FINAL STUDY REPORT

STUDY TITLE

Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Utilizing Feline Calicivirus as a Surrogate Virus for Norovirus

PRODUCT IDENTITY

Axen 30
Lot #2006.003.001 and Lot #2005.269.001

DATA REQUIREMENT

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

AUTHOR

Kelleen Gutzmann, M.S.
Study Director

STUDY COMPLETION DATE

August 18, 2006

PERFORMING LABORATORY

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

SPONSOR

Pure Bioscience
1725 Gillespie Way
El Cajon, CA 92020

PROJECT NUMBER

A04151

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: Pure Bioscience

Company Agent: DOLANA BLOUNT

DIRECTOR OF REGULATORY AFFAIRS
Title

Signature

Date: 8.22.06

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 test substance(s).

Submitter: _____ Date: 8.22.06

Sponsor: _____ Date: 8.22.06

Study Director: Kelleen Gutzmann Date: 8-18-06
Kelleen Gutzmann, M.S.

QUALITY ASSURANCE UNIT SUMMARY

Study: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Utilizing Feline Calicivirus as a Surrogate Virus for Norovirus

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 dates listed below. This study was inspected at time intervals to assure the integrity of the study.

Phase Inspected	Date	Study Director	Management
Critical Phase	August 9, 2006	August 9, 2006	August 18, 2006
Final Report	August 17, 2006	August 17, 2006	

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

Quality Assurance Auditor: Judy Heidemann Date: 8-18-06

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STUDY PERSONNEL

STUDY DIRECTOR: Kelleen Gutzmann, M.S.

Professional Personnel Involved:
Karen M. Ramm, B.A. - Technical Director
Shanen Conway, B.S. - Research Assistant II

STUDY REPORT

GENERAL STUDY INFORMATION

Study Title: Virucidal Efficacy of a Disinfectant for Use on Inanimate Environmental Surfaces Utilizing Feline Calicivirus as a Surrogate Virus for Norovirus

Project Number: A04151

Protocol Number: IMS01010906.FCAL.2

Sponsor: Pure Bioscience
1725 Gillespie Way
El Cajon, CA 92020

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

TEST SUBSTANCE IDENTITY

Test Substance Name: Axen 30

Lot/Batch(s): Lot #2006.003.001 and Lot #2005.269.001

Test Substance Characterization
Test substance characterization as to content, stability, etc., (40 CFR, Part 160, Subpart F [160.105]) is the responsibility of the Sponsor.

STUDY DATES

Date Sample Received: July 25, 2006
Study Initiation Date: July 31, 2006
Experimental Start Date: August 9, 2006
Experimental End Date: August 16, 2006
Study Completion Date: August 18, 2006

OBJECTIVE

The purpose of this study was to evaluate the virucidal efficacy of a disinfectant against Feline Calicivirus, used as a surrogate virus for Norovirus, according to test criteria and methods approved by the U.S. Environmental Protection Agency for registration of a product as a virucide.

Norovirus, a member of the *Caliciviridae* family, is a non-enveloped RNA-containing virus and is an important cause of gastroenteritis in humans. Little is known about disinfectant efficacy against this virus due to the inability to propagate this virus in-vitro. Feline Calicivirus, also a member of the *Caliciviridae* family, serves as a valuable model virus for efficacy testing of Norovirus, since these viruses share many similar characteristics and Feline Calicivirus can be propagated in cell cultures.

SUMMARY OF RESULTS

Test Substance:	Axen 30, Lot #2006.003.001 and Lot #2005.269.001
Dilution:	Ready to use (RTU), trigger spray
Virus:	Feline Calicivirus, as a surrogate virus for Norovirus
Exposure Time:	Ten minutes
Exposure Temperature:	Room temperature (20.0°C)
Organic Soil Load:	5% fetal bovine serum
Efficacy Result:	Two lots of Axen 30 (Lot #2006.003.001 and Lot #2005.269.001) met the test criteria specified in the study protocol. Under these test conditions, the results indicate **complete inactivation** of Feline Calicivirus as required by the U.S. EPA for claims of virucidal activity.

TEST SYSTEM

1. Virus

The F-9 strain of Feline Calicivirus used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-782). The stock virus was prepared by collecting the supernatant culture fluid from 75-100% infected culture cells. The cells were disrupted and cell debris removed by centrifugation at approximately 2000 RPM for five minutes at approximately 4°C. The supernatant was removed, aliquoted, and the high titer stock virus was stored at ≤ -70°C until the day of use. On the day of use two aliquots of stock virus (ATS Labs lot FC-36) were removed, thawed, combined and refrigerated until use in the assay. The stock virus culture contained 5% fetal bovine serum as the organic soil load. The stock virus tested demonstrated cytopathic effects (CPE) typical of Feline Calicivirus on Crandel Reese feline kidney cells. The cytopathic effect observed was small, rounding of the cells, with a slight granular look.

2. Indicator Cell Cultures

Cultures of Crandel Reese feline kidney (CRFK) cells were originally obtained from the American Type Culture Collection, Manassas, VA (ATCC CCL-94). The cells were propagated by ATS Labs personnel. The cells were seeded into multiwell cell culture plates and maintained at 36-38°C in a humidified atmosphere of 5-7% CO_2. The confluency of the cells was appropriate for the test virus. This cell line has historically been used as the cell line for propagation and detection of Feline Calicivirus. The cultures were commercially available, were serially propagated, and were capable of showing cytopathic effect in the presence of the virus.

All cell culture documentation is retained for the cell cultures used in this assay with respect to source, passage number, growth characteristics, seeding densities and the general condition of the cells.

3. Test Medium

The test medium used for this assay was Minimum Essential Medium (MEM) supplemented with 5% (v/v) heat inactivated fetal bovine serum. The medium was also supplemented with 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 numbers of cultures used. See text for a more detailed explanation.

SAMPLES TESTED FOR THE PRESENCE OF VIRUS			
Test or Control Group	**Dilutions Assayed Per Carrier (log_{10})**	**Cultures per Dilution**	**Total Cultures Inoculated**
Negative Controls	N/A	2-4	2-4/group
Input Virus Control	-4,-5,-6,-7,-8	4	20
Dried Virus Control (performed in duplicate)	-4,-5,-6,-7,-8	4	40
Test Substance – Batch #1 (performed in duplicate)	-1,-2,-3,-4	4	32
Test Substance – Batch #2 (performed in duplicate)	-1,-2,-3,-4	4	32
Cytotoxicity Control – Test Substance Batch #1	-1,-2,-3	2	6
Cytotoxicity Control – Test Substance Batch #2	-1,-2,-3	2	6
Neutralization Control - Test Substance Batch #1	-1,-2,-3	2	6
Neutralization Control - Test Substance Batch #2	-1,-2,-3	2	6

TEST METHOD

1. Preparation of Test Substance
Two lots of Axen 30 (Lot #2006.003.001 and Lot #2005.269.001) were used as received from the Sponsor. On the day of testing, 100 mL of test substance was transferred to a trigger spray bottle provided by the Sponsor on July 27, 2006. Following testing, the remaining test substance in the trigger spray bottle was discarded. The test substance was applied according to the use directions provided by Sponsor. (See section on Treatment of Virus Films with Test Substance)

2. Preparation of Virus Films
Films of virus were prepared at staggered intervals by spreading 0.2 mL of virus inoculum uniformly over the bottoms of six separate 100 X 15mm sterile glass petri dishes. The virus films were air-dried at 20.0°C in a relative humidity of 49% 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, loaded with approximately 2.0 mL of virus-test substance mixture and immediately passed through the column utilizing the syringe plunger.

4. Input Virus Control
On the day of test, the stock virus utilized in the assay was titered by 10-fold serial dilution and assayed for infectivity to determine the starting titer of the virus. The results of this control are for informational purposes only.

5. Treatment of Virus Films with Test Substance

For each of the two lots of test substance, two dried virus films were exposed for the Sponsor specified exposure time of ten minutes at room temperature (20.0°C) to the amount of spray released under use conditions. The carriers were sprayed until thoroughly wet with three sprays at a distance of 6-8 inches from the surface. The virus films were completely covered with the test substance as determined by visual observation. Following the exposure time, each plate was individually scraped with a cell scraper to resuspend the contents. The virus-test substance mixtures were immediately passed through individual Sephadex columns utilizing the syringe plunger in order to detoxify the mixture. This dilution was considered the 10^{-1} dilution. A 0.2 mL aliquot of the test virus (the virus film) was resuspended in approximately 2.0 mL of test substance which equals a 1:10 dilution. The filtrates were then titered by 10-fold serial dilution and assayed for infectivity and/or cytotoxicity.

6. Treatment of Virus Control Films

Two virus films were prepared as previously described. The control films were individually exposed to 2.0 mL of test medium for ten minutes at room temperature (20.0°C). The virus films were individually scraped with a plastic cell scraper and passed through individual Sephadex columns in the same manner as the test virus. The filtrates (10^{-1} dilution) were then titered by 10-fold serial dilution and assayed for infectivity.

7. Cytotoxicity Assay

Each lot of the test substance was sprayed as previously described onto a separate sterile petri dish. The plates were individually scraped with a cell scraper, the contents were transferred to a Sephadex column and immediately passed through the column utilizing a syringe plunger. The filtrate (10^{-1} dilution) was then titered by 10-fold serial dilution and assayed for cytotoxicity. Cytotoxicity of the CRFK cell cultures was scored at the same time as the virus-test substance and virus control cultures.

8. Assay of Non-Virucidal Level of Test Substance

Each dilution of the Sephadex-filtered test substance (cytotoxicity control dilutions) was mixed with an aliquot of low titer stock virus. The resulting mixtures of dilutions were assayed for infectivity and/or cytotoxicity 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.

9. Infectivity Assays

The CRFK cell line, which exhibits cytopathic effect (CPE) in the presence of Feline Calicivirus, 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 virus control groups. The cytotoxicity and neutralization control dilutions were inoculated in duplicate. Uninfected indicator cell cultures (cell controls) were inoculated with test medium alone. Cultures were incubated at 31-35°C in a humidified atmosphere of 5-7% CO_2 in sterile disposable cell culture labware. The cultures were microscopically scored periodically for seven days for the absence or presence of CPE, cytotoxicity and for viability.

PROTOCOL CHANGES

Protocol Amendments
No protocol amendments were required for this study.

Protocol Deviations:
No protocol deviations occurred during this study.

DATA ANALYSIS

Calculations

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.

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

Statistical Analysis

The $\log_{10}$ reduction in infectivity was calculated using the revised EPA approved method for calculating the Most Probable Number (MPN) as obtained from the EPA on January 4, 2001.

STUDY ACCEPTANCE CRITERIA

A valid test requires 1) that at least 4 $\log_{10}$ of infectivity be recovered from the dried virus control films; 2) that when cytotoxicity is evident, at least a 3-log reduction in viral titer is demonstrated beyond the cytotoxic level; and 3) that the cell controls be negative for infectivity. **Note:** An efficacious product must demonstrate complete inactivation of the virus at all dilutions.

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 returned 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. Environmental Protection Agency Federal Register: August 25, 2000 (Volume 65, Number 166).
7. Statistical Analysis of Hepatitis B Carrier Test Data Revised (1-01) Template for Calculating the Log Reduction (LR) and Associated Standard Error (SE). M. Hamilton, Center for Biofilm Engineering, Montana State University, January 9, 2001, Published January 4, 2001.
8. Inactivation of feline Calicivirus, a Norwalk virus surrogate, Journal of Hospital Infection (1999) 41: 51-57.
9. Virucidal Efficacy of Four New Disinfectants, Journal of the American Animal Hospital Association, Vol. 38 No. 3, May/June 2002, Pages 231-234.
10. Efficacy of Commonly Used Disinfectants for the Inactivation of Calicivirus on Strawberry, Lettuce, and Food-Contact Surface, Journal of Food Protection, Vol. 64, No. 9, 2001, Pages 1430-1434.
11. Concentration and Detection of Caliciviruses from Food Contact Surfaces, Journal of Food Protection, June 2002; 65 (6).

STUDY RESULTS

Results of tests with two lots of Axen 30 (Lot #2006.003.001 and Lot #2005.269.001), a trigger spray, exposed to Feline Calicivirus in the presence of a 5% fetal bovine serum soil load at room temperature (20.0°C) for ten minutes are shown in Tables 1-2. The input titer (not dried) of the virus was 6.75 $\log_{10}$. The titer of the dried virus control was 5.0 $\log_{10}$ for Replicate #1 and 4.75 $\log_{10}$ for Replicate #2. The MPN for the two dried virus control replicates is 62168 and 36164, respectively. Following exposure, test virus infectivity was not detected in either replicate of either lot of the virus-test substance mixture at any dilution tested ($\leq$0.5 $\log_{10}$). The MPN for both test replicates for both lots is <1.000. Test substance cytotoxicity was not observed 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 $\log_{10}$. Utilizing the statistical program provided by the EPA, the log reduction in viral titer is ≥4.68 and the standard error of the log reduction is 0.12.

STUDY CONCLUSION

Under the conditions of this investigation, in the presence of a 5% fetal bovine serum soil load, Axen 30, (Lot #2006.003.001. and Lot #2005.269.001), a trigger spray, demonstrated complete inactivation of Feline Calicivirus following a ten minute exposure time at room temperature (20.0°C) as required by the U.S. EPA for virucidal label claims.

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

TABLE 1: Virus Controls and Test Substance Assay Results

Effects of Axen 30 (Lot #2006.003.001 and Lot #2005.269.001) Following a Ten Minute Exposure to Feline Calicivirus Dried on an Inanimate Surface

Dilution	Input Virus Control	Dried Virus Control		Feline Calicivirus + Lot # 2006.003.001		Feline Calicivirus + Lot # 2005.269.001	
		Replicate #1	Replicate #2	Replicate #1	Replicate #2	Replicate #1	Replicate #2
Cell Control	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-1}	NT	NT	NT	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-2}	NT	NT	NT	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0
10^{-3}	NT	NT	NT	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
10^{-5}	+ + + +	+ 0 0 +	0 0 + 0	NT	NT	NT	NT
10^{-6}	+ + + +	0 0 0 0	0 0 0 0	NT	NT	NT	NT
10^{-7}	+ 0 0 0	0 0 0 0	0 0 0 0	NT	NT	NT	NT
10^{-8}	0 0 0 0	0 0 0 0	0 0 0 0	NT	NT	NT	NT
$TCID_{50}$/0.1 mL	$10^{6.75}$	$10^{5.0}$	$10^{4.75}$	$\leq 10^{0.5}$	$\leq 10^{0.5}$	$\leq 10^{0.5}$	$\leq 10^{0.5}$
MPN	NA	62168	36164	<1.000*	<1.000*	<1.000*	<1.000*
Log_{10} MPN	NA	4.79357	4.55828	0.00000	0.00000	0.00000	0.00000
Log Reduction	NA	NA		≥4.68			

TABLE 2: Test Substance Cytotoxicity and Neutralization Control Results

Dilution	Cytotoxicity Control Lot #2006.003.001	Cytotoxicity Control Lot #2005.269.001	Neutralization Control Lot #2006.003.001	Neutralization Control Lot #2005.269.001
Cell Control	0 0	0 0	0 0	0 0
10^{-1}	0 0	0 0	+ +	+ +
10^{-2}	0 0	0 0	+ +	+ +
10^{-3}	0 0	0 0	+ +	+ +
TCD_{50}/0.1 mL	$\leq 10^{0.5}$	$\leq 10^{0.5}$	See below	See below

(+) = Positive for the presence of test virus
(0) = No test virus recovered and/or no cytotoxicity present
(NT) = Not tested
(NA) = Not applicable
(MPN) = Most probable number
(*) = For calculating the log reduction utilizing the MPN statistical method, MPN values of zero are reported as <1.000.

The results of the neutralization control indicate that both batches of the test substance were neutralized at the dilution equivalent to ≤0.5 log_{10} $TCID_{50}$/0.1 mL as compared to the treated test samples.