

**Final Report submitted to Virox Technologies Inc.  
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**ASSESSMENT OF THE BROAD-SPECTRUM  
MICROBICIDAL ACTIVITY OF ACCEL C.  
*DIFFICILE* GEL**

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## A. OBJECTIVE

The objective of this study was to evaluate the bactericidal, fungicidal and sporicidal activities of an accelerated hydrogen peroxide (AHP)-based gel (Accel *C. difficile* Gel) using the first tier (QCT-1) of a quantitative carrier test method (Springthorpe and Sattar, 2005) and its effectiveness as a virucide using ASTM International's protocol #E-1053 of (ASTM 2002). QCT-1 (#E-2111) is also a standard of ASTM International (ASTM 2000) and it incorporates all important elements specified in the Canadian General Standard Board's document number CAN/CGSB-2.161-97 entitled *Assessment of Efficacy of Antimicrobial Agents for use on Environmental Surfaces and Medical Devices* (CGSB 1997).

## B. MATERIALS AND METHODS

### **The Product:**

Three separate lots of Accel *C. difficile* Gel were provided by the sponsor for testing in this study. Upon arrival in our laboratory, the bottles were stored at room temperature in a place with restricted access.

### **Carriers:**

For bactericidal, fungicidal and sporicidal tests using QCT-1, the inside bottom surface of glass vials (Galaxy Co., Newfield, New Jersey) was the carrier. The carriers for virucidal tests using ASTM-1053 were glass Petri dishes. The carriers were reused after their decontamination, cleaning and autoclave sterilization.

### **Soil Load:**

For inoculation of the carriers, all the test organisms were first suspended in a tripartite soil load: 25  $\mu$ L of bovine serum albumin, 100  $\mu$ L of mucin and 35  $\mu$ L of Tryptone were added to 340  $\mu$ L of the bacterial suspension. This soil load mixture contained a level of protein roughly equal to that in 5% bovine serum.

### **Neutralizer, Microbial Diluent and Filter Rinse:**

Lethen Broth (with 1% sodium thiosulphate pentahydrate) was used as the neutralizer and to rinse the membrane filters and the filter holder unit. Normal saline was used to make dilutions of the bacterial suspensions and as the final rinse of the carrier vials and the filter holder unit to aid in rinsing off the froth created by the Lethen broth.

For the virucidal tests Lethen Broth (with 1% sodium thiosulphate pentahydrate) was also used as the neutralizer, and Earle's Balanced Salt Solution (EBSS) was used as the diluent.

### **Test Organisms:**

The organisms used for this study and their specific strain numbers, where available, are given below:

1. *Staphylococcus aureus* (ATCC 6538)
2. *Pseudomonas aeruginosa* (ATCC 15442)
3. *Salmonella choleraesuis* (ATCC 10708)
4. *Bacillus subtilis* (ATCC 19659)
5. *Clostridium sporogenes* (ATCC 7955)

6. *Clostridium difficile* (clinical isolate)
7. *Trichophyton mentagrophytes* (ATCC 9533)
8. The Sabin vaccine strain of Poliovirus type 1 (ATCC VR-192)

a) ***Bacillus subtilis* (ATCC 19659)**: *B. Subtilis* spores were grown aerobically in a 1:10 dilution of Columbia broth (Difco), with manganese, for 72 hours at 37°C. To yield a concentration of 10<sup>9</sup> spores/mL, the spore suspension was centrifuged, washed and resuspended in sterile distilled, deionized water.

b) ***Clostridium sporogenes* (ATCC 7955)**: *C. sporogenes* spores were grown anaerobically in undiluted Columbia broth for 5 days at 30°C. To yield a concentration of 10<sup>9</sup> spores/mL the spore suspension was centrifuged, washed and resuspended in sterile distilled, deionized water.

c) ***Clostridium difficile* (Clinical isolate obtained from Dr. Frank Chan of the Children's Hospital of Eastern Ontario)**: *C. difficile* spores were grown anaerobically in undiluted Columbia broth initially for 7 days at 36±1°C and further for 15 days at 25°C. The spore suspension was centrifuged, washed and resuspended in sterile distilled, deionized water to yield a concentration of 5 x 10<sup>8</sup> - 10<sup>9</sup> spores/mL.

**All the spore suspensions were heated at 80°C for 10 minutes to ensure the absence of any vegetative cells.**

d) ***Pseudomonas aeruginosa* (ATCC 15442), *Staphylococcus aureus* (ATCC 6538) and *Salmonella choleraesuis* (ATCC 10708)**: Stock suspensions of the three vegetative bacteria were prepared by culturing them in tryptic soy broth (TSB; Difco) for 24 hours at 37°C.

e) ***Trichophyton mentagrophytes* (ATCC 9533)**: A stock suspension of the conidia was obtained by inoculating the center of a Sabouraud Dextrose Agar (SDA) plate and incubating it at 28°C for 10 days. Mycelial mats were harvested from the agar surface, homogenized with sterile glass beads in normal saline and filtered through sterile cotton gauze to remove the hyphae.

f) **The Sabin vaccine strain of Poliovirus type 1 (ATCC VR-192)**: A stock of the virus was prepared by infecting a monolayer of Vero cells in 75 cm<sup>2</sup> flasks. The virus was allowed to adsorb to cells for 60 minutes at 37°C and the infected monolayer kept in minimum essential medium, without any antibiotics or fetal bovine serum (FBS), until approximately 75% of the monolayer had been affected by the virus cytopathic effects. The culture was then frozen (-20°C) and thawed three times and the suspension was centrifuged at 1,000x g for 10 minutes to remove cellular debris. The supernatant was used as the virus pool.

## C. TESTS TO ASSESS THE MICROBICIDAL ACTIVITIES:

### 1. Quantitative Carrier Test:

QCT-1 used in this evaluation has been designed to: (a) permit the determination of the exact number of colony forming units (CFU) placed on each carrier and the CFU remaining after the drying of the inoculum, (b) avoid wash-off of any of the test organism, (c) allow complete recovery of the inoculum from the carrier surface, (d) arrest the test product's activity by immediately at the end of the contact time, (e) capture all the cells of the test organism on a membrane filter before and after exposure to the test product, (f) removal of any residual microbicidal activity by a thorough rinsing of the membrane filter, (g) allow a ratio of 1:100 between the volume of the test microbial inoculum and the volume of the product being evaluated, (h) incorporation of glass inserts to eliminate any false-positive results due to the generation of micro-aerosols in the carriers and (i) give a precise determination of  $\log_{10}$  reduction in CFU of the test organism after exposure to the product under test. This test method, therefore, eliminates the deficiencies associated with the AOAC Use- Test (Springthorpe and Sattar 2005).

### Recovery Media and Detection of Viable Organisms:

The control suspensions and the test samples were passed through 47 mm diameter membrane filters (Millipore; 0.22  $\mu\text{m}$  pore diameter). The filters were placed on TSA plates, incubated at 37°C, and the colony forming units (CFU) recorded at 24 hour intervals for a total of 5 days. For *T. mentagrophytes*, the filters were placed on SDA and incubated at 28°C, monitored, and the CFU recorded at 4 days, and every 24 hour interval thereafter for a total of 10 days

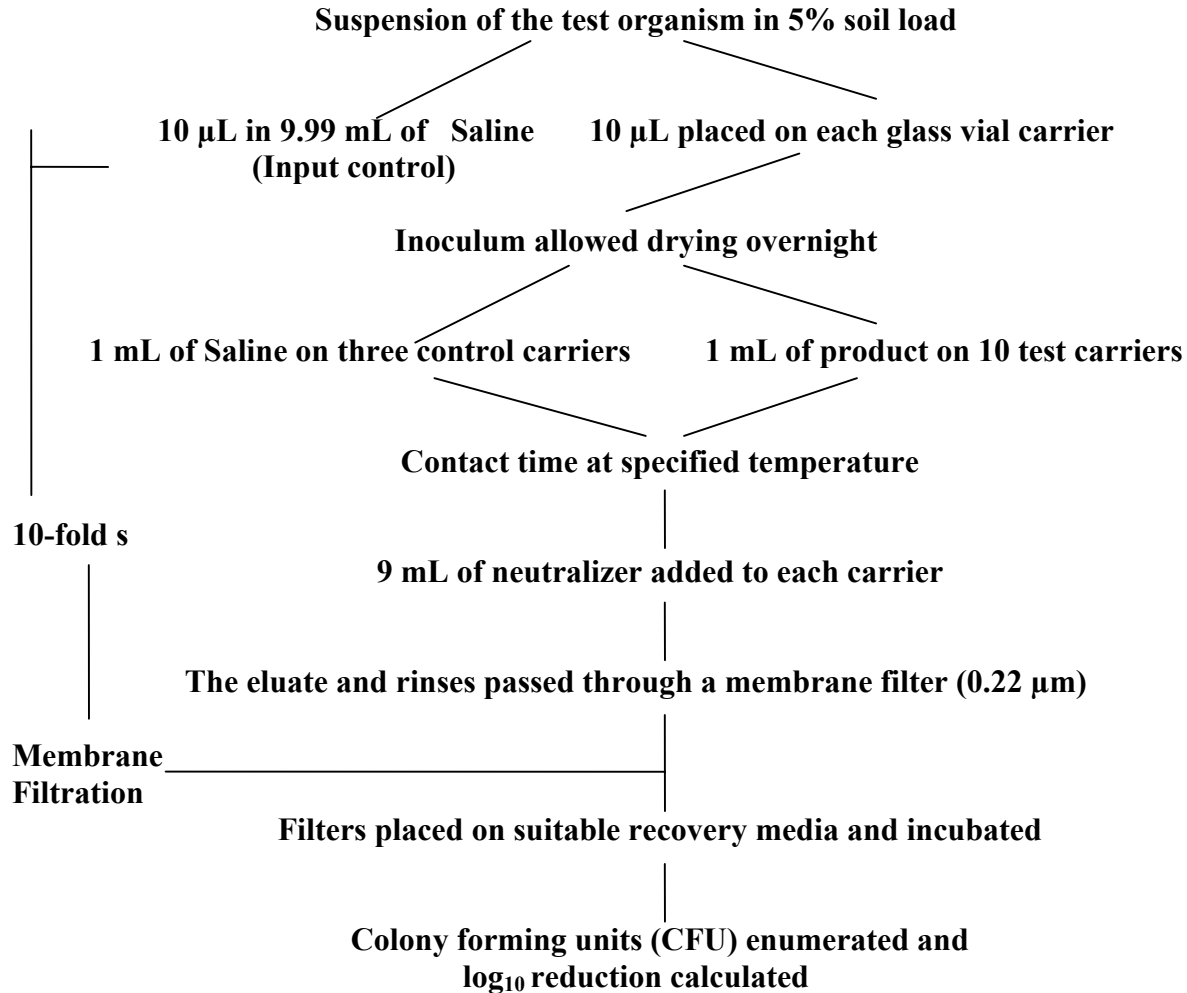
### Controls:

In QCT-1 for sporicidal, fungicidal and bactericidal activities, control carriers were used in the same manner as test carriers except that normal saline was applied to the dried inoculum instead of the test product.

**The Method for Testing Bactericidal Activity:** The general procedure for testing is given in Flow Chart 1.

## FLOW CHART 1

**THE BASIC QUANTITATIVE CARRIER METHOD FOR TESTING THE BACTERICIDAL ACTIVITIES OF LIQUID CHEMICAL GERMICIDES**



*The test involved drying a microbial suspension on a hard surface carrier and covering the dried inoculum with the use of the disinfectant for the specified contact time at room temperature. At the end of the contact time, an eluent/rinse was used to recover the reaction mixture from the carrier and the eluate was passed through a membrane filter (0.22 µm pore diameter) to capture the test organism. The filters were then placed on plates of suitable recovery agar medium and incubated to allow viable organisms to form visible colonies. The numbers of colony forming units (CFU) were recorded and the level of inactivation of the test organism was calculated.*

## 2. The Virucidal Test:

The test method used in this study was ASTM International's *Standard Method for Efficacy of Virucidal Agents Intended for Inanimate Environmental Surfaces* (ASTM 2002). The poliovirus suspension (0.2 mL) was spread over the surface of a sterile glass Petri dish with a pipette tip and allowed to air dry for about 30-35 minutes at ambient temperature. The dried virus films were then exposed to 2.0 mL of the disinfectant for the required exposure time at room temperature (23±1°C). Twenty seconds before the end of the contact time, the inoculum was scraped with a rubber policeman and remained in suspension until the end of the contact time. At the end of the contact time, the virus-disinfectant mixture was swirled gently to mix in the Petri dish and 0.2 mL from the mixture was transferred into 1.8 mL of neutralizer (Lethen broth+1% sodium thiosulphate pentahydrate). A control experiment was run in parallel and treated in the same manner except that 2.0 mL of Earle balanced salt solution (EBSS) was used in place of the disinfectant. To remove any cytotoxicity in the neutralized mixture, the neutralized samples were passed through a column of Sephadex LH-20 as described in the ASTM method E-1482 (ASTM 2004). The filtrates were transferred into sterile labelled vials. The control and test filtrates were serially diluted and inoculated into cell culture monolayer for virus plaque assays. Plaque forming units (PFU) were determined and log<sub>10</sub> reductions calculated.

### Plaque Assay

Confluent monolayer of Vero cells were trypsinized and dispensed into 12-well cell culture plates (Corning cat # 08-757-16B) for all plaque assays. The cells were dispensed at a density (approximately 1 x 10<sup>6</sup> cells/well) to allow for formation of confluent monolayer within 24-48 hour. Each assay included three wells as cell controls and each of the samples tested was inoculated into at least three wells.

The medium from each plate was aspirated and 100 µL of the appropriate of the test virus suspension was then dispensed directly onto each monolayer. Each was titrated in triplicate. The plates were incubated for 60 minutes at 37°C in a 5% CO<sub>2</sub> atmosphere to allow for virus adsorption. Each monolayer was overlaid with 2 mL of an overlay medium containing 2X MEM supplemented with HEPES, L-glutamine, non-essential amino acids (NEAA), and 2% FBS, 26 Mm MgCl<sub>2</sub> and Noble Difco Agar. The ratio of the agar and the supplemented medium was 1:1. Once the overlay had solidified, the plates were held for 30 hrs in a 5% CO<sub>2</sub> atmosphere at 37°C.

At the end of the required incubation period for the plaque assay, 2 mL of a 3.7% solution of formaldehyde in saline was added to each well and the plates were left for three to four hours to fix the cells and inactivate the virus. The fixative and the agar overlay were then removed from each plate and each well received 2 mL of a 0.1% aqueous solution of crystal violet to stain the cells. Following a contact time of about five minutes, the stain was aspirated; the wells washed in tap water and the plates allowed to dry to determine the plaque counts.

### Microbicidal Neutralization Control

#### Bactericidal Test:

One part of the use-dilution of the product was mixed with 9 parts of the neutralizer. The test

organism was added to the neutralized solution to give an estimated 20-100 CFU. The neutralizer alone was used as the control solution. At the end of a contact time of 5 minutes at 20°C, the mixture was passed through a membrane filter to capture the bacteria. The filters were placed on the appropriate recovery medium. The plates were incubated for 24 hours at 37°C and the colonies counted.

The time of 5 minutes was selected in these experiments because it is the maximum delay that may occur between the initial of the product in the carrier vial and the last lot of rinse passed through the membrane filter.

### **Virucidal Test:**

This was to determine if the neutralization of the sample, followed by detoxification, was sufficient to render it ineffective against the test virus. The test virus (200 µL) was added to 1.8 mL of the neutralized sample (in the ratio of 1:9). The mixture was then passed through a Sephadex column. The same amount of virus was added to 1.8 mL of the neutralizer control. The virus eluates were then inoculated onto cell monolayer, followed by adsorption for 60 minutes and subsequent addition of overlay medium and incubation.

### **Cytotoxicity and Interference with Plaque Formation**

To determine the effect of the detoxified test product on cell monolayers and the plaque-forming ability of the test virus, 1.2 mL of a 1/10 and 1/100 of the test product in neutralizer were first passed through the Sephadex column to remove cytotoxicity. The filtrates were then placed into three wells each of a 12-well cell culture plate while the other six wells received neutralizer which was also passed through the column and EBSS, respectively, as controls and allowed to incubate for 30 minutes. The monolayers were observed under an inverted microscope for signs of toxicity of the test product. In the absence of any apparent cytotoxicity, the monolayers were then washed once with EBSS. Virus, diluted to give countable plaques/well, was added to each well. The virus was allowed to adsorb for 60 minutes. Each cell monolayer was then overlaid with an agar overlay and the plates held at 37°C for the development of virus plaques.

## **PRODUCT PERFORMANCE CRITERIA**

The numbers of test carriers in the virucidal test were 3 and 10 for each sporicidal, bactericidal and fungicidal test. Each test also included three control carriers. The results are reported as log<sub>10</sub> reductions in viability in reference to the control carriers. Under the conditions of this test, for the product to be considered microbicidal, it was expected to reduce the viability of the test organisms by a minimum of 6 log<sub>10</sub> in the sporicidal and bactericidal tests, a minimum of 5 log<sub>10</sub> in the fungicidal test and a  $\geq 3$  log<sub>10</sub> for the virucidal activity.

## **D. RESULTS**

**Activity against *Staphylococcus aureus* (QCT-1):** Table 1 summarizes the results of tests against *S. aureus*. All three lots of the product were able to bring about a  $>6\log_{10}$  reduction in the viability titre of the organism in a contact time of 1 minute at room temperature, indicating

bactericidal activity.

**Table 1: The activity of full-strength Accel *C. difficile* Gel against *Staphylococcus aureus* in a contact time of 1 minute**

Lot Number	Date of Experiment	CFU/Control Carrier	CFU/Test Carrier	Log <sub>10</sub> Reduction
5725	10/08/06	1.00 x 10 <sup>6</sup>	0	6.03
5726	10/08/06	1.00 x 10 <sup>6</sup>	0	6.03
5727	10/08/06	1.00 x 10 <sup>6</sup>	0	6.03

**Activity against *Pseudomonas aeruginosa* (QCT-1):** Table 2 summarizes the results of tests against *P. aeruginosa*. All three lots of the product were able to bring about a >6 log<sub>10</sub> reduction in the viability titre of the organism in a contact time of 1 minute at room temperature, indicating bactericidal activity.

**Table 2: The activity of full-strength Accel *C. difficile* Gel against *Pseudomonas aeruginosa* in a contact time of 1 minute**

Lot Number	Date of Experiment	CFU/Control Carrier	CFU/Test Carrier	Log <sub>10</sub> Reduction
5725	24/08/06	2.64 x 10 <sup>6</sup>	0	6.42
5726	24/08/06	2.64 x 10 <sup>6</sup>	0	6.42
5727	24/08/06	2.64 x 10 <sup>6</sup>	0	6.42

**Activity against *Salmonella choleraesuis* (QCT-1):** Table 3 summarizes the results of tests against *Salmonella choleraesuis*. All three lots of the product were able to bring about a >6 log<sub>10</sub> reduction in the viability titre of the organism in a contact time of 1 minute at room temperature indicating bactericidal activity.

**Table 3: The Activity of full-strength Accel *C. difficile* Gel against *Salmonella choleraesuis* in a contact time of 1 minute**

Lot Number	Date of Experiment	CFU/Control Carrier	CFU/Test Carrier	Log <sub>10</sub> Reduction
5725	16/08/06	8.17 x 10 <sup>6</sup>	0	6.91
5726	16/08/06	8.17 x 10 <sup>6</sup>	0	6.91
5727	16/08/06	8.17 x 10 <sup>6</sup>	0	6.91

**Activity against the spores of *B. subtilis* (QCT-1):** Table 4 summarizes the results of tests against *B.subtilis*. All three lots of the product were able to bring about a >6log<sub>10</sub> reduction in the viability titre of in a contact time of 10 minutes at room temperature indicating sporicidal activity.

**Table 4: The activity of full-strength Accel *C. difficile* Gel against the spores of *B. subtilis* in a contact time of 10 minutes**

Lot Number	Date of Experiment	CFU/Control Carrier	CFU/Test Carrier	Log <sub>10</sub> Reduction
5725	12/07/06	1.05 x 10 <sup>7</sup>	1.18 x 10 <sup>1</sup>	6.05
5726	12/07/06	1.05 x 10 <sup>7</sup>	5.30 x 10 <sup>0</sup>	6.42
5727	12/07/06	1.05 x 10 <sup>7</sup>	0	7.02

**Activity against the spores of *C. sporogenes* (QCT-1):** Table 5 summarizes the results of tests against *C. sporogenes*. All three lots of the product were able to bring about a >7 log<sub>10</sub> reduction in the viability titre of in a contact time of 10 minutes at room temperature indicating sporicidal activity.

**Table 5: The activity of full-strength Accel *C. difficile* Gel against the spores of *C. sporogenes* in a contact time of 10 minutes**

Lot Number	Date of Experiment	CFU/control carrier	CFU/test Carrier	Log <sub>10</sub> Reduction
5725	25/07/06	1.11 x 10 <sup>7</sup>	0	7.05
5726	25/07/06	1.11 x 10 <sup>7</sup>	0	7.05
5727	25/07/06	1.11 x 10 <sup>7</sup>	0	7.05

**Activity against the spores of *C. difficile* (QCT-1):** Table 6 summarizes the results of the tests against *C. difficile*. All three lots were able to bring about a >7 log<sub>10</sub> reduction in the viability titre of *C. difficile* in a contact time of 10 minutes at room temperature indicating sporicidal activity.

**Table 6: The activity of full-strength Accel *C. difficile* Gel against the spores of *C. difficile* in a contact time of 10 minutes**

Lot Number	Date of Experiment	CFU/control	CFU/test	Log <sub>10</sub> Reduction
5725	29/07/06	8.03 x 10 <sup>7</sup>	0	7.90
5726	29/07/06	8.03 x 10 <sup>7</sup>	0	7.90
5727	29/07/06	8.03 x 10 <sup>7</sup>	0	7.90

**Activity of the product against *Trichophyton mentagrophytes* (QCT-1):** Table 7 summarizes the results of the tests against *T. mentagrophytes*. All three lots of the product were able to bring about a >5 log<sub>10</sub> reduction in the viability titre in a contact time of 1 minute at room temperature indicating fungicidal activity.

**Activity against the Sabin vaccine strain of Poliovirus type 1 (E-1053):** Table 8 summarizes the results of the tests against the poliovirus. All three lots of the product were able to bring about a >5 log<sub>10</sub> reduction in the viability titre of the virus in a contact time of 1 minute at room temperature indicating virucidal activity.

**Table 7: The activity of full-strength Accel *C. difficile* Gel against *T. mentagrophytes* in a contact time of 1 minute**

Lot Number	Date of Experiment	CFU/Control Carrier	Average CFU Test Carrier	Log <sub>10</sub> Reduction
5725	18/08/06	3.90 x 10 <sup>5</sup>	0	5.55
5726	18/08/06	3.90 x 10 <sup>5</sup>	0	5.55
5727	18/08/06	3.90 x 10 <sup>5</sup>	0	5.55

**Activity of full-strength Accel *C. difficile* Gel against the poliovirus in a contact time of 1 minute**

Lot number	Date of experiment	PFU/control carrier	PFU/test carrier	Log <sub>10</sub> Reduction
5725	22/08/06	2.76 x 10 <sup>5</sup>	0	5.69
5726	22/08/06	2.76 x 10 <sup>5</sup>	0	5.69
5727	22/08/06	2.76 x 10 <sup>5</sup>	0	5.69

**Neutralization verification results to arrest activity of Accel *C. difficile* Gel:** Table 9 summarizes the results of the neutralization tests of the product. The absence of any significant difference in the number of CFU of the test organism in the test and control samples was taken to mean that a 1:100 of the product in the neutralizer was sufficient to arrest its microbicidal activity.

**Table 9: Neutralization Verification of Accel *C. difficile* Gel**

Test Organism	Number of CFU on plates after exposure to a 10-fold dilution of the test solution in the neutralizer	Number of CFU on plates after exposure to the neutralizer
<i>P. aeruginosa</i>	37/34	66/62
<i>S. choleraesuis</i>	20/10	48/38
<i>S. aureus</i>	118/112	148/140
<i>C. sporogenes</i>	53/51	48/53
<i>T. mentagrophytes</i>	101/89	91/96

**Cytotoxicity of the test product:** A 1:10 of the product in the neutralizer, followed by gel filtration, showed no apparent toxicity for the cell line used for the study.

**Interference with Plaque Formation:** Pre-exposure of the cell monolayer to a 1:10 of the test product in the neutralizer, followed by gel filtration, did not interfere with the plaque formation by the virus tested in the study. Any interference by the residual amounts of the product would have resulted in significantly lower numbers of plaques in the monolayer pre-treated with its when compared to the number of plaques in the control monolayer.

**Neutralization of the product to arrest virucidal activity:** Adding the virus separately to a 1:10 of the product in the neutralizer followed by gel filtration did not result in any loss in its infectivity, which indicates that the neutralization of the test product at the end of the contact time, followed by gel filtration, was sufficient to arrest its virucidal activity.

#### **E. CONCLUDING REMARKS**

All three lots of the formulation tested were able to meet the product performance criteria under the conditions of the testing carried out in this study.

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