

Volume \_\_\_\_\_

**FINAL REPORT**

**Study Title**

**Evaluation of Virucidal Efficacy on Surface by EcoloxTech's Hypochlorous Acid Solution against Murine Norovirus (Surrogate for Human Norovirus)**

**Test Substance**

**Hypochlorous acid solution (HAS)  
(Generated by EcoloxTech 240 System using NaCl solution)**

**Test Surfaces**

**Ceramic Tile**

**Test Organism**

**Murine Norovirus, Strain MNV-G; Yale University**

**Test Guidelines**

**EPA Guidelines 810.2000 and 810.2200 (G)**

**Author**

**Cory Chiossone**

**Study Completion Date**

**09/04/18**

**Performing Laboratory**

**Microbac Laboratories, Inc.  
105 Carpenter Drive  
Sterling, VA 20164**

**Laboratory Project Identification Number**

**974-101**

**Protocol Identification Number**

**ECO.1.06.22.18**

**Sponsor**

**IET, Inc. dba EcoloxTech  
102 NW 22 Ave  
Miami, FL 33125**

**Page 1 of 34**

**STATEMENT OF NO DATA CONFIDENTIALITY**

Title: Evaluation of Virucidal Efficacy on Surface by EcoloxTech's Hypochlorous Acid Solution against Murine Norovirus (Surrogate for Human Norovirus)

Performed by: Microbac Laboratories, Inc.  
105 Carpenter Drive  
Sterling, VA 20164

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Submitter signature: \_\_\_\_\_ Date: \_\_\_\_\_

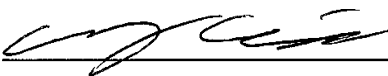
Typed Name of Signer: Scott Hartnett

Typed Name of Company: EcoloxTech

### COMPLIANCE STATEMENT

This study was conducted in accordance with 40 CFR Part 160.

Information of the identity, strength, purity, stability, uniformity and dose solution analysis of the test substance resides with the sponsor of the study.

Study Director Signature:  Date: 8/4/18  
Typed Name: Cory Chiossone  
Typed Name of Laboratory: Microbac Laboratories, Inc.

Sponsor signature: \_\_\_\_\_ Date: \_\_\_\_\_  
Typed Name of Signer: Scott Hartnett  
Typed Name of Company: EcoloxTech

Submitter signature: \_\_\_\_\_ Date: \_\_\_\_\_  
Typed Name of Signer: Scott Hartnett  
Typed Name of Company: EcoloxTech

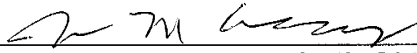
### QUALITY ASSURANCE UNIT STATEMENT

Title: Evaluation of Virucidal Efficacy on Surface by EcoloxTech's Hypochlorous Acid Solution against Murine Norovirus (Surrogate for Human Norovirus)

The Quality Assurance Unit of Microbac has inspected Project Number 974-101 in compliance with current Good Laboratory Practice regulations, (40 CFR § 160).

The dates that inspections were made and the dates that findings were reported to management and to the study director are listed below.

<u>PHASE INSPECTED</u>	<u>DATE OF INSPECTION</u>	<u>DATE REPORTED TO STUDY DIRECTOR</u>	<u>DATE REPORTED TO MANAGEMENT</u>
Protocol	08/08/18	08/08/18	08/08/18
In Process (Test)	08/08/18	08/08/18	08/08/18
Final Report	08/25/18	08/25/18	08/25/18

  
\_\_\_\_\_  
Jeanne M. Anderegg, RQAP-GLP  
Quality Assurance Manager

09-04-2018  
Date

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## TEST SUMMARY

**Title:** Evaluation of Virucidal Efficacy on Surface by EcoloxTech's Hypochlorous Acid Solution against Murine Norovirus (Surrogate for Human Norovirus)

**Study design:** This study was performed according to the signed protocol and project sheet(s) issued by the Study Director (See Appendix I).

Test substances supplied by the sponsor of the study:

<b>Equipment</b>	<b>Disinfectant</b>	<b>Dose</b>	<b>Contact time</b>
Ecolox 240 (DS No. I497, Lot No. 39362661, Received at Microbac on 08/08/18)	Hypochlorous acid solution (HAS) (Generated by Ecolox 240 using NaCl solution)	50 ppm of free chlorine at pH 8 (25% hypochlorous acid)	1 minute

Test surfaces:

<b>Test Surface (approx. 2" x 2")</b>	<b>Lot No.</b>	<b>Date Received</b>	<b>Assigned DS. No.</b>
Ceramic Tile	N/A	07/27/18	I493

**Sponsor:** IET, Inc. dba EcoloxTech  
102 NW 22 Ave  
Miami, FL 33125

**Study Personnel:** Semhar Fanuel, Cory Chiossone.

## TEST CONDITIONS

Challenge virus:

Murine Norovirus, Strain MNV-G; Yale University

Host:

RAW 264.7 cells, ATCC TIB-7.1

Active ingredients:

Hypochlorous acid, 50 ppm of free chlorine at pH 8 (25% hypochlorous acid)

Dilution medium:

Dulbecco's Modified Eagle Medium (DMEM) + 2% Fetal Bovine Serum (FBS)

Neutralizer:

DMEM + 10% FBS + 0.5% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>

Dilution(s)

Ready-to-Use

Contact time:

1 minute

Contact temperature and relative humidity (RH):

Room Temperature 20±1°C (Actual: 21°C)

Test coupon preparation:

Coupons were steam sterilized for 15 minutes and the UV irradiated for 15 minutes per side on the day of testing.

Carrier inoculation and dry time:

Test surface coupons were inoculated with 0.4mL of virus in a 4 in<sup>2</sup> area and dried for 30 minutes at 21°C.

### **TEST CONDITIONS (continued)**

Test substance application:

2.0 mL of test substance was added to the dried virus on the test surface coupons.

Organic load:

5% serum in viral inoculum

Media and reagents:

DMEM + 2% FBS  
DMEM + 10% FBS + 0.5% Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub>  
DMEM + 5% FBS

### **STUDY DATES AND FACILITIES**

The laboratory phase of this test was performed at Microbac Laboratories Inc., 105 Carpenter Drive, Sterling, VA 20164, from 08/08/18 to 08/15/18. The study director signed the protocol on 08/08/18. The study completion date is the date the study director signed the final report.

All changes or revisions of the protocol were documented, signed by the study director, dated and maintained with the protocol.

### **RECORDS TO BE MAINTAINED**

All testing data, protocol, protocol modifications, test substance records, the final report, and correspondence between Microbac and the sponsor will be stored in the archives at Microbac Laboratories Inc., 105 Carpenter Drive, Sterling, VA 20164, or at a controlled facility off site.



## CALCULATION OF TITER

The 50% Tissue Culture Infectious Dose per mL (TCID<sub>50</sub>/mL) was determined using the Spearman-Kärber method using the following formula:

$$m = x_k + \left(\frac{d}{2}\right) - d \sum p_i$$

where:

m = the logarithm of the titer at which half of the wells are infected relative to the test volume

x<sub>k</sub> = the logarithm of the smallest dosage which induces infection in all cultures

d = the logarithm of the dilution factor

p<sub>i</sub> = the proportion of positive results at dilution i

∑p<sub>i</sub> = the sum of p<sub>i</sub> (starting with the highest dilution producing 100% infection)

The values were converted to TCID<sub>50</sub>/mL using a sample inoculum of 1.0 mL.

The Virus Load was calculated in the following manner:

$$\text{Virus Load (Log}_{10} \text{ TCID}_{50}) = \text{Virus Titer (Log}_{10} \text{ TCID}_{50}/\text{mL}) + \text{Log}_{10} [\text{Volume per sample (mL)}]$$

The Log<sub>10</sub> Reduction Factor (LRF) was calculated in the following manner:

$$\text{Log}_{10} \text{ Reduction Factor} = \text{Initial viral load (Log}_{10} \text{ TCID}_{50}) - \text{Output viral load (Log}_{10} \text{ TCID}_{50})$$

## RESULTS

Results are presented in Tables 1–5.

Key (for all tables):

T/y = Cytotoxicity observed in y wells inoculated; viral cytopathic effects (CPE) could not be determined

X/y = X wells out of y wells inoculated exhibited positive viral cytopathic effect

0/y = 0 out of y wells inoculated exhibited positive viral CPE; no cytotoxicity or bacterial contamination was observed in any of the wells inoculated

## RESULTS (continued)

**Table 1**  
**Test Substance**

Dilution*	50 ppm of free chlorine at pH 8 (25% hypochlorous acid)	
	Replicate 1	Replicate 2
10 <sup>-1</sup>	4/4	4/4
10 <sup>-2</sup>	1/4	2/4
10 <sup>-3</sup>	0/4	0/4
10 <sup>-4</sup>	0/4	0/4
10 <sup>-5</sup>	0/4	0/4
10 <sup>-6</sup>	0/4	0/4
Titer (Log <sub>10</sub> TCID <sub>50</sub> /mL)	1.75	2.00
Load (Log <sub>10</sub> TCID <sub>50</sub> ) Per Carrier (0.4 mL of Undilute)	1.35	1.60
<b>Log<sub>10</sub> Reduction</b>	<b>4.07</b>	<b>3.82</b>

\*Dilution refers to the fold of dilution from the virus inoculum.

**Table 2**  
**Neutralizer Effectiveness/Viral Interference and Cytotoxicity Controls**

Dilution*	50 ppm of free chlorine at pH 8 (25% hypochlorous acid)	
	Neutralizer Effectiveness/Viral Interference Control	Cytotoxicity Control
10 <sup>-1</sup>	4/4	0/4
10 <sup>-2</sup>	4/4	0/4
10 <sup>-3</sup>	4/4	0/4

\*Dilution refers to the fold of dilution from the mock inoculum.

## RESULTS (continued)

**Table 3**  
**Coupon Recovery Control**

Dilution*	Coupon Recovery Control	
	Replicate 1	Replicate 2
10 <sup>-3</sup>	4/4	4/4
10 <sup>-4</sup>	4/4	4/4
10 <sup>-5</sup>	3/4	4/4
10 <sup>-6</sup>	1/4	2/4
10 <sup>-7</sup>	0/4	0/4
10 <sup>-8</sup>	0/4	0/4
Titer (Log <sub>10</sub> TCID <sub>50</sub> /mL)	5.50	6.00
Load (Log <sub>10</sub> TCID <sub>50</sub> ) Per Carrier (0.4 mL of Undilute)	<b>5.10</b>	<b>5.60</b>
Average Load (Log <sub>10</sub> TCID <sub>50</sub> ) Per Carrier	<b>5.42</b>	

\*Dilution refers to the fold of dilution from the virus inoculum.

**Table 4**  
**Virus Stock Titer Control**

Dilution*	Virus Stock Titer Control
10 <sup>-4</sup>	4/4
10 <sup>-5</sup>	4/4
10 <sup>-6</sup>	3/4
10 <sup>-7</sup>	0/4
10 <sup>-8</sup>	0/4
10 <sup>-9</sup>	0/4
Titer (Log <sub>10</sub> TCID <sub>50</sub> /mL)	6.25

\*Dilution refers to the fold of dilution from the virus inoculum.

**Table 5**  
**Cell Viability Control**

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

## CONCLUSIONS

According to the US Environmental Protection Agency, the test substance passes the Virucidal Hard-Surface Efficacy Test if the product demonstrates a  $\geq 3 \log_{10}$  reduction on each surface in the presence or absence of cytotoxicity. When cytotoxicity is present, the virus control titer should be increased, if necessary, to demonstrate a  $\geq 3 \log_{10}$  reduction in viral titer on each surface beyond the cytotoxicity level.

When tested as described, Hypochlorous acid solution (HAS) passed the Virucidal Hard-Surface Efficacy Test when Murine Norovirus (surrogate for Human Norovirus), containing 5% serum, was exposed to the test substance for 1 minute at 21°C. All of the controls met the criteria for a valid test. These conclusions are based on observed data.

## APPENDIX I



## Microbac Protocol

# Evaluation of Virucidal Efficacy on Surface by EcoloxTech's Hypochlorous Acid Solution against Murine Norovirus (Surrogate for Human Norovirus)

Prepared for  
IET, Inc. dba EcoloxTech  
102 NW 22 Ave  
Miami, FL 33125

Testing Facility  
Microbac Laboratories, Inc.  
105 Carpenter Drive  
Sterling, Virginia 20164

June 22, 2018

Microbac Protocol: ECO.1.06.22.18

Microbac Project: 974-101

Microbac Laboratories, Inc.  
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## **I. OBJECTIVE**

This study is designed to evaluate the virucidal efficacy of EcoloxTech's hypochlorous acid solution (HAS), when applied in liquid onto surface coupons, against Human Norovirus (HuNoV) using Murine Norovirus (MNV) as a surrogate virus.

The test will simulate consumer use and conforms to the EPA OCSP 810.2000 and 810.2200 Test substance Performance Test Guidelines. It also follows the procedure outlined in the ASTM International test method designated E1053-11, "Standard Test Method to Assess Virucidal Activity of Chemicals Intended for Disinfection of Inanimate, Nonporous Environmental Surfaces". This study will be run according to Good Laboratory Practice Standards (40 CFR Part 160).

## **II. SUMMARY OF TESTING CONDITIONS**

HAS will be generated at the Microbac facility by EcoloxTech's personnel using EcoloxTech's electrolyzing machine and NaCl solution.

One batch of NaCl solution will be used to generate the HAS, at one dose and one pH value [50 ppm of free chlorine at pH 8 (25% hypochlorous acid)]. The generated HAS will be tested for efficacy against MNV at one contact time (1 minute) using coupons representing one surface (ceramic tile), at two replicates (N=2).

For each run, the test surface coupon ("carrier") will be inoculated with an aliquot of virus inoculum, dried and treated with a specific dose of the disinfectant and held for the specified contact time. Then virus on each carrier will be recovered into an appropriate volume of a virus recovery solution (= neutralizer). The "post neutralized sample" (PNS) will be serially diluted in dilution medium (DM) and selected dilutions will be inoculated onto host cells to determine the titer of infectious virus. The titer will be compared to a coupon recovery control wherein the same amount of virus is added to the carrier, dried, treated with DM (in lieu of disinfectant) and recovered.

### III. SELECTION OF VIRUS FOR EVALUATION

When selecting a target virus for disinfection efficacy studies, the potential of contamination and its implication to public health shall be considered. The ability of virus to grow to high titer in serum-free or low protein medium, and its ease of detection in a sensitive and reliable assay are also important aspects of virus selection.

According to the World Health Organization (WHO), viral-induced diarrheal diseases lead to an annual loss of 73 million years of healthy life; and viral infections are estimated to kill nearly 6 million persons per year globally. Additionally, viruses are among the most frequent nosocomial pathogens. Human norovirus is the leading cause of acute nonbacterial gastroenteritis. The U.S. Centers for Disease Control and Prevention estimate that approximately 23 million people suffer from Human norovirus gastroenteritis each year. The significance of regular and proper hand decontamination in infection control is well recognized.

The virus to be tested in this study will be MNV, a surrogate for HuNoV. Both viruses belong to the *Caliciviridae* family. Due to lack of an efficient *in-vitro* culture system for HuNoV, MNV is selected. It is phylogenetically close to HuNoV.

A summary of the biological properties of the virus and their resistance to physical-chemical inactivation is presented in Table 1.

**Table 1**  
**Biological Properties of the Challenging Virus**

<b>Virus</b>	<b>Family</b>	<b>Genus</b>	<b>Genome</b>	<b>Envelope</b>	<b>Size (nm)</b>	<b>Source</b>	<b>Resistance</b>
MNV*	<i>Caliciviridae</i>	<i>Norovirus*</i>	ssRNA	No	30-40	Murine	High

\* HuNoV also belongs to the *Norovirus* genus.



#### IV. MATERIALS AND EQUIPMENT

- A. Responsible parties (Microbac and Sponsor) will supply the necessary equipment and supplies for the studies.

The test substance will be tested as supplied by the sponsor unless directed otherwise. Specialized storage conditions must be specified by the sponsor before initiation of testing.

The sponsor assures Microbac testing facility management that the test article has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

Microbac will retain all unused test articles for a period of one year upon completion of the test, then return them to the sponsor of the study or discard them in a manner that meets the approval of the safety officer.

- B. Materials supplied by Microbac, including, but not limited to:

1. Challenge virus (requested by the Sponsor of the study):
  - MNV, strain MNV-G; Yale University

**Note:** an organic load of 5% serum in the inocula will be present.

2. Host cells:
  - RAW 264.7 cells (for MNV)
3. Laboratory equipment and supplies.
4. Media and reagents:

Media and reagents relevant to the virus-host system and test substance being tested will be documented in the first project sheet and data pack.

C. The Sponsor will provide the following:

1. **Surface coupons (carriers)**, each 1" x 4" or 2" x 2":
  - Ceramic tile
  
2. **Disinfectant:**
  - Hypochlorous acid solution (HAS) will be generated at Microbac on each day of testing by EcoloxTech's personnel using an Ecolox 1200 (or equivalent model) electrolyzing machine and NaCl solution
  
  - One batch of NaCl solution will be used, at two replicates (N=2)

Note: No wiping will be used.

## V. TEST SYSTEM IDENTIFICATION

All applicable dilution tube racks and host-containing apparatus will be labeled with the following information: virus and project number.

## VI. EXPERIMENTAL DESIGN

Microbac will provide the spiking virus and will contaminate the coupons (carriers). Microbac will operate the coupon disinfection procedure using the HAS generated and supplied by the sponsor representative (EcoloxTech's personnel), neutralize and collect the samples, and titrate the samples to endpoint to determine the infectious virus titer.

A) Inoculum preparation:

Viral stocks are originally obtained from reputable sources and are propagated at Microbac. The original source of virus will be included in the final report. Records are maintained that demonstrate the origin of the virus. The stock virus is titered and stored in an ultra-low temperature freezer.

Frozen viral stocks will be thawed on the day of the test (freshly prepared viral stocks may also be used). The organic soil concentration will be adjusted to 5%

(if not already 5%) for the virus stock unless otherwise directed by the Sponsor and pre-approved by Microbac.

Note: a level of at least 4.8-Log<sub>10</sub> virus load challenge per carrier (as indicated by the coupon recovery control load), and at least 3.0-Log<sub>10</sub> beyond the level of cytotoxicity, when present, should be achieved.

B) Coupon (carrier) preparation and viral contamination:

The carriers will be treated by one or more of the following methods to reduce the bioburden prior to use:

- (1) incubating in a 180°C hot oven for 10-15 minutes
- (2) exposure to Ultra-Violet (UV) light for 15 minutes per side
- (3) steam sterilization for 10-15 minutes at 121°C

The exact treatment and sterilization procedure utilized will be described in the final report.

On the day of testing, a 0.4-mL aliquot of virus inoculum will be applied onto the designated side of each carrier using a micro-pipettor and spread over the surface of the carrier. The carrier may be placed in a Petri dish during the inoculation. Then the virus will be allowed to dry for 20 – 40 minutes at room temperature. The drying time and temperature will be recorded.

A total of 4 carriers will be prepared using virus - 2 carriers for HAS treatment and 2 for the coupon recovery control (CRC). Additionally, 1 carrier will be prepared for the neutralizer effectiveness/viral interference (NE/VI) and cytotoxicity (CT) controls using dilution medium (DM) in lieu of virus as the inoculum.

C) Disinfectant preparation:

The HAS will be generated at Microbac facility by EcoloxTech's personnel. The NaCl, HCl and the related equipment will be provided by EcoloxTech. One batch of NaCl will be used to generate the HAS. The free available chlorine will be confirmed by EcoloxTech.

D) Virucidal efficacy evaluation:

Procedures involved in performance of this study are described in a detailed series of SOPs that are maintained at Microbac. SOPs and Logs are referred to in the raw data. The performance and sample collection for the study are diagrammed in Figures 1 - 2, with details described below.

For each run, after the virus inoculum has dried for about 20 – 40 minutes, the carrier will be treated with 2.0 mL HAS in liquid using a pipette. The HAS should cover the entire dried inoculum. Use a micropipette tip or a spreader to spread the HAS across the surface if required. Note: No wiping will be used.

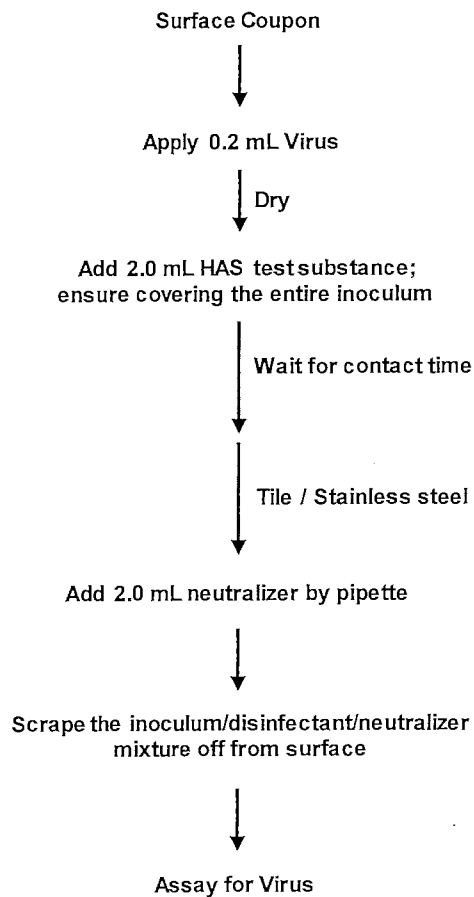
Then the carrier will remain at the contact temperature and for the time specified by the sponsor. After the contact period, each tile coupon will be neutralized with 2.0 mL of an appropriate neutralizer via pipetting and the inoculum/disinfectant/neutralizer mixture will be scraped off from the surface with a cell scraper and collected into a sterile test tube. This “post neutralized sample” (PNS) will be considered undiluted.

If Sephacryl columns are used to aid in the neutralization and to further reduce the cytotoxicity, 0.8 mL aliquots of the inoculum/test substance/neutralizer mixture sample will be loaded onto a pre-spun Sephacryl column and centrifuged at 1,000 RPM for 3 minutes. Following the passage through columns, the eluates will be aseptically collected and serially ten-fold diluted in DM. If columns are not used, serial ten-fold dilutions of the inoculum/disinfectant test substance/neutralizer mixture will directly be prepared in DM.

Each PNS will then be analyzed by a cell culture-based infectivity assay to determine the titer of infectious virus, as described below.

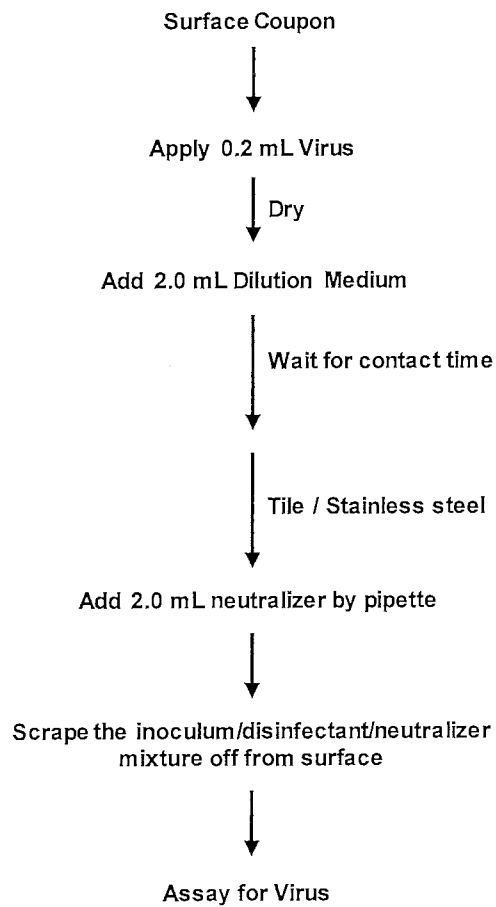
### FIGURE 1 – Disinfectant Treatment

<b>DISINFECTANT TYPE:</b>	Hypochlorous acid solution (HAS)	
<b>DISINFECTANT BATCH:</b>	1	
<b>DISINFECTANT DOSES:</b>	1	[50 ppm of free chlorine at pH 8 (25% hypochlorous acid)]
<b>COUPON SURFACE:</b>	1	(Ceramic Tile)
<b>CONTACT TIME:</b>	1	(1 minute)
<b>CHALLENGE VIRUS:</b>	1	(MNV)
<b>REPLICATES:</b>	Two replicates (N=2)	



## FIGURE 2 – Coupon recovery control

<b>COUPON SURFACE:</b>	1	(Ceramic Tile)
<b>CONTACT TIME:</b>	1	(1 minute)
<b>CHALLENGE VIRUS:</b>	1	(MNV)
<b>REPLICATES:</b>	Two replicates (N=2)	



E) Infectivity assay:

The collected PNS sample, considered undiluted, will be further serially diluted in DM and selected dilutions will be inoculated onto host cells.

For MNV (RAW 264.7 cells, 24-well format), each sample will be inoculated at 1.0 mL per well and 4 wells per dilution (24-well plates) and incubated at  $36\pm 2^{\circ}\text{C}$  with  $5\pm 3\%$   $\text{CO}_2$  for a period of 4-7 days. Note: The cell seeding medium will be aspirated prior to inoculation.

At the end of the incubation period, the inoculated cultures will be examined microscopically for viral-induced cytopathic effect (CPE) as indication of infectious virus. These observations will be recorded.

F) Controls

1. Coupon recovery control (CRC) – MNV:

This control will be performed in duplicate runs (N=2) (see Figure 2).

For each run, after the virus inoculum has dried for 20 – 40 minutes, the coupon will be applied with 2.0 mL dilution medium (DM) (in lieu of the HAS disinfectant). The DM should cover the entire dried inoculum. Then the coupon will be held at room temperature for the contact time.

After the contact period, each tile coupon will be neutralized with 2.0 mL of neutralizer using a pipette and the inoculum/DM/neutralizer mixture will be scraped off from the surface with a cell scraper and collected into a sterile test tube. This “post neutralized sample” (PNS) will be considered undiluted.

The PNS will then be serially diluted in DM and selected dilutions will be inoculated onto host cells to determine the titer of infectious virus as described in the “Infectivity Assay” section. This control will determine the relative loss in viral infectivity resulting from the drying of virus and the neutralization procedure.

The results from the CRC will be compared with the disinfectant-treated samples to calculate the Log<sub>10</sub> viral reduction for each test surface and virus.

2. Neutralizer effectiveness/Viral interference control (NE/VI) – MNV:

This control will determine if residual active ingredient is present after the neutralization, and if the neutralized disinfectant interferes with the virus infection system. This control will be performed for the test substance at one replicate (N=1).

For each type of test surface, the disinfectant test substance will be applied exactly as the test procedure but in lieu of virus inoculum, dried DM will be exposed to the test substance and assayed as previously described. Post-treatment and neutralization, the neutralized DM/test substance mixture will be divided into two portions, one for cytotoxicity control and the other for neutralizer effectiveness/viral interference control, and processed as the test.

If columns are used, each portion will be passed through individual columns and the eluate will be serially diluted ten-fold in DM. If columns are not used, each portion will be directly diluted using serial ten-fold dilutions in DM.

The neutralizer effectiveness/viral interference control sample will be diluted as follows: using dilution test tubes and appropriate pipette, an aliquot of the PNS will be used for making serial 10-fold dilutions in DM (for example, 0.5 mL sample + 4.5 mL DM). Following serial dilution, 0.1 mL of a low titered virus, containing up to approximately 1,000 – 5,000 infectious units of virus, will be added to 4.5 mL of each dilution and held for a period of no shorter than the contact time. Then these samples will be used to inoculate host cells as described for the test procedure.

Selected dilutions of the sample will be added to cultured cell monolayers at a minimum of four wells per dilution per sample, as described in the “Infectivity Assay” section.



3. Cytotoxicity control (CT) – RAW 264.7 (24-well) cells:

This control will be performed for the test substance at one replicate (N=1).

The cytotoxicity sample, acquired from the neutralizer effectiveness/viral interference control run, will be diluted and have no virus added. Selected dilutions will be inoculated and incubated in the same manner as the rest of the test and control samples. These effects are distinct from virus-induced cytopathic effects, which will be evident in the coupon recovery control cultures.

4. Cell viability control – RAW 264.7 (24-well) cells:

Four wells (for 24-well format) will be inoculated with DM during the incubation phase of the study. This control will demonstrate that cells remain viable throughout the course of the assay period; and will be used to compare with the cytotoxicity samples. This control will be performed on each day samples are assayed.

5. Virus Stock Titer control (assay positive control) – MNV:

An aliquot of the virus stock used in the study will be serially diluted with DM and inoculated onto the indicator cells. This control will demonstrate that the viral infectivity assay is performed appropriately. This control will be performed on each day viral-containing samples are assayed in singlet.

## VII. TITER CALCULATION

The 50% tissue culture infective dose per mL (TCID<sub>50</sub>/mL) will be determined using the method of Spearman-Kärber (Kärber G., Arch. Exp. Pathol. Pharmacol. 1931, 162: 480-483) or other appropriate methods such as Reed and Muench (Am. J. of Hyg. 1938, 27:493). These analyses will be described in detail in the final report. The test results will be reported as reduction of the virus titer post treatment with the test article expressed as log<sub>10</sub>.

The Virus Load will be calculated in the following manner:

Virus Load ( $\text{Log}_{10} \text{TCID}_{50}$ ) = Virus Titer ( $\text{Log}_{10} \text{TCID}_{50}/\text{mL}$ ) +  $\text{Log}_{10}$  [Volume per sample (mL)]

The  $\text{Log}_{10}$  Reduction Factor (LRF) will be calculated in the following manner:

$\text{Log}_{10}$  Reduction Factor = Initial viral load ( $\text{Log}_{10} \text{TCID}_{50}$ ) – Output viral load ( $\text{Log}_{10} \text{TCID}_{50}$ )

## VIII. TEST ACCEPTANCE CRITERIA

The study will be acceptable for evaluation of the test results if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance.

- The infectious virus recovered from the CRC control from each surface must be  $\geq 4.8\text{-log}_{10} \text{TCID}_{50}$  units.
- Viral-induced cytopathic effect (CPE) must be distinguishable from test substance induced cytotoxic effects (if any).
- Virus must be recovered from the neutralizer effectiveness/viral interference control (not exhibiting cytotoxicity).
- The cell viability control must remain viable and exhibit no viral induced CPE.

## IX. TEST SUBSTANCE EVALUATION CRITERIA:

According to the US Environmental Protection Agency, the test substance passes the test if (1) the product demonstrates a  $\geq 3 \text{ log}_{10}$  reduction on each surface in the presence or absence of cytotoxicity; and (2) if cytotoxicity is present, the virus control titer should be increased if necessary to demonstrate a  $\geq 3 \text{ log}_{10}$  reduction in viral titer on each surface beyond the cytotoxic level.

## **X. PERSONNEL AND TESTING FACILITIES**

A study director will be assigned before initiation of the test. Resumes for technical personnel are maintained and are available on request. This study will be conducted at Microbac, 105 Carpenter Drive, Sterling VA 20164.

## **XI. PROTOCOL AMENDMENTS AND DEVIATIONS:**

Any protocol amendment(s) and protocol deviation(s) identified will be reported in project sheet(s) and included in the final report.

## **XII. REPORT FORMAT**

The report will contain all items required by 40 CFR Part 160.185 and EPA 810.2200 and be in compliance with EPA PR Notice 2011-3 (replaced PRN 86-5). Microbac employs a standard report format for each test design. Each final report will provide at least the following information:

- Sponsor identification
- Test substance identification
- Type of assay and project number
- Study start and end time (clock time)
- Interpretation of results and conclusions
- Test results presented in tabular form
- Methods and evaluation criteria, if applicable
- Dates of study initiation and completion (GLP studies only)
- Signed Quality Assurance and Compliance Statements (GLP studies only)
- Certificate of Analysis (for GLP studies only; if provided by the Sponsor)

### **XIII. REGULATORY REQUIREMENTS AND QUALITY ASSURANCE**

This study will be performed in compliance with the US Environmental Protection Agency's Good Laboratory Practices (GLP) regulations, 40 CFR 160 (note: information on the identity, strength, purity, stability, uniformity, and dose solution analysis of the test substance resides with the sponsor of the study unless otherwise stated).

The Quality Assurance Unit of Microbac will inspect the conduct of the study for GLP compliance. The dates of the inspections and the dates that findings are reported to the study management and study director will be included in the final report.

### **XIV. RECORDS TO BE MAINTAINED**

For all GLP studies, the original signed final report will be sent to the Sponsor. All raw data, protocol, protocol modifications, test substance records, copy of final report, and correspondence between Microbac and the sponsor will be stored in the archives at Microbac, 105 Carpenter Drive, Sterling, Virginia 20164 or in a controlled facility off site.

All changes or revisions to this approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of any change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test substance; challenge virus and host cell line used and the type of neutralizers employed in the test will be addressed in a project sheet issued separately for each study. The date the study director signs the protocol will be the initiation date. All project sheets issued will be forwarded to the study sponsor for appropriate action.

**Table: Summary of samples to be assayed for liquid – MNV:**

Sample #	Test Surface	HAS dose	Contact time	Rep.	Sample Description
1	Tile	50 ppm (pH 8)	1 min	Rep. 1	MNV, Tile, HAS 50 ppm (pH 8), 1 min, Rep. 1
2				Rep. 2	MNV, Tile, HAS 50 ppm (pH 8), 1 min, Rep. 2
3		N/A	1 min	Rep. 1	MNV, Tile, CRC, Rep. 1
4				Rep. 2	MNV, Tile, CRC, Rep. 2
5		50 ppm (pH 8)	1 min	Rep. 1	MNV, Tile, NEVI Control
6					MNV, Tile, TOX Control
7	N/A				Cell Viability Control
8	N/A				Virus Stock Titer Control

*CRC: Coupon Recovery Control*

*NEVI Control: Neutralizer Effectiveness/Viral Interference Control*

*TOX Control: Cytotoxicity Control*

**XV. MISCELLANEOUS INFORMATION**

The following is to be completed by Sponsor (please check all applicable open boxes):

A. Name and address: IET, Inc. dba EcoloxTech  
 102 NW 22 Ave  
 Miami, FL 33125

**B. Disinfectants:**

Equipment Model No.	Disinfectant	Dose	Contact time	NaCl Batch No.
Ecolox 1200 (to be confirmed)	Hypochlorous acid solution (HAS)  (Generated by Ecolox 1200 using NaCl solution)	50 ppm of free chlorine at pH 8 (25% hypochlorous acid)	1 minute	To be recorded

**C. Test Surface:**

Test surface description	Lot No.	Side to be tested
Ceramic Tile	N/A	

D. Contact temperature: Ambient room temperature (20±1°C)  
 Disinfectant application: Direct liquid application  
 Organic load: 5% serum in viral inoculum

E. Precautions/storage – MSDS or CofA of disinfectant provided:  Yes  No

**REPORT HANDLING:**

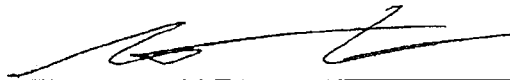
The sponsor intends to submit this information to:  CDC; or  Other: \_\_\_\_\_

**STUDY CONDUCT:** GLP

*Continued on next page*

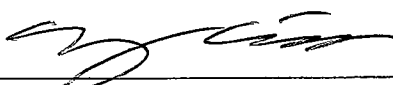
**XV. MISCELLANEOUS INFORMATION: (Continued)**

**PROTOCOL APPROVAL BY SPONSOR:**

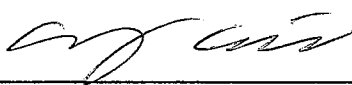
Sponsor Signature:  Date: 7/25/2018

Printed Name: Scott Hartnett



**PROTOCOL APPROVAL BY STUDY DIRECTOR (Microbac):**

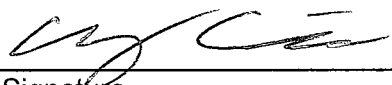
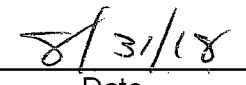
Study Director Signature:  Date: 8/8/18

Printed Name: Cory Chiosone

Date Issued: 08/08/18 Project Sheet No. 1 Page No. 1 Laboratory Project Identification No. 974-101			
<b>STUDY TITLE:</b> Evaluation of Virucidal Efficacy on Surface by EcoloxTech's Hypochlorous Acid Solution against Murine Norovirus (Surrogate for Human Norovirus)		<b>STUDY DIRECTOR:</b> S. Steve Zhou, Ph.D.	
		 <span style="float: right; font-size: 1.2em;">8/8/18</span>	
		Signature	Date
<b>TEST MATERIAL(S):</b> EcoloxTech 240 System Saltwater Additive	<b>LOT NO.</b> 39362661 N/A	<b>DATE RECEIVED:</b> 08/08/18 08/08/18	<b>DS NO.</b> 1497 1498
	<b>TEST SURFACE COUPON(S):</b> Ceramic Tile	N/A	07/27/18
<b>PERFORMING DEPARTMENT(S):</b> Virology and Toxicology		<b>STORAGE CONDITIONS:</b> Location: I6 <input type="checkbox"/> Dark <input checked="" type="checkbox"/> Ambient Room Temperature <input type="checkbox"/> Desiccator <input type="checkbox"/> Freezer <input type="checkbox"/> Refrigerator <input type="checkbox"/> Other:	
<b>PROTECTIVE PRECAUTION REQUIRED:</b> MSDS <input type="checkbox"/> Yes / <input checked="" type="checkbox"/> No			
<b>PHYSICAL DESCRIPTION:</b> <input checked="" type="checkbox"/> Solid (coupon materials) <input checked="" type="checkbox"/> Liquid (disinfectants) <input type="checkbox"/> Aerosol			
<b>PURPOSE:</b> See attached protocol. <b>AUTHORIZATION:</b> See client signature.			
<b>PROPOSED EXPERIMENTAL START DATE:</b> 08/08/18 <b>TERMINATION DATE:</b> 08/17/18			
<b>CONDUCT OF STUDY:</b> <input type="checkbox"/> FDA <input type="checkbox"/> EPA <input type="checkbox"/> R&D <input checked="" type="checkbox"/> GLP <input type="checkbox"/> GCP <input type="checkbox"/> Other:			
<b>SPONSOR:</b> IET, Inc. dba EcoloxTech 102 NW 22 Ave Miami, FL 33125		<b>CONTACT PERSON:</b> Scott Hartnett Email: scott@ecolox.com	
<b>TEST CONDITIONS:</b>			
<b>Challenge organisms:</b>		Murine Norovirus, Strain MNV-G; Yale University	
<b>Host:</b>		RAW 264.7 cells, ATCC TIB-7.1	
<b>Organic load:</b>		5% serum in viral inoculum	
<b>Equipment</b>	<b>Disinfectant</b>	<b>Dose</b>	<b>Contact time</b>
Ecolox 1200	Hypochlorous acid solution (HAS) (Generated by Ecolox 1200 using NaCl solution)	50 ppm of free chlorine at pH 8 (25% hypochlorous acid)	1 minute
<b>Disinfectant application:</b>		Direct liquid application.	
<b>Dilution medium:</b>		Dulbecco's Modified Eagle Medium (DMEM) + 2% Fetal Bovine Serum (FBS)	
<b>Neutralizer(s):</b>		DMEM + 10% FBS + 0.5% Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub>	
<b>Contact temperature:</b>		Ambient room temperature (20±1°C)	
<b>Incubation time(s):</b>		4 – 7 days	
<b>Incubation temperature(s):</b>		36±2C in 5±3% CO <sub>2</sub>	



Date Issued: 08/08/18    Project Sheet No. 1    Page No. 2    Laboratory Project Identification No. 974-101			
<b>STUDY TITLE:</b> Evaluation of Virucidal Efficacy on Surface by EcoloxTech's Hypochlorous Acid Solution against Murine Norovirus (Surrogate for Human Norovirus)		<b>STUDY DIRECTOR:</b> Cory Chiossone	
		 	
		<hr/> Signature <span style="float: right;">Date</span>	
<b>TEST MATERIAL(S):</b> EcoloxTech 240 System Saltwater Additive	<b>LOT NO.</b> 39362661 N/A	<b>DATE RECEIVED:</b> 08/08/18 08/08/18	<b>DS NO.</b> I497 I498
<b>TEST SURFACE COUPON(S):</b> Ceramic Tile	N/A	07/27/18	I493
<b>PERFORMING DEPARTMENT(S):</b> Virology and Toxicology	<b>STORAGE CONDITIONS:</b> Location: A2 (I497, I498), I6 (I493) <input type="checkbox"/> Dark <input checked="" type="checkbox"/> Ambient Room Temperature <input type="checkbox"/> Desiccator <input type="checkbox"/> Freezer <input type="checkbox"/> Refrigerator <input type="checkbox"/> Other:		
<b>CONDUCT OF STUDY:</b> <input type="checkbox"/> FDA <input type="checkbox"/> EPA <input type="checkbox"/> R&D <input checked="" type="checkbox"/> GLP <input type="checkbox"/> GCP <input type="checkbox"/> Other:			
<b>SPONSOR:</b> IET, Inc. dba EcoloxTech 102 NW 22 Ave Miami, FL 33125		<b>CONTACT PERSON:</b> Scott Hartnett Email: scott@ecolox.com	
<b>PROTOCOL AMENDMENTS:</b>			
<ol style="list-style-type: none"> <li>1. The miscellaneous information section on protocol page 17 has blank spaces for "side to be tested", "Precautions/storage" and "report handling". The side to be tested is the top, a MSDS or CofA was not provided and the report is not being submitted to anyone other than the client. This amendment serves to fill in the blank spaces on protocol page 17.</li> <li>2. Protocol pages 8 and 9 state to apply 0.2 mL of virus to the test surface. The statement should be to add 0.4 mL to the test surface. This amendment serves to correct the volume of virus applied to the test surface on protocol page 8 and 9.</li> </ol>			

Date Issued: 08/31/18 Project Sheet No. 2 Page No. 1 Laboratory Project Identification No. 974-101			
<b>STUDY TITLE:</b> Evaluation of Virucidal Efficacy on Surface by EcoloxTech's Hypochlorous Acid Solution against Murine Norovirus (Surrogate for Human Norovirus)		<b>STUDY DIRECTOR:</b> Cory Chiossone	
		 Signature	
		 Date	
<b>TEST MATERIAL(S):</b> EcoloxTech 240 System Saltwater Additive	<b>LOT NO.</b> 39362661 N/A	<b>DATE RECEIVED:</b> 08/08/18 08/08/18	<b>DS NO.</b> I497 I498
<b>TEST SURFACE COUPON(S):</b> Ceramic Tile	N/A	07/27/18	I493
<b>PERFORMING DEPARTMENT(S):</b> Virology and Toxicology	<b>STORAGE CONDITIONS:</b> Location: A2 (I497, I498), I6 (I493) <input type="checkbox"/> Dark <input checked="" type="checkbox"/> Ambient Room Temperature <input type="checkbox"/> Desiccator <input type="checkbox"/> Freezer <input type="checkbox"/> Refrigerator <input type="checkbox"/> Other:		
<b>CONDUCT OF STUDY:</b> <input type="checkbox"/> FDA <input type="checkbox"/> EPA <input type="checkbox"/> R&D <input checked="" type="checkbox"/> GLP <input type="checkbox"/> GCP <input type="checkbox"/> Other:			
<b>SPONSOR:</b> IET, Inc. dba EcoloxTech 102 NW 22 Ave Miami, FL 33125		<b>CONTACT PERSON:</b> Scott Hartnett Email: scott@ecolox.com	
<b>PROTOCOL AMENDMENTS:</b>			
3. Protocol page 17 and the table on Project Sheet one state that the equipment used for this study would be the EcoloxTech 1200. Per sponsor email the device was an EcoloxTech 240. This amendment serves to correct the name of the equipment used for this study listed on protocol page 17 and Project Sheet 1.			
4. Project Sheet one lists the study director as S. Steve Zhou, Ph.D. The study director should be listed as Cory Chiossone. This amendment serves to correct the study director name listed on Project Sheet one.			