

FINAL STUDY REPORT

PROTOCOL TITLE

Virucidal Efficacy of Disinfectants for Use on Inanimate Environmental Surfaces

Virus: Human Immunodeficiency Virus Type 1

DATA REQUIREMENT

U.S. EPA 40 CFR Part 158,
"Data Requirements for Registration"
Subdivision G: Product Performance, 91-2(f)

PRODUCT IDENTITY

Virox 5
Lot #2060, #2061, and #2081

PROTOCOL NUMBER

VX010599.HIV

PROJECT NUMBER

7431

AUTHOR

Karen M. Ramm, B.A.
Study Director

STUDY COMPLETION DATE

June 18, 1999

PERFORMING LABORATORY

ViroMed Biosafety Laboratories
6101 Blue Circle Drive
Minneapolis, MN 55343

SPONSOR

Virox
6705 Millcreek Drive
Mississauga, Ontario
Canada

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10 (d) (1) (A), (B), or (C).

Company: _____

Company Agent: _____ Date: _____

CERTIFICATION OF GOOD LABORATORY PRACTICE

The study referenced in this report was conducted in compliance with U.S. Environmental Protection Agency Good Laboratory Practice (GLP) regulations set forth in 40 CFR part 160.

The studies not performed by or under the direction of ViroMed Laboratories, Inc. are exempt from this Good Laboratory Practice statement and include: characterization and stability of the compound(s).

Study Director:	<u>Karen M. Ramm</u> Karen M. Ramm, B.A.	<u>6-18-99</u> Date
Submitter:	_____	_____ Date
Sponsor Representative:	_____	_____ Date

QUALITY ASSURANCE UNIT SUMMARY

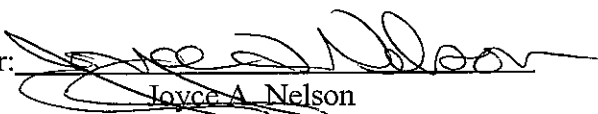
Study: Virucidal Efficacy of Disinfectants for Use on Inanimate Environmental Surfaces

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of nonclinical laboratory studies. These studies have been performed under Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures and standard protocols. The Quality Assurance Unit maintains copies of study protocols and standard operating procedures and has inspected this study on the date(s) listed below. Studies are inspected at time intervals to assure the integrity of the study. The findings of these inspections have been reported to management and the Study Director.

Phase Inspected:

Project Set-up	Date: May 25, 1999
In Process	Date: May 28, 1999
Final Reading	Date: June 4, 1999
Final Report	Date: June 17, 1999
Study Director Review	Date: June 18, 1999
Management Review	Date: June 18, 1999

Documentation of the above Quality Assurance audits has been reviewed.

Quality Assurance Director:  Date: 18 June 1999
Joyce A. Nelson

Professional personnel involved:

Bonita L. Baskin, Ph.D.	- Laboratory Director
Karen M. Ramm, B.A.	- Virology Laboratory Manager
Mary J. Miller, MT	- Research Scientist II
Katherine A. Paulson, M.L.T.	- Research Assistant I

REPORT**VIRUCIDAL EFFICACY OF DISINFECTANTS FOR USE ON
INANIMATE ENVIRONMENTAL SURFACES****TEST OBJECTIVE**

The purpose of this study was to evaluate the virucidal efficacy of a disinfectant against Human Immunodeficiency Virus type 1 according to test criteria and methods approved by the U.S. Environmental Protection Agency for registration of a product as a virucide (2). In addition, per Sponsor request, additional carriers and an additional batch of test substance were assayed to develop data to support a Human Immunodeficiency Virus type 1 registration in Canada.

TESTING FACILITY: ViroMed Biosafety Laboratories
2540 Executive Drive
St. Paul, MN 55120

SPONSOR: Virox
6705 Millcreek Drive
Mississauga, Ontario
Canada

SAMPLE NAME OR CODE: Virox 5
Lots #2060, #2061, and #2081

DATE SAMPLES RECEIVED BY VIROMED: April 8, 1999-Lot #2060 and #2061
April 29, 1999-Lot #2081

VIROMED PROTOCOL NUMBER: VX010599.HIV
VIROMED PROJECT NUMBER: 7431

STUDY INITIATION DATE: May 3, 1999
EXPERIMENTAL START DATE: May 25, 1999
EXPERIMENTAL COMPLETION DATE: June 4, 1999
STUDY COMPLETION DATE: June 18, 1999

TEST SUBSTANCE CHARACTERIZATION

The identity, strength, purity, stability, solubility, and chemical composition of the test material are the responsibility of the Sponsor.

DATA AND TEST SUBSTANCE RETENTION

A certified copy of this report as well as all raw data pertinent to this study will be stored at ViroMed Laboratories, Inc., 2540 Executive Drive, St. Paul, MN 55120. As stated in the study protocol, test substance retention is the responsibility of the Sponsor. Unused test substances will be discarded following study completion.

SUMMARY OF RESULTS:

Test Substance: Virox 5, Lot #2060, #2061 and #2081
Dilution: 1:16 in 200ppm AOAC Synthetic Hard Water
Virus: Human Immunodeficiency Virus Type 1, Strain HTLV-III_B
Exposure Time: Five minutes
Exposure Temperature: Room temperature
Organic Soil Load: 5% Fetal Bovine Serum
Efficacy Result: Three lots of Virox 5 met the test criteria specified in the study protocol. The results indicate **complete inactivation** of Human Immunodeficiency virus type 1 under these test conditions as required by the U.S. EPA for claims of virucidal activity.

TEST SYSTEM

- Virus
The HTLV-III_B strain of Human Immunodeficiency virus type 1 (HIV-1) used for this study was obtained from Advanced Biotechnologies Incorporated, Columbia, MD. Stock virus was prepared by collecting the supernatant culture fluid from infected culture cells as determined by an indirect immunofluorescence assay specific for the HIV-1 antigen. The cells were disrupted and cell debris removed by centrifugation at approximately 1200 RPM for ten minutes. The virus was further concentrated by ultra-centrifugation at 17,000 RPM for approximately 3½ hours. The virus pellet was resuspended in RPMI-1640 medium, aliquoted and the high titer stock virus was stored at ≤ -60°C until the day of use. On the day of use an aliquot of stock virus (ViroMed Lot HT-III_B-2A) was removed, thawed and refrigerated until use in the assay. The stock virus cultures contained 5% fetal bovine serum as the organic soil load. The stock virus tested demonstrated cytopathic effects (CPE) typical of HIV on MT-2 cells.
- Test Cell Cultures
MT-2 cells (human CD4+ lymphocytes) were originally obtained from the National Cancer Institute, Frederick, MD. Cultures were grown and propagated in-house and used in suspension in disposable tissue culture labware. On the day of testing, cells were observed as having proper cell integrity and therefore, were acceptable for use in this study.
- Test Medium
The test medium used in this study was RPMI 1640 supplemented with 15% (v/v) fetal bovine serum (fbs) heat-inactivated at approximately 56°C for 30 minutes. The medium was also supplemented with 2 mM L-glutamine and 50 µg/mL gentamicin.

METHODS1. Preparation of Test Substance

Virox 5 was tested at a 1:16 dilution in 200ppm AOAC Synthetic Hard Water (1.0mL product + 15.0mL hard water) as requested by the Sponsor. The test substance was in solution as observed by visual observation and used on the day of preparation.

The 200ppm AOAC Synthetic Hard Water was prepared using 2.0mL of Solution I, 4.0mL of Solution II and 994.0mL of sterile deionized water. The 200ppm hard water was prepared, titrated (at 200ppm) and used on day of test set-up.

2. Preparation of Virus Films

Films of virus were prepared by spreading 0.2mL of virus inoculum uniformly over the bottoms of twenty separate 100 X 15mm sterile glass petri dishes. The virus films were air-dried at room temperature (20°C) until visibly dry (20 minutes) and then incubated at 37°C for an additional 30 minutes to increase the level of dryness.

3. Sephadex Gel Filtration

To reduce the cytotoxic level of the virus-disinfectant mixture prior to assay of virus and/or to reduce the virucidal level of the disinfectant, virus was separated from disinfectant by filtration through Sephadex gel. Columns of Sephadex LH-20-100 were equilibrated with phosphate buffered saline containing 1% albumin, centrifuged for three minutes to clear the void volume, loaded with 2.0mL of virus-disinfectant mixture and immediately passed through the column utilizing the syringe plunger.

4. Treatment of Virus Films with Test Substance (TABLES 2-4)

For each lot of disinfectant, separate dried virus films were exposed to 2.0mL of the use dilution for five minutes at room temperature (20°C). Following the exposure time, the plates were scraped with a cell scraper to resuspend the contents of the plate and the virus-disinfectant mixture was immediately passed through a Sephadex column utilizing the syringe plunger in order to detoxify the mixture. The filtrate (10^{-1} dilution) was then titered by serial dilution for infectivity.

5. Treatment of Virus Control Films (TABLE 1)

A virus film was prepared as previously described (paragraph 2). The control film was exposed to 2.0mL test medium for the same amount of time as the test film was exposed to the disinfectant. The virus was then scraped and passed through a Sephadex column in the same manner as the test virus and the filtrate (10^{-1} dilution) was then titered by serial dilution and assayed for infectivity (paragraph 4).

6. Cytotoxicity Assay (TABLE 5)

A 2.0mL aliquot of each lot of the disinfectant was filtered through a Sephadex column and the filtrate was diluted serially in medium and inoculated into MT-2 cell cultures. Cytotoxicity of the MT-2 cell cultures was scored at the same time as virus-disinfectant and virus control cultures.

7. Assay of Non-Virucidal Level of Test Substance (TABLE 6)
Each dilution of the Sephadex-filtered disinfectant (disinfectant control for cytotoxicity assay) was mixed with an aliquot of low titer stock virus, and the resulting mixtures of dilutions were assayed for infectivity in order to determine the dilution(s) of disinfectant at which virucidal activity, if any, was retained. Dilutions that showed virucidal activity were not considered in determining the reduction in infectivity by the test substance.

8. Infectivity assays
The MT-2 (human CD4+lymphocytes) cell line, which exhibits CPE in the presence of HIV-1, was used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes were inoculated in quadruplicate with 0.2mL of the dilutions prepared from test and control groups. Uninfected indicator cell cultures (cell controls) were inoculated with test medium alone. Cultures were incubated at 36.7-37.3°C in a humidified atmosphere of 5.5-6.0% CO₂ in sterile disposable cell culture labware. The cultures were scored periodically for ten days for the absence or presence of CPE, cytotoxicity, and for viability.

9. Statistical Methods: N/A

CALCULATION OF TITERS

Viral and cytotoxicity titers are expressed as $-\log_{10}$ of the 50 percent titration endpoint for infectivity (TCID₅₀) or cytotoxicity (TCD₅₀), respectively, as calculated by the method of Spearman Karber.

$$-1 - \left[\frac{(\text{Sum of \% mortality at each dilution}) - 0.5 \times (\text{logarithm of dilution})}{100} \right]$$

ANALYSIS AND CONCLUSIONS

Results of tests with three lots of Virox 5 (Lot #2060, #2061, and #2081) exposed to HIV-1 for five minutes are shown in Tables 1-6. The titer of the five virus control replicates are as follows: 5.5 log₁₀, 5.75 log₁₀, 6.0 log₁₀, 6.0 log₁₀ and 6.25 log₁₀. The average viral titer of the five replicates is 5.97 log₁₀. The average of the five virus control replicates was used to calculate the log reduction in viral titer. Following exposure, test virus infectivity was not detected in any replicate of the virus-test substance mixtures for any of the three lots at any dilution tested ($\leq 1.5 \log_{10}$). Test substance cytotoxicity was observed for all three lots at 1.5 log₁₀. The neutralization control (non-virucidal level of the test substance) indicates that the test substance was neutralized at $\leq 1.5 \log_{10}$ for all three lots. Taking the cytotoxicity and neutralization control results into consideration, the average reduction in virus titer was $\geq 4.47 \log_{10}$ for all three lots. Under these test conditions, three lots of Virox 5 **demonstrated complete inactivation** of the HIV-1 as required by the U.S. E.P.A. for virucidal claims.

TABLE 1: Dried Virus Controls

Dried Virus Control					
Dilution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻¹	++++	++++	++++	++++	++++
10 ⁻²	++++	++++	++++	++++	++++
10 ⁻³	++++	++++	++++	++++	++++
10 ⁻⁴	++++	++++	++++	++++	++++
10 ⁻⁵	+++0	++++	++++	++++	++++
10 ⁻⁶	+000	00+0	++00	+00+	++0+
10 ⁻⁷	0000	0000	0000	0000	0000
TCID ₅₀ /0.2mL	10 ^{5.5}	10 ^{5.75}	10 ^{6.0}	10 ^{6.0}	10 ^{6.25}
Average TCID ₅₀ /0.2mL of the five replicates is 10 ^{5.97}					

TABLE 2: Effects of Virox 5 (Lot #2060) Following a Five Minute Exposure to HIV-1 Dried on an Inanimate Surface

HIV-1 + Virox 5 Lot 2060					
Dilution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻¹	TTTT	TTTT	TTTT	TTTT	TTTT
10 ⁻²	0000	0000	0000	0000	0000
10 ⁻³	0000	0000	0000	0000	0000
10 ⁻⁴	0000	0000	0000	0000	0000
10 ⁻⁵	0000	0000	0000	0000	0000
10 ⁻⁶	0000	0000	0000	0000	0000
10 ⁻⁷	0000	0000	0000	0000	0000
TCID ₅₀ /0.2mL	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present

TABLE 3: Effects of Virox 5 (Lot #2061) Following a Five Minute Exposure to HIV-1 Dried on an Inanimate Surface

HIV-1 + Virox 5 Lot 2061					
Dilution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻¹	T T T T	T T T T	T T T T	T T T T	T T T T
10 ⁻²	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻³	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻⁴	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻⁵	0 0 0 0	0 0 0 0	0 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	0 0 0 0	0 0 0 0
10 ⁻⁷	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
TCID ₅₀ /0.2mL	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}

TABLE 4: Effects of Virox 5 (Lot #2081) Following a Five Minute Exposure to HIV-1 Dried on an Inanimate Surface

HIV-1 + Virox 5 Lot 2081					
Dilution	Replicate 1	Replicate 2	Replicate 3	Replicate 4	Replicate 5
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻¹	T T T T	T T T T	T T T T	T T T T	T T T T
10 ⁻²	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻³	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻⁴	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻⁵	0 0 0 0	0 0 0 0	0 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	0 0 0 0	0 0 0 0
10 ⁻⁷	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
TCID ₅₀ /0.2mL	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}	≤10 ^{1.5}

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present
(T) = Cytotoxicity present

REFERENCES

1. ASTM Standards on Materials and Environmental Microbiology, 1994, E1053-91.
2. U.S. Environmental Protection Agency Pesticide assessment Guidelines, Subdivision G: Product Performance, 91-2(f), November, 1982. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, DIS/TSS-7, November 12, 1981.
3. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Schmidt, N.J. and Emmons, R.W. editors. Sixth edition, 1989. p. 18-20.
4. Blackwell, J.H., and J.H.S. Chen. 1970. Effects of various germicidal chemicals on HEP-2 cell culture and Herpes simplex virus. J. AOAC 53:1229-1236.
5. Techniques in HIV Research, A. Aldovini and B. Walker, 1990.

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TABLE 5: Cytotoxicity of Virox 5 on MT-2 Cell Cultures

Dilution	Cytotoxicity Control Lot #2060	Cytotoxicity Control Lot #2061	Cytotoxicity Control Lot # 2081
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻¹	T T T T	T T T T	T T T T
10 ⁻²	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻³	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻⁴	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻⁵	0 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	0 0 0 0	0 0 0 0
TCD ₅₀ /0.2mL	10 ^{1.5}	10 ^{1.5}	10 ^{1.5}

TABLE 6: Non-Virucidal Level of Test Substance (Neutralization Control)

Dilution	Virus Control + Cytotoxicity Control Lot #2060	Virus Control + Cytotoxicity Control Lot #2061	Virus Control + Cytotoxicity Control Lot #2081
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0
10 ⁻¹	T T T T	T T T T	T T T T
10 ⁻²	+	+	+
10 ⁻³	+	+	+
10 ⁻⁴	+	+	+
10 ⁻⁵	+	+	+
10 ⁻⁶	+	+	+
10 ⁻⁷	+	+	+

(+) = Positive for the presence of test virus after low titer stock virus added (neutralization control)

(0) = No test virus recovered and/or no cytotoxicity present

(T) = Cytotoxicity present

Results of the Non-virucidal level control indicate that the test substance was neutralized at TCID₅₀ of ≤1.5 log₁₀.

In the opinion of the Study Director, there were no circumstances which may have affected the quality or integrity of the data.