FINAL STUDY REPORT

PROTOCOL TITLE

Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Virus: Avian Influenza A (H3N2) virus (Avian Reassortant)

DATA REQUIREMENT

U.S. EPA 40 CFR Part 158,
"Data Requirements for Registration"
Pesticide Assessment Guidelines - Subdivision G, 91-2(f)

PRODUCT IDENTITY

Virox 5
Lot # 4585, Lot # 4586 and Lot # 4587

PROTOCOL NUMBER

VIR07042005.AFLU.2

PROJECT NUMBER

A03027

AUTHOR

Kelleen Gutzmann, M.S.
Study Director

STUDY COMPLETION DATE

July 27, 2005

PERFORMING LABORATORY

ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

SPONSOR

Virox Technologies Inc.
2815 Bristol Circle, Unit 4
Oakville, Ontario L6H 6X5
CANADA

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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: Virox Technologies Inc.

Company Agent: ______________________________

____
Title

______________________________ Date:______________
Signature

GOOD LABORATORY PRACTICE STATEMENT

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 procedures not performed by or under the direction of ATS Labs are exempt from this Good Laboratory Practice Statement and include: characterization and stability of the compound(s).

Submitter:______________________________ Date:________________

Sponsor:______________________________ Date:________________

Study Director: Kelleen Gutzmann Date: 7-27-05
Kelleen Gutzmann, M.S.

QUALITY ASSURANCE UNIT SUMMARY

Study: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

The objective of the Quality Assurance Unit is to monitor the conduct and reporting of non-clinical laboratory studies. This study has been performed under Good Laboratory Practice regulations (40 CFR Part 160) and in accordance to standard operating procedures and a standard protocol. 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.

Phase Inspected	Date	Study Director	Management
Critical Phase	July 13, 2005	July 13, 2005	July 27, 2005
Final Report	July 21, 2005	July 21, 2005	

The findings of these inspections have been reported to management and the Study Director.

Quality Assurance Auditor: [signature] Date: 7/27/05

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STUDY PERSONNEL

STUDY DIRECTOR: Kelleen Gutzmann, M.S.

Professional Personnel Involved:

Karen M. Ramm, B.A.	- Technical Director
Mary J. Miller, M.T.	- Research Scientist II
Katherine A. Paulson, M.L.T.	- Research Assistant II
Matthew Cantin, B.S.	- Research Assistant II

STUDY REPORT

GENERAL STUDY INFORMATION

Study Title: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces

Project Number: A03027

Protocol Number: VIR07042005.AFLU.2

Sponsor: Virox Technologies Inc.
2815 Bristol Circle, Unit 4
Oakville, Ontario L6H 6X5
CANADA

Testing Facility: ATS Labs
1285 Corporate Center Drive, Suite 110
Eagan, MN 55121

TEST SUBSTANCE IDENTITY

Test Substance: Virox 5

Lot/Batch(s): Lot # 4585, Lot # 4586 and Lot # 4587

Test Substance Characterization
Test substance characterization as to content, stability, solubility, storage, etc., is the responsibility of the Sponsor.

STUDY DATES

Date Sample Received: June 21, 2005
Study Initiation Date: June 22, 2005
Experimental Start Date: July 13, 2005
Experimental End Date: July 20, 2005
Study Completion Date: July 27, 2005

OBJECTIVE

The objective of this study was to evaluate the virucidal efficacy of a disinfectant against Avian Influenza A (H3N2) virus (Avian Reassortant) according to test criteria and methods approved by the U.S. Environmental Protection Agency and Health Canada for registration of a product as a virucide.

SUMMARY OF RESULTS

Test Substance:	Virox 5, Lot # 4585, Lot # 4586 and Lot # 4587
Dilution:	1:16 in 400 ppm AOAC Synthetic Hard Water
Virus:	Avian Influenza A (H3N2) virus (Avian Reassortant), ATCC VR-2072, Strain A/Washington/897/80 X A/Mallard/New York/6750/78
Exposure Time:	Five minutes
Exposure Temperature:	18-20°C
Organic Soil Load:	5% fetal bovine serum
Efficacy Result:	Three batches of Virox 5 (Lot # 4585, Lot # 4586 and Lot # 4587) met the test criteria specified in the study protocol. The results indicate **complete inactivation** of Avian Influenza A (H3N2) virus (Avian Reassortant) under these test conditions as required by the U.S. EPA for claims of virucidal activity. At least a 3 log reduction in viral titer was demonstrated as required by Health Canada for claims of virucidal activity.

TEST SYSTEM

1. Virus
   The A/Washington/897/80 X A/Mallard/New York/6750/78 strain of Avian Influenza A (H3N2) stock virus (Avian Reassortant) used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-2072). Stock virus was prepared by collecting the allantoic fluid from inoculated 11 day old fertilized, embryonated chicken eggs. The fluid was clarified by centrifugation, aliquoted and was stored at ≤-70°C until the day of use. On the day of use, two aliquots of stock virus (ATS Labs Lot IA-49) were removed, thawed, combined and refrigerated until use in the assay. The stock virus culture was adjusted to contain 5% fetal bovine serum as the organic soil load. The stock virus tested demonstrated cytopathic effects (CPE) typical of Influenza virus on Rhesus monkey kidney cells.

2. Test Cell Cultures
   Rhesus monkey kidney (RMK) cells were obtained from ViroMed Laboratories, Inc., Cell Culture Division. Cultures were maintained and used as monolayers 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.

3. Test Medium
   Test medium used in this study was Minimum Essential Medium (MEM) supplemented with 1% heat-inactivated fetal bovine serum (FBS), 10 µg/mL gentamicin, 100 units/mL penicillin, and 2.5 µg/mL amphotericin B.

The following table lists the test and control groups, the dilutions assayed, and the number of cultures used. See text for a more detailed explanation.

NUMBER OF DILUTIONS AND CULTURES FOR VIRUCIDAL EFFICACY STUDY			
Test or Control Group	**Dilutions Assayed ($\log_{10}$)**	**Cultures per dilution**	**Total Cultures**
Cell Control	N/A	4	4/group
Dried Virus Control (Group A)	-1,-2,-3,-4,-5,-6,-7	4	28
Sample lot #1 + virus (Group B)	-1,-2,-3,-4,-5,-6,-7	4	28
Sample lot #2 + virus (Group B)	-1,-2,-3,-4,-5,-6,-7	4	28
Sample lot #3 + virus (Group B)	-1,-2,-3,-4,-5,-6,-7	4	28
Cytotoxicity of lot #1 (Group C)	-1,-2,-3,-4,-5,-6,-7	4	28
Cytotoxicity of lot #2 (Group C)	-1,-2,-3,-4,-5,-6,-7	4	28
Cytotoxicity of lot #3 (Group C)	-1,-2,-3,-4,-5,-6,-7	4	28
Non-Virucidal level - lot #1 (Group D)	-1,-2,-3,-4,-5,-6,-7	4	28
Non-Virucidal level - lot #2 (Group D)	-1,-2,-3,-4,-5,-6,-7	4	28
Non-Virucidal level - lot #3 (Group D)	-1,-2,-3,-4,-5,-6,-7	4	28

METHODS

1. Preparation of Test Substance

   Three lots of Virox 5 (Lot # 4585, Lot # 4586 and Lot # 4587) were tested at a 1:16 dilution in 400 ppm AOAC Synthetic Hard Water (1.0 mL product + 15.0 mL hard water) as requested by the Sponsor. The test substance was in solution and used on the day of preparation.

   The 400 ppm AOAC Synthetic Hard Water was prepared using 4.3 mL of Solution I, 4.0 mL of Solution II and 991.9 mL of sterile deionized water. The 400 ppm hard water was prepared, titrated (at 402 ppm) and used on day of test set-up.

2. Preparation of Virus Films

   Films of virus were prepared by spreading 0.2 mL of virus inoculum uniformly over the bottoms of four separate 100 x 15mm sterile glass petri dishes. The virus films were dried at 18.5°C in a relative humidity of 51% until visibly dry (20 minutes).

3. Sephadex Gel Filtration

   To reduce the cytotoxic level of the virus-test substance mixture prior to assay of virus and/or to reduce the virucidal level of the test substance, virus was separated from test substance 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. The column was then ready to be used in the assay.

4. Treatment of Virus Films with Test Substance (GROUP B, TABLE 1)
For each lot of test substance, separate dried virus films were exposed to 2.0 mL of the use dilution for five minutes at 18.5°C. Following the exposure time, the plates were scraped with a cell scraper to resuspend the contents of the plate and the virus-test substance 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 10-fold serial dilution and assayed for infectivity.

5. Treatment of Virus Control Films (GROUP A, TABLE 1)
A virus film was prepared as previously described (paragraph 2). The control film was exposed to 2.0 mL of test medium for the same amount of time and at the same temperature as the test film was exposed to the test substance. 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 10-fold serial dilution and assayed for infectivity.

6. Cytotoxicity Assay (GROUP C, TABLE 2)
A 2.0 mL aliquot of the use dilution of each lot of the test substance was filtered through a Sephadex column and the filtrate was diluted serially in medium and inoculated into RMK cell cultures. Cytotoxicity of the RMK cell cultures was scored at the same time as the virus-test substance and virus control cultures.

7. Assay of Non-Virucidal Level of Test Substance (GROUP D, TABLE 3)
Each dilution of the Sephadex-filtered test substance (test substance 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 test substance 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 RMK cell line, which exhibits CPE in the presence of Avian Influenza A (H3N2) virus (Avian Reassortant), was used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes were inoculated in quadruplicate with 0.1 mL 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 36-38°C in a humidified atmosphere of 5-7% CO_2 in sterile disposable cell culture labware. The cultures were scored periodically for seven days for the absence or presence of CPE, cytotoxicity, and for viability.

9. Statistical Methods: N/A

PROTOCOL CHANGES

Protocol Amendments:
No protocol amendments were required for this study.

Protocol Deviations:
No protocol deviations occurred during this study.

DATA ANALYSIS

Calculation of Titers

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

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

Calculation of Log Reduction

Dried Virus Control $TCID_{50}$ – Test Substance $TCID_{50}$ = Log Reduction

STUDY RETENTION

Record Retention

All of the original raw data developed exclusively for this study shall be archived at ATS Labs, 1285 Corporate Center Drive, Suite 110, Eagan, MN 55121. These original data include, but are not limited to, the following:

1. All handwritten raw data for control and test substances including, but not limited to notebooks, data forms and calculations.
2. Any protocol amendments/deviation notifications.
3. All measured data used in formulating the final report.
4. Memoranda, specifications, and other study specific correspondence relating to interpretation and evaluation of data, other than those documents contained in the final study report.
5. Original signed protocol.
6. Certified copy of final study report.
7. Study-specific SOP deviations made during the study.

Test Substance Retention

The test substance will be discarded following study completion per Sponsor approved protocol. It is the responsibility of the Sponsor to retain a sample of the test material.

REFERENCES

1. Annual Book of ASTM Standards 2000, Section 11 Water and Environmental Technology Volume 11.05 Biological Effects and Environmental Fate: Biotechnology; Pesticides, E1053-97.

2. U.S. Environmental Protection Agency Pesticide Assessment Guidelines, Subdivision G: Product Performance, 91-2(f), November 1982.

3. U.S. Environmental Protection Agency, Registration Division, Office of Pesticide Programs, DIS/TSS-7, November 12, 1981.

4. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Schmidt, N.J. and Emmons, R.W. editors. Sixth edition, 1989. p. 18-20.

5. 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.

6. Assessment of Efficacy of Antimicrobial Agents for Use on Environmental Surfaces and Medical Devices, CAN/CGSB-2. 161-96, August 1997.

STUDY RESULTS

Results of tests with three lots of Virox 5 (Lot # 4585, Lot # 4586 and Lot # 4587), diluted 1:16 in 400 ppm AOAC Synthetic Hard Water, exposed to Avian Influenza A (H3N2) virus (Avian Reassortant) in the presence of a 5% fetal bovine serum soil load at 18.5°C for five minutes are shown in Tables 1-3. All cell controls were negative for test virus infectivity. The titer of the dried virus control was 6.75 $\log_{10}$. Following exposure, test virus infectivity was not detected in the virus-test substance mixture for any of the three lots at any dilution tested ($\leq$0.5 $\log_{10}$). Test substance cytotoxicity was not observed in any of the three lots at any dilution tested ($\leq$0.5 $\log_{10}$). The neutralization control (non-virucidal level of the test substance) indicates that the test substance was neutralized at $\leq$0.5 $\log_{10}$ for all three lots. Taking the cytotoxicity and neutralization control results into consideration, the reduction in viral titer was $\geq$6.25 $\log_{10}$ for all three lots.

STUDY CONCLUSION

Under the conditions of this investigation, in the presence of a 5% fetal bovine serum soil load, Virox 5 (Lot # 4585, Lot # 4586 and Lot # 4587), diluted 1:16 in 400 ppm AOAC Synthetic Hard Water, demonstrated complete inactivation of Avian Influenza A (H3N2) virus (Avian Reassortant) following a five minute exposure time at 18.5°C as required by the U.S. EPA for virucidal label claims. At least a 3 log reduction in viral titer was demonstrated as required by Health Canada for claims of virucidal activity.

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

The use of the ATS Labs name, logo or any other representation of ATS Labs without the written approval of ATS Labs is prohibited. In addition, ATS Labs may not be referred to in any form of promotional materials, press releases, advertising or similar materials (whether by print, broadcast, communication or electronic means) without the express written permission of ATS Labs.

TABLE 1: Virus Control and Test Results

Effects of Virox 5 (Lot # 4585, Lot # 4586 and Lot # 4587) Following a Five Minute Exposure to Avian Influenza A (H3N2) Virus (Avian Reassortant) Dried on an Inanimate Surface

Dilution	Dried Virus Control (GROUP A)	Avian Influenza A (H3N2) virus (Avian Reassortant) + Lot # 4585 (GROUP B)	Avian Influenza A (H3N2) virus (Avian Reassortant) + Lot # 4586 (GROUP B)	Avian Influenza A (H3N2) virus (Avian Reassortant) + Lot # 4587 (GROUP B)
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	+ + + +	0 0 0 0	0 0 0 0	0 0 0 0
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 0 0 0
$TCID_{50}$/0.1 mL	$10^{6.75}$	$\leq 10^{0.5}$	$\leq 10^{0.5}$	$\leq 10^{0.5}$

TABLE 2: Cytotoxicity Control Results

Cytotoxicity of Virox 5 on RMK Cell Cultures

Dilution	Cytotoxicity Control Lot # 4585 (GROUP C)	Cytotoxicity Control Lot # 4586 (GROUP C)	Cytotoxicity Control Lot # 4587 (GROUP C)
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	0 0 0 0	0 0 0 0	0 0 0 0
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}/0.1 mL	$\leq 10^{0.5}$	$\leq 10^{0.5}$	$\leq 10^{0.5}$

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

TABLE 3: Neutralization Control Results

Non-Virucidal Level of Test Substance (Neutralization Control)

Dilution	Test Virus + Cytotoxicity Control Lot # 4585 (GROUP D)	Test Virus + Cytotoxicity Control Lot # 4586 (GROUP D)	Test Virus + Cytotoxicity Control Lot # 4587 (GROUP D)
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	+ + + +	+ + + +	+ + + +
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

Results of the non-virucidal level control indicate that the test substance was neutralized at $TCID_{50}$ of ≤ 0.5 $\log_{10}$ for all three lots.