinet.
- The test substance is shaken or otherwise mixed well immediately prior to use (if applicable).
- The test substance is handled safely in accordance with the chemical risks it may pose, stated in the MSDS or by the Study Sponsor during the course of pre-study communication.

PROTOCOL (CONT.)

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VI. Study Parameters, Incorporate d by References

Carrier Type — **50 mm x 50 mm ± 2 mm plastic carrier**
Control Surface Replicates – **3 per test microorganism per lot of test substance (provided by test facility, ~50 mm x 50 mm acrylic surface)**
Test Surface Replicates – **3 per test microorganism per lot of test substance**
Test Substance Form — **50 mm x 50 mm sample**
Contact Time(s) — **8 ± 1 hour, 12 ± 1 hour**
Test Temperature — **Ambient (room) temperature**
Neutralization and Recovery Medium — **0.1% sodium thiosulfate in 2% FBS EMEM supplemented with antibiotics**

Proposed Experimental Start Date: **22JUN2016**
Proposed Experimental Termination Date: **29JUN2016**

VII. Test System (Microorganism)

Feline calicivirus, Strain F-9, ATCC VR-782 (U.S. EPA-Approved Human Norovirus Surrogate)

VIII. Materials

- Sufficient quantity of test substance surfaces (treated and control).
- Crandell-Rees Feline Kidney (CRFK) cells, ATCC CCL-94 (host cell system for Feline calicivirus) prepared to suitable confluence in an appropriate number of multi-well cell culture trays.
- Incubator capable of maintaining the temperature range (37 ± 2°C) and atmospheric conditions (5 ±1% CO_2) appropriate for Feline calicivirus-CRFK host cell assay incubation.
- Sufficient sterile tubes containing 0.9 ml sterile 0% FBS EMEM for dilution of neutralized microbial suspensions prior to plating.
- Sterile 50 ml centrifuge tubes containing 3 ml sterile neutralization and recovery medium.
- Sufficient quantity of sterile plastic films cut to 40 x 40 ± 2 mm.
- Sufficient volume of Phosphate Buffered Saline (PBS).
- Parafilm.
- Forceps.
- Vortex mixer.
- Bunsen burner, microbiological incinerator, or micro-torch.
- Micropipettes and a sufficient quantity of appropriately sized sterile micropipette tips.
- Automatic pipettor (PipetAid or similar) and various sizes of sterile serological pipets.
- Appropriate volume of 95% ethanol.
- Incubators capable of sustaining temperatures of 36 ± 1°C.
- Certified satellite clock.
- Calibrated digital timer.
- Calibrated hygrometer.
- Calibrated thermometer.
- Autoclave.
- Sufficient quantity of sterile Petri dishes.

PROTOCOL (CONT.)

IX. Procedure

Preparation of Vessels Containing Neutralizer and Recovery Media

- Before the test begins, 3 ml of the appropriate neutralization and recovery media is added to sterile 50 ml centrifuge tubes.

Preparation of Stock Virus

- The Feline calicivirus strain (F-9) to be used in the study is obtained from the American Type Culture Collection (ATCC) located in Manassas, Virginia.
- The virus strain to be used in this study is readied by combining the supernatant from multiple cell culture flasks displaying cytopathic effect on ≥ 90% of the permissive host cell monolayers.
- After subjection to several freeze-thaw cycles, the supernatants are centrifuged in order to remove cell debris. The supernatant is removed and the viruses pelleted using a PEG (polyethylene glycol) extraction. The stock virus is titered on the appropriate host cell line.
- One milliliter aliquots are stored at approximately -70°C until the day of use, at which time the appropriate number of stock aliquots are removed, thawed, and used promptly in the assay. Viral stocks may be diluted as needed to reach an acceptable viral inoculum titer for study purposes.
- Prior to carrier inoculation, the test suspension is vortex mixed and diluted in sterile PBS (or another appropriate diluent), if applicable, to achieve approximately 1.0 x 10^5 infective units/carrier from initial recovery control carriers.

Preparation of Test Substances, Control Substances, and Plastic Films

- Test samples are aseptically cut down to an appropriate size (50 mm x 50 mm) if necessary. Three test samples per microorganism tested are placed into sterile Petri dishes, with one carrier per Petri dish.
- Before testing begins, control carriers are processed as follows:
  - Acrylic strips are cut down to approximately 50 mm x 50 mm.
  - The re-sized control carriers are washed as follows: rinsing or soaking in 95% ethanol followed by multiple tap water rinses, further followed by multiple reverse osmosis water rinses.
  - The washed control carriers are then dried via incubation in an appropriate incubator, or wiped with a clean cloth.
  - Dried control carriers are then placed under a UV lamp in a biological safety cabinet for a minimum of 15 minutes per side to sterilize.
  - Control carriers are then observed for any defects (e.g cracks), and defective carriers are discarded.
- Before the test begins, plastic films are processed as follows:
  - Plastic films are cut down to approximately 40 mm x 40 mm.
  - The re-sized plastic films are washed as follows: rinsing or soaking in 95% ethanol followed by multiple reverse osmosis water rinses.
  - The washed plastic films are then dried by via incubation in an appropriate incubator.
  - Dried plastic films are then placed under a UV lamp in a biological safety cabinet for a minimum of 15 minutes per side to sterilize.
  - Plastic films are then observed for any defects (e.g creases), and defective films are discarded.

PROTOCOL (CONT.)

Inoculation of Test and Control Carriers

- Three treated and six control carriers, per microorganism, are inoculated with 0.400 ml of the appropriate test inoculum suspension.
- The inoculum is covered with a 40 mm x 40 mm ± 2 mm sterile plastic film, so that the inoculum spreads to fill the entire area under the film but does not leak beyond the film. The plastic film may be gently pressed down if necessary to ensure complete spreading of the inoculum.
- Excluding the carriers used in the determination of the initial carrier microorganism concentration, all carriers are sealed inside Petri dishes or other appropriate containers with 0.200 ml of PBS to reduce desiccation and incubated under ambient (room temperature) conditions for the duration of the selected contact time(s).

Determination of Initial Recovery Control Carrier Viral Concentrations

- Immediately after inoculation, three control carriers for each microorganism are eluted with 3 ml neutralizing and recovery media. To ensure complete microbial recovery from the carriers the carrier surface and plastic film are subject to a minimum of four washes using a sterile serological pipette.
- Following recovery of the microbe-recovery media suspension, the resultant volume is subject to sequential 1:10 serial dilutions in sterile 0% FBS EMEM, and appropriate dilutions are plated in quadruplicate to determine the viable control virus concentration.

Determination of Viable Viral Concentrations Following Each Contact Time

- At the conclusion of the selected contact time(s), appropriate control and test carriers for each microorganism are eluted with 3 ml neutralizing and recovery media. To ensure complete microbial recovery from the carriers the carrier surface and plastic film are subject to a minimum of four washes using a sterile serological pipette.
- Following recovery of the microbe-recovery media suspension, the resultant volume is subject to sequential 1:10 serial dilutions in sterile 0% FBS EMEM, and appropriate dilutions are plated in quadruplicate to determine the viable viral concentrations.

Cell Culture Control

- To ensure that the host cell monolayers are not contaminated with bacteria, fungi, or any cytopathogenic viruses other than those used in the test, and to confirm the viability of the cells during the incubation period of the assay, at least four host cell monolayers are left untreated on the day of testing. These wells receive an aliquot of test culture/assay medium only.

Cytotoxicity Control

- A cytotoxicity control will be run to determine if any active contained within the neutralization and recovery media is toxic to the selected host cell line.
- One test surface is processed exactly as in the test procedure, but instead of viral inoculum cell culture media will be applied and spread using a plastic film. Following neutralization, two aliquots of the neutralization and recovery media will be prepared- one for use in the cytotoxicity control, one for use in the neutralization effectiveness control.

PROTOCOL (CONT.)

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- The aliquot reserved for the cytotoxicty control will be subject to serial 10-fold dilutions in 0% FBS EMEM through to the appropriate dilution and applied in quadruplicate per dilution to the appropriate host cell culture monolayers prepared to suitable confluency in multi-well trays.

Neutralization Effectiveness Control

- An aliquot of the Cytotoxicity Control filtrate (neutralized test substance) generated from the submitted test substance is used to determine the effectiveness of the prescribed neutralization method.
- An aliquot of PBS is prepared as a control substance to determine if comparable levels of infectious viral units are recovered from the control and the neutralized test substance filtrates.
- Each viral stock is diluted in order to add a low number (e.g. 1000 to 5000) of infective units of the respective test system into each neutralized test substance filtrate and PBS control substance preparation.
- The PBS control and neutralized test substance filtrate preparations are each inoculated with 0.100 ml of the low virus titer suspension and allowed to sit undisturbed for 10 to 20 minutes at room temperature.
- The two mixtures are then diluted and enumerated to determine the comparative levels of infectious viruses by plating to the appropriate test culture monolayers (quadruplicate per dilution).

Infectivity Assay Incubation

- All assay trays may be incubated at 37 ± 2 °C (5 ± 1% CO_2) for approximately 30 minutes to facilitate virus-host cell adsorption. The trays may also be placed upon an orbital rotator during this incubation period, if feasible. Following either plating or the adsorption period, each well receives ~1.0 ml of test/cell culture medium via pipette delivery. The cell culture assay trays are incubated at 37 ± 2 °C (5 ± 1% CO_2) for a minimum of 7 days in a humidified CO_2 incubator.
- Assay trays may be examined regularly, with changes to healthy monolayers including viral cytopathic effects (CPE), cytotoxicity, and contamination clearly documented as such changes are observed.

X. Calculations

- The Spearman-Karber Method is used to calculate the Plate Recovery Control titer ($TCID_{50}$), the viral titer following test substance exposure ($TCLD_{50}$), and the titer of host cell cultures exhibiting cytotoxicity following test substance exposure ($TCCD_{50}$).
- The $TCID_{50}$ (Tissue Culture Infectivity Dose) represents the endpoint dilution where 50% of the cell cultures exhibit cytopathic effects due to infection by the test virus. The dose required to kill 50% of the test viruses after the given exposure time is referred to as the Tissue Culture Lethal Dose ($TCLD_{50}$), and the endpoint dilution at which 50% of the host cell monolayers exhibit cytotoxicity is termed the Tissue Culture Cytotoxic Dose ($TCCD_{50}$). The $TCID_{50}$, $TCLD_{50}$, and $TCCD_{50}$ are determined according to the method of Spearman-Karber as follows:

[-Log of 1st dilution inoculated – [(Sum of % mortality at each dilution / 100) – 0.5 x (logarithm of dilution)]

Calculation of Virus Inactivation Due to Test Substance Exposure

Mean Plate Recovery Control Log_{10} $TCID_{50}$ – Mean Virus-Test Substance Film Log_{10} $TCLD_{50}$ = Log_{10} Reduction of Virus Due to Inactivation by Test Substance

PROTOCOL (CONT.)

XI. Success Criteria

- The experimental success (controls) criteria follow:
  1. A minimum of 4.00 $\log_{10}$ infectious viruses/carrier ($TCID_{50}$) is recovered from each plate recovery control film.
  2. Quantification of the plate recovery control titer, the virus titer following test substance exposure, cytotoxicity levels, and test substance neutralization controls is conducted at a minimum of four determinations per dilution for each assay system.
  3. The test results are reported as the reduction of the virus titer due to the activity of the test substance [$TCID_{50}$ of the plate recovery control less the $TCLD_{50}$ of the virus test carrier(s)] expressed as the logarithm to the base of 10 ($\log_{10}$) and calculated by a statistical method (e.g. Spearman-Karber).
  4. Assay wells designated as cell viability (sterility) controls be absent of infectivity, contamination, and cytotoxicity.

- Test substance performance criteria
  1. To be determined by Study Sponsor.

XII. Reporting

- The report will include, but is not limited to, identification of the sample, date received, dates on which the test was initiated and completed, identification of the bacterial strains used, description of media and reagents, description of the methods employed, tabulated results and conclusion as it relates to the purpose of the test, and all other items required by ISO/IEC 17025:2005. A draft final report will be provided to the Sponsor for review prior to finalization.

XIII. Data and Sample Retention

- The study report, and corresponding data will be held in the archives of Microchem Laboratory for at least 2 years after the date of the final report. After 2 years, documentation may be returned to the Study Sponsor for archiving.
- The test substance may be returned to the Study Sponsor at Sponsor's request and expense within 30 days of study completion. If the Study Sponsor does not request return of the sample, it will be destroyed >30 days after study completion. Archiving of test substances is the responsibility of the Sponsor.

XIV. Quality Control

- The study will be conducted in accordance with the performing laboratory's quality management system and will undergo a full quality assurance review. All protocol amendments will be fully recorded and reported, as well as any deviations from the protocol.

XV. References

- International Organization for Standardization. ISO 22196 "Measurement of antibacterial activity on plastics and other non-porous surfaces." (2011). ISO 22196:2011 (E)
- International Organization for Standardization. ISO/IEC 17025:2005 "General Requirements for the Competence of Testing and Calibration Laboratories."

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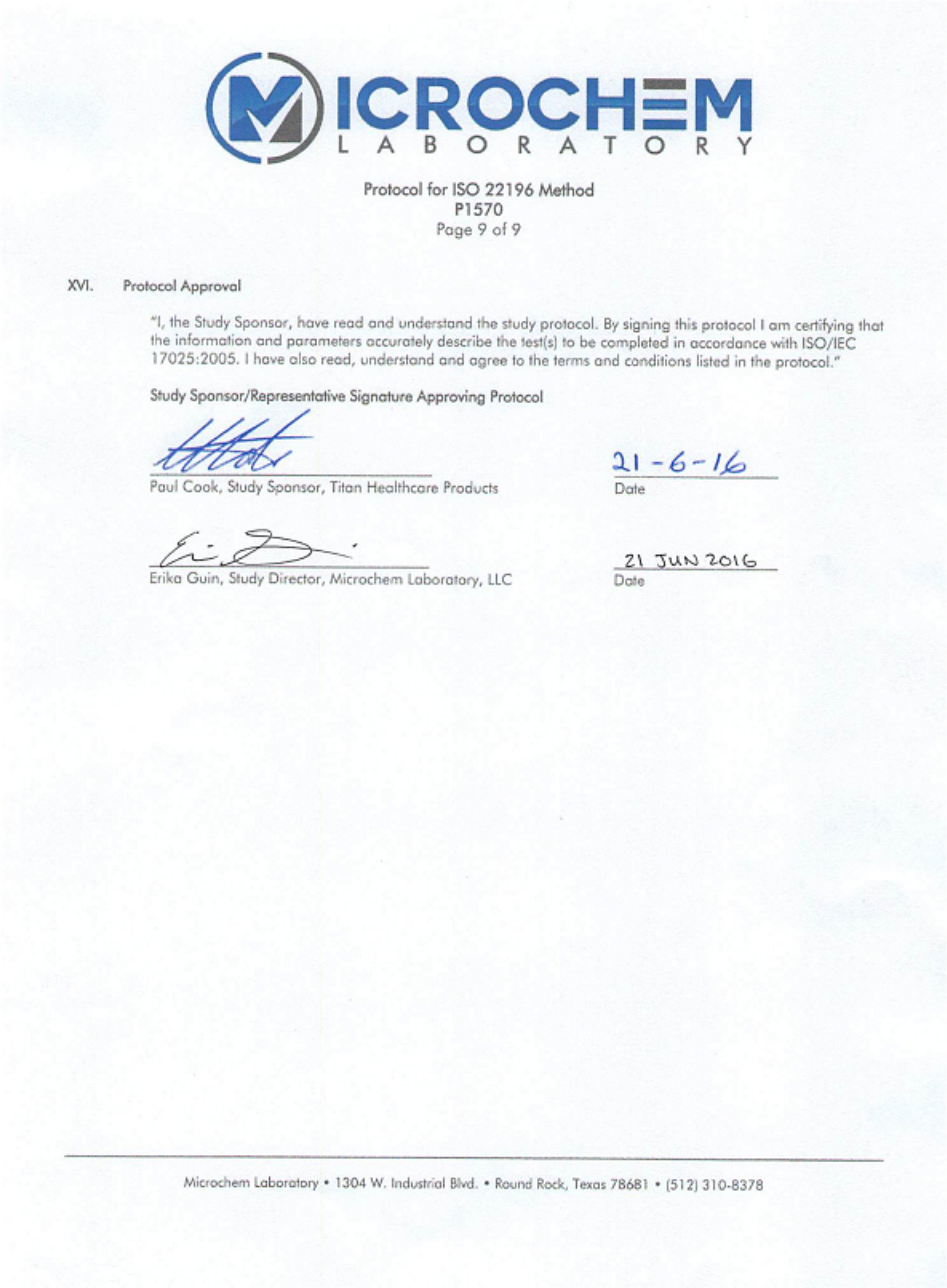
PROTOCOL (CONT.)



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XVI. Protocol Approval

"I, the Study Sponsor, have read and understand the study protocol. By signing this protocol I am certifying that the information and parameters accurately describe the test(s) to be completed in accordance with ISO/IEC 17025:2005. I have also read, understand and agree to the terms and conditions listed in the protocol."

Study Sponsor/Representative Signature Approving Protocol

Paul Cook, Study Sponsor, Titan Healthcare Products — Date: 21-6-16

Erika Guin, Study Director, Microchem Laboratory, LLC — Date: 21 JUN 2016

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