BIODOX™
CONCENTRATED LIQUID STERILIZER

FINAL STUDY REPORT

Study Title
Virucidal Efficacy of a Test Substance for Use on Inanimate, Nonporous Surfaces

Product Identity
BIODOX Chlorine Dioxide 4000 ppm
Lot Numbers: 000136, 21.05.19.01

Test Microorganism
Human coronavirus, 229E strain, ATCC VR-740

Data Requirements
U.S. EPA OCSPP 810.2200

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Study Completion Date
205692021

Testing Facility

Microchem Laboratory
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Study Sponsor
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FINAL STUDY REPORT SUMMARY

Study Title: Virucidal Efficacy of a Test Substance for Use on Inanimate Nonporous Surfaces

Study Identification Number: GLP2845

Test Microorganism: Human coronavirus, 229E strain, ATCC VR-740

Host Cell: MRC-5 cells (ATCC CCL-171)

Test Substance: **BIODOX** Chlorine Dioxide 4000 ppm Lot Numbers: 000136,21.05.19.01

Test Substance Dilution: Diluted 30 ml test substance into 970 ml of 200 ppm autoclave-sterilized tap water

Test Substance Application: 2.0 ml aliquot of the use dilution of the liquid test substance applied via pipette

Organic Soil Load: No additional supplementation of organic soil load incorporated into the test inoculum

Inoculum Volume: 0.200 ml

Carrier Type: Sterile glass Petri dish (100 mm x 15 mm)

Number of Carriers per Lat: One

Contact Time: 10 minutes

Exposure Temperature: Ambient room temperature (24.0-24.6◦C) and 31% relative humidity (RH)

Neutralization Method: Sephadex LH - 20 gel filtration column

Study Results

Description	*Assay Results Lot: 000136*	*Lot:21.05.19.01*	*Plate Recovery Control*
Log_{10} $TCID_{50}$ / 0.1 ml	***< 0.50 log***	***0.75 log_{10}***	***5.05 log_{10} ($TCID_{50}$/ Carrier)***
Log_{10} $TCID_{50}$ /Carrier	***< 0.80 log_{10}***	***1.05 log^{10}***	
Log_{10} Reduction/Carrier	***> 4.25 log_{10}***	***4.00 log_{10}***	

STUDY DATES

Study Initiation Date: 04AUG2021
Experimental Start Date/Time: 05AUG2021 / 1455
Experimental End Date/Time: 12AUG2021 / 0911
Study Completion Date: 20SEP2021

TEST SUBSTANCE

Name: BioDox Chlorine Dioxide 4000 ppm

Lot: 000136
Active Ingredients (concentration): Chlorine dioxide (0.4%)*
Date of Manufacture: 10APR2021
Date Received: 04MAY2021
Expiration Date: 10NOV2021*

Lot: 21.05.19.01

Active Ingredients (concentration): Chlorine dioxide (0.4%)*
Date of Manufacture: 19MAY2021
Date Received: 01 JUN2021
Expiration Date: 19MAY2022
**As indicated in the approved protocol.*

Form: Liquid; dilution required.

Storage Conditions: Ambient room temperature under fluorescent lighting.

Test Substance Preparation: The test substance was used as directed by the Study Sponsor. Each lot of the test substance was prepared by adding 30ml of test substance 970ml of autoclave sterilized tap water. The prepared test substance appeared to be in solution as determined by visual observation on the day of use.

The 200 ppm autoclave - sterilized tap water (180 - 210 ppm range) used as the test substance diluent was titrated using a calibrated buret on the day of use. The titration result was 182 ppm.

The test substance was equilibrated to the requested exposure temperature prior to use.

PROTOCOL CHANGES

Protocol Amendment(s)

Protocol Amendment #1

On 20SEP2021, the approved/signed protocol P3254 has been amended to reflect that the Certificates of Analysis for each lot of test substance will not be provided.

All remaining testing parameters are to be followed as stated in the protocol.

Protocol Deviation(s)

There were no deviations from the approved protocol during the conduct of this study.

TEST OBJECTIVE

The purpose of this study was to document the virucidal efficacy of the test substance against the test system (microorganism) under the test parameters specified in this protocol. The test protocol was in compliance with the requirements of and may be submitted to one or more of the following agencies as indicated by the Study Sponsor: U.S. Environmental Protection Agency (EPA) and Health Canada.

TEST PRINCIPLE

The test substance was applied to a film of virus that has been dried onto the surface of a glass carrier (representing a hard, nonporous surface) and held for the Sponsor-specified contact time. At the conclusion of the contact time, the recovered virus-test substance mixture was neutralized and the mixture was assayed for infectivity. Plate recovery, cytotoxicity, neutralization, virus inoculum titer and cell culture controls are performed concurrently with the test.

TEST PROCEDURE

Test System (Microorganism)

Human coronavirus, 229E strain, ATCC VR-740, originally received from the American Type Culture Collection (ATCC), Manassas, VA,, was used in this study. The Microchem Laboratory lot number used in testing was HCoV_21MAR2020C.

ATCC microorganisms are used under commercial license. The ATCC trademark and trade name and any and all ATCC catalog numbers are trademarks of the American Type Culture Collection.

TEST PROCEDURE (cont.)

Preparation of the Test Virus

The test virus was propagated internally by Microchem Laboratory personnel by inoculating the virus into cell culture flasks containing the appropriate host cell line and incubating at the appropriate conditions. Once the cell cuore flasks displayed approximately 75-100% cytopathic effect (as determined by microscopic evaluation), the flasks were subjected to freeze-thaw cycles to release virus from infected cells. The contents of the cell culture flasks were collected and centrifuged in order to remove the cell debris. The test virus was then aliquoted and stored at s- 70 °C.

On the day of testing, the appropriate number of virus stock suspension vials were removed from cryostorage and thawed for use in the assay. The test virus contained 2% fetal bovine serum (FBS) organic soil load. The test virus was not adjusted to incorporate any additional organic soil load into the inoculum.

Host Cell-Line

MRC-5 cells (ATCC CCL-171), originally received from the ATCC, were utilized in the assay. The cells were subcultured by Microchem Laboratory personnel and seeded into 24-well cell culture plates. The plates were incubated at 36±2°C in a humidified atmosphere of 6±1% CO_2 until they reached the desired confluence required for testing. On the day of use, the cells were microscopically examined to verify the appropriate confluency and health of the cells. Cell culture passage documentation including cell culture source, passage number, seeding densities, etc. was retained.

ATCC microorganisms are used under commercial license. The ATCC trademark and trade name and any and all ATCC catalog numbers are trademarks of the American Type Culture Collection.

Test Medium

The test medium utilized in the assay was Eagle's Minimum Essential Medium (EMEM) supplemented with 2% FBS, 40 mM HEPES buffer, 125 µM non-essential amino acids, 1 mM sodium pyruvate, plus antibiotics [antibiotic-antimycotic solution (100 units/ml penicillin G, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B)]. Concentrations based on preparation of 1 L of Eagle's Minimal Essential Medium.

Preparation of the Test Substance

The test substance was used as directed by the Study Sponsor. Each lot of the test substance was prepared by adding 30 ml of test substance to 970 ml of autoclave-sterilized tap water. The prepared test substance appeared to be in solution as determined by visual observation on the day of use. The 200 ppm autoclave-sterilized tap water (180-210 ppm range) used as the test substance diluent was titrated using a calibrated buret on the day of use. The titration result was 182 ppm. The test substance was equilibrated to the requested exposure temperature prior to use.

TEST PROCEDURE (cont.)

Preparation of Sephadex LH-20 Gel Filtration Columns

Sephadex LH-20 gel filtration columns were utilized to neutralize and/or to reduce the cytotoxicity of the test substance following exposure to the test virus by separating the virus from the test substance via filtration. On the day of testing, the prepared Sephadex slurry was aseptically added to prepared column units (sterile syringe) to completely fill the column. Just prior to testing, the syringe was centrifuged at approximately 100 x g for 3-4 minutes to clear the void volume.

Preparation of Virus Films

The test virus was vortexed thoroughly and a 0.200 ml aliquot of virus was placed on the inside bottom surface of three 100 mm x 15 mm sterile glass Petri dishes which served as the test carriers and plate recovery control. The inoculum was spread over the entire area of the carriers using a sterile bent pipette tip without touching the sides of the Petri dish. The virus films were dried in an environmental chamber for 20 minutes at 20.0 °C in a relative humidity of 30%.

Exposure of Virus Films to the Test Substance

For each lot of the test substance, one dried virus film carrier was treated with a 2.0 ml aliquot of the use dilution of the liquid test substance following Study Sponsor instructions. The carriers were gently rotated to ensure complete coverage of the test substance over the entirety of each test surface. The carriers were held covered at the Sponsor-requested exposure temperature of 24.0-24.6 °C in a relative humidity of 31% for the requested contact time of 10 minutes. Just prior to the completion of the contact time, a sterile cell scraper was used to re-suspend each viral film and the solution was immediately transferred into a gel filtration column. The syringe plunger was used to pass the contents of the re-suspended test carrier through the column. Serial 10-fold dilutions (e.g. 0.1 ml filtrate + 0.9 ml test media) of the filtrate (10 dilution) were prepared to the appropriate dilution.

STUDY CONTROLS

Plate Recovery Control

One plate recovery control film was prepared to determine the baseline dried virus titer. The plate recovery control film was generated as described above in "Preparation of Virus Films." Following drying, a 2.0 ml aliquot of test medium was overlaid on the control film. The carrier was then gently rotated to ensure complete coverage of the solution over the entirety of the surface. The carrier was held covered at the Sponsor-requested exposure temperature of 24.1-24.4 °C in a relative humidity of 31% for the requested contact time of 10 minutes. Just prior to the completion of the study contact time, a sterile cell scraper was used to re-suspend each viral film and the solution was immediately transferred into a gel filtration column. The re-suspended content of the carrier was passed through the gel filtration column using the syringe plunger. Serial 10-fold dilutions (e.g. 0.1 ml filtrate + 0.9 ml test media) of the filtrate (10) dilution) were prepared to the appropriate dilution. The results of the plate recovery control were microscopically evaluated at the same time as the results of the test substance and all other controls. The results of this control were used to calculate the log reduction in viral titer following exposure of the test substance to the test virus.

STUDY CONTROLS (cont.)

Cytotoxicity Control

For each lot of test substance assayed, one sterile glass Petri dish carrier (containing no virus film) was treated in the same manner as the test carriers. A 2.0 ml aliquot of the use dilution of the use dilution of the test substance was added to the sterile Petri dish and held covered at the Sponsor-requested contact time of 10 minutes at the requested exposure temperature of 23.9- 24.5 °C in a relative humidity of 30-31%. Just prior to the completion of the study contact time, the carrier was scraped using a sterile cell scraper, and the test substance suspension was promptly transferred into a gel filtration column. The re-suspended test substance was passed through the gel filtration column using the syringe plunger. Serial 10-fold dilutions (e.g. 0.1 ml filtrate + 0.9 ml test media) of the filtrate (10 dilution) were prepared to the appropriate dilution. The results of the cytotoxicity control were microscopically evaluated at the same time as the results of the test substance and all other controls.

Test Substance Neutralization Control

For each lot of test substance assayed, one sterile glass Petri dish carer (containing no virus film) was treated in the same manner as the test carriers. A 2.0 ml aliquot of the use dilution of the test substance was added to the sterile Petri dish. The carrier was scraped using a sterile cell scraper, and the test substance suspension was promptly transferred into a gel filtration column. The re-suspended test substance was passed through the gel filtration column using the syringe plunger. A 2.0 ml aliquot of test media, or other media as appropriate, was passed through the gel filtration column in the same manner as the test to serve as a neutralization control substance, to determine if comparable levels of infectious viral units were recovered from the control and the neutralized test substance filtrate. The filtrate is considered the 10' dilution.

To verify that the test substance had been neutralized, the filtrate (neutralized test substance) and the neutralization control substance were each challenged with a 0.1 ml aliquot of low titer (e.g. 1000-5000) infective units of the test system and held for at least 10 minutes at an exposure temperature of 24.1-24.7 °C in a relative humidity of 30-31%. Serial 10-fold dilutions were prepared using test media by adding 0.100 ml filtrate to 0.900 ml test media. The results of the neutralization control were microscopically evaluated at the same time as the results of the test substance and all other controls.

Cell Culture Control

To ensure that the host cells were not contaminated with bacteria, fungi, or any cytopathogenic viruses, and to confirm the viability of the cells during the incubation period of the assay, af least four cell monolayers were left untreated and microscopically examined periodically throughout the incubation period. Any obvious contamination or degeneration in such monolayers could invalidate the virucidal efficacy assay.

Virus Inoculum Titer Control

To confirm that the host cell-line monolayers were susceptible to the test virus and to confirm the titer of the viral inoculum, an aliquot of the test virus inoculum was serially diluted (10-fold) in test media. This control was also used to confirm the level of virus inoculated in the Neutralization Control.

STUDY CONTROLS (cont.)

Infectivity Assay

A 0.1 ml aliquot of all test and control dilutions was inoculated into the host cell cultures (which contained test medium) in quadruplicate. The cell culture control wells contained just test medium. The assay plates were incubated at 33±2°C in a humidified atmosphere of 6±1% CO_2 for ~7 days. The assay plates were examined microscopically periodically throughout the incubation period with any changes to the monolayers including viral cytopathic effects (CPE), cytotoxicity, or contamination clearly documented in the raw data. Data obtained from the final reading are documented in the Results section of this report.

Table 1: Plate Recovery Control and Test Results

		Recovery Control	Test Results Lot: 000136	Lot: 21.05.19.01
Cell Control		0 0 0 0	N/A	N/A
Dilution	10^{-1}	+ + + +	0 0 0 0	0 0 + 0
	10^{-2}	+ + + +	0 0 0 0	0 0 0 0
	10^{-3}	+ + + +	0 0 0 0	0 0 0 0
	10^{-4}	+ + + +	0 0 0 0	0 0 0 0
	10^{-5}	0 0 + 0	0 0 0 0	0 0 0 0
	10^{-6}	0 0 0 0	0 0 0 0	0 0 0 0
	10^{-7}	0 0 0 0	N/A	N/A
$TCID_{50}$ /0.1ml		4.75 log_{10}	< 0.50 log_{10}	0.75 log_{10}
$TCID_{50}$ / Carrier		5.05 log_{10}	< 0.80 log_{10}	1.05 log_{10}
Log Reduction / Carrier		N/A	> 4.25 log_{10}	4.00 log_{10}

Table 2: Cytotoxicity Control Results

		Cytotoxicity Control	
		Lot: 000136	Lot:21.05.19.01
Dilution	10^{-1}	0 0 0 0	0 0 0 0
	10^{-2}	0 0 0 0	0 0 0 0
	10^{-3}	0 0 0 0	0 0 0 0
		< 0.50 log_{10}	< 0.50 log_{10}

Table 3: Test Substance Neutralization Control Results

		Neutralization Control	
	Lot: 000136	Lot: 21.05.19.01	Control Substance
Dilution 10^{-1}	+ + + +	+ + + +	+ + + +
10^{-2}	+ + + +	+ + + +	+ + + +
10^{-3}	+ + + +	+ + + +	+ + + +
10^{-4}	+ + + +	+ + + +	+ + + +
10^{-5}	+ + + +	+ + + +	+ + + +
10^{-6}	0000	0 0 + 0	+ 0 0 0
$TCID_{50}$ /0.1ml	5.50 log_{10}	5.75 log_{10}	5.75 log_{10}

Table 4: Virus Inoculum Titer Control

	Virus Inoculum Titer Control
Dilution 10^{-1}	+ + + +
10^{-2}	+ + + +
10^{-3}	+ + + +
10^{-4}	0 + + +
10^{-5}	+ 0 0 0
10^{-6}	0 0 0 0
$TCID_{50}$ /0.1ml	5.50 log_{10}

STUDY CONCLUSION

The purpose of the study was to determine the virucidal efficacy of BioDox Chlorine Dioxide 4000 ppm (Lots: 000136 and 21.05.19.01) against Human coronavirus, 229E strain, ATCC VR-740, with no additional supplementation of organic soil load incorporated into the test inoculum,at a contact time of 10 minutes and exposure temperature of room temperature.

The Plate Recovery Control demonstrated a viral titer of 4.75 $\log_{10}$ $TCID_{50}$ per 0.1 ml and 5.05 $\log_{10}$ $TCID_{50}$ per carrier, thereby satisfying U.S. EPA study acceptance criteria of a minimum of 4.80 $\log_{10}$ infective units per control carrier.

Taking the cytotoxicity and neutralization control results into consideration, the evaluated test substance, BioDox Chlorine Dioxide 4000 ppm, demonstrated a > 4.25 $\log_{10}$ reduction in viral titer for Lot: 000136 and a 4.00 $\log_{10}$ reduction in viral titer for Lot: 21.05.19.01.

No test substance cytotoxicity was detected in either lot of test substance assayed, <0.50 $\log_{10}$ TCD_{50} per 0.1 ml for Lot: 000136 and < 0.5 $\log_{10}$ TCD_{50} per 0.1 ml for Lot: 21.05.19.01.

The test substance and control substance demonstrated comparable levels of infective unitsrecovered in the Neutralization Control.

No microbial contamination of any host cell cultures was observed during the course of the study.

BioDox Chlorine Dioxide 4000 ppm (Lots: 000136 and 21.05.19.01) met the U.S. EPA Product Performance Guidelines for Disinfectants for Use on Hard Surfaces outlined in U.S. EPA OCSPP 810.2200 and the success criteria detailed in the approved protocol when tested against Human coronavirus, 229E strain, ATCC VR-740 at contact time of 10 minutes.

This study was carried out in compliance with the approved protocol. All experimental controls met the established acceptance criteria unless otherwise noted in the Protocol Changes section of this report.

There were no circumstances that may have affected the quality or the integrity of the data.

REFERENCES

- *Annual Book of ASTM Standards, Standard Test Method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces, Designation E1053, current edition. American Society for Testing Materials, 100 Barr Habor Drive, West Conshohocken, PA 19428.*

- *Annual Book of ASTM Standards, Standard Practice for Use of Gel Filtration Columns for Cytotoxicity Reduction and Neutralization, Designation E1482, current edition. American Society for Testing Materials, 100 Barr Harbor Drive, West Conshohocken, PA 19428.*

- *Official Methods of Analysis of the AOAC International, Chapter 6, Disinfectants, Official Method 960.09 Germicidal and Detergent Sanitizing Action of Disinfectants. Revised 2013.*

- *U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines OCSPP 810.2000: General Considerations for Testing Public Health Antimicrobial Pesticides - Guidance for Efficacy Testing. February 2018.*

- *U.S. Environmental Protection Agency, Office of Chemical Safety and Pollution Prevention, Product Performance Test Guidelines OCSPP 810.2200: Disinfectants for Use on Environmental Surfaces - Guidance for Efficacy Testing. February 2018.*

- *U.S. Environmental Protection Agency, Frequent Questions for the 2018 series 810- Product Performance Test Guidelines: Antimicrobial Efficacy Test Guidelines. 2019.*

- *Guidance Document - Disinfectant Drugs. Health Canada. April 2020.*

- *Guidance Document - Safety and Efficacy Requirement for Hard Surface Disinfectant Drugs. Health Canada. April 2020.*

Manufactured in the USA by BioCentric Solutions
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The BioCentric Solutions Ethos

BioDox™ was developed by BioCentric Solutions, a company that believes in creating the most effective solutions to dangerous pathogens without harming people or our planet. Our mission is to create safe and effective solutions that improve the health of the world around us.