(For Laboratory Use Only)

ATS Labs Project #_____

ATS LABS

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AVIAN VIRUS
USA STRAIN.

PROTOCOL

Evaluation of Antiviral Properties of a Product Using a Virucidal Suspension Assay

Virus: Influenza A virus

COMPLETE KILL

PROTOCOL

TTI01021213.FLUA

PREPARED FOR

Trevi Technology, Inc.
2451 Marblevista Blvd.
Columbus, OH 43204

PERFORMING LABORATORY

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

PREPARED BY

Shanen Conway, B.S.
Senior Virologist

DATE

February 12, 2013

1285 Corporate Center Drive, Suite 110 • Eagan, MN 55121 • 877.287.8378 • 651.379.5510 • Fax: 651.379.5549

Evaluation of Antiviral Properties of a Product Using a Virucidal Suspension Assay

SPONSOR: Trevi Technology, Inc.
2451 Marblevista Blvd.
Columbus, OH 43204

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

PURPOSE

The purpose of this study is to evaluate the antiviral properties of a product against Influenza A virus when exposed (in suspension) for a specified exposure period(s). This protocol is a modification of the Standard Test Method for Efficacy of Antimicrobial Agents Against Viruses in Suspension (ASTM E 1052).

TEST SUBSTANCE CHARACTERIZATION

Test substance characterization as to content, stability, solubility, storage, etc., (21 CFR, Part 58, Subpart F [58.105]) is the responsibility of the Sponsor. The test substance shall be characterized before the experimental start date of this study. Pertinent information, which may affect the outcome of this study, shall be communicated in writing to the Study Director upon sample submission to ATS Labs.

SCHEDULING AND DISCLAIMER OF WARRANTY

Experimental start dates are generally scheduled on a first-come/first-serve basis once ATS Labs receives the Sponsor approved/completed protocol, signed fee schedule and corresponding test substance(s). Based on all required materials being received at this time, the proposed experimental start date is February 26, 2013. Verbal results may be given upon completion of the study with a written report to follow on the proposed completion date of March 26, 2013. To expedite scheduling, please be sure all required paperwork and test substance documentation is complete/accurate upon arrival at ATS Labs.

If a test must be repeated, or a portion of it, because of failure by ATS Labs to adhere to specified procedures, it will be repeated free of charge. If a test must be repeated, or a portion of it, due to failure of internal controls, it will be repeated free of charge. "Methods Development" fees shall be assessed, however, if the test substance and/or test system require modifications due to complexity and difficulty of testing.

If the Sponsor requests a repeat test, they will be charged for an additional test.

Neither the name of ATS Labs nor any of its employees are to be used in advertising or other promotion without written consent from ATS Labs.

The Sponsor is responsible for any rejection of the final report by the United States FDA of its submission concerning report format, pagination, etc. To prevent rejection, Sponsor should carefully review the ATS Labs final report and notify ATS Labs of any perceived deficiencies in these areas before submission of the report to the regulatory agency. ATS Labs will make reasonable changes deemed necessary by the Sponsor, without altering the technical data.

TEST SYSTEM JUSTIFICATION

This *in-vitro* virucidal suspension assay is designed to evaluate the antiviral properties of a product against Influenza A virus. The presence of virus (infectivity) is determined by monitoring the virus specific cytopathic effect (CPE) on the appropriate indicator cell line, Rhesus monkey kidney. The indicator cell line chosen is capable of supporting the growth of Influenza A virus.

EXPERIMENTAL DESIGN

Protocol Summary

A suspension of virus is exposed to the use dilution of the product. At each pre-determined exposure time an aliquot is removed, neutralized by serial dilution, and assayed for the presence of virus. The positive virus controls, cytotoxicity controls, and neutralization controls are assayed in parallel. Antiviral properties of the product will be evaluated and compared at the specified concentrations and time intervals.

Test Parameters

The following is a list of the test and control groups, usual dilutions and number of cultures to be assayed.

Cell Control	Dilutions to be assayed	Cultures/diln
Virus Control (for each exposure time)	NA	4
Test (for each exposure time and/or product concentration)	10^{-2} to 10^{-7}*	4
Cytotoxicity Control (for each product concentration)	10^{-2} to 10^{-7}*	4
Neutralization Control (for each product concentration)	10^{-2} to 10^{-4}*	4
	10^{-2} to 10^{-4}*	4

* Alternate dilutions may be assayed as determined by the stock virus titer.

CULTURE MATERIALS

Virus

The Hong Kong strain of Influenza A virus to be used for this study was obtained from the American Type Culture Collection, Manassas, VA (ATCC VR-544). The stock virus is prepared by collecting the supernatant culture fluid from 75-100% infected culture cells. The cells are disrupted and cell debris removed by centrifugation. The supernatant is removed, aliquoted, and the high titer stock virus is stored at ≤-70°C until the day of use. On the day of use, the appropriate number of aliquots of virus are removed, thawed, combined (if applicable) and maintained at a refrigerated temperature until used in the assay. **Note:** If the Sponsor requests an organic soil load challenge, fetal bovine serum (FBS) or the requested organic soil will be incorporated into the stock virus aliquot. The stock virus aliquot will be adjusted to yield the percent organic soil load requested.

Indicator Cell Cultures

Rhesus monkey kidney (RMK) cells are received from Diagnostic Hybrids, Athens, OH. Cultures are maintained and used at the appropriate density in tissue culture labware at 36-38°C in a humidified atmosphere of 5-7% CO_2. RMK cells obtained from an alternate, reputable source may be used. The source of the cells will be specified in the final report.

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.

Test Medium

The test medium used for the virucidal assays is Minimum Essential Medium (MEM) supplemented with 1-10% (v/v) heat inactivated FBS. The medium may also be supplemented with one or more of the following: 10 µg/mL gentamicin, 100 units/mL penicillin, and 2.5 µg/mL amphotericin B. The composition of the test medium may be altered based on the virus and/or cells. The composition of the medium will be specified in the final report.

TEST METHOD

Preparation of Test Substance

The test substance(s) will be prepared according to the directions for the intended use and furnished by the Sponsor. The test substance(s) will be pre-equilibrated to the exposure temperature if applicable.

Treatment of Virus Suspension
A 1.8 mL aliquot of each concentration of each test substance is dispensed into separate tubes and each is mixed with 200 µL aliquot of the stock virus suspension. This is considered the 10^{-1} dilution of the virus. The mixtures are vortex mixed for a minimum of 10 seconds and held for the remainder of the specified exposure times at the appropriate temperature. A calibrated timer will be used for timing the exposure. Immediately following each exposure time, a 100 µL aliquot is removed from each tube and the mixtures are titered by 10-fold serial dilutions (100 µL + 0.9 mL test medium) and assayed for the presence of virus. Note: To decrease the product cytotoxicity, the first dilution may be made in fetal bovine serum or other appropriate neutralizer with the remaining dilutions in test medium. Sterile glass beads may be added to aid in mixing of viscous products.

Alternate volumes of the virus and test substance may be utilized as long as the 1:10 virus to test substance ratio is maintained. All controls will be adjusted accordingly.

If excessive cytotoxicity to the indicator cell cultures is caused by the test substance or suspected, the affected dilution(s) may be passed through individual Sephadex gel filtration columns following titration to aid in reducing the toxicity. If this procedure is performed, the same dilutions of the controls must also be passed through individual columns.

Treatment of Virus Control
A 200 µL aliquot of the stock virus suspension is exposed to a 1.8 mL aliquot of test medium in lieu of the test substance and treated as previously described in Treatment of Virus Suspension section. A virus control will be performed for each exposure time tested. A calibrated timer will be used for all exposure times. All controls will employ the neutralizer utilized in the test. The virus control titer will be used as a baseline to compare the percent and log reduction of each test parameter following exposure to the test substance(s).

Cytotoxicity Control
A 1.8 mL aliquot of each concentration of each test substance is mixed with a 200 µL aliquot of test medium containing the Sponsor requested organic soil load (if applicable) in lieu of virus and treated as previously described. When multiple exposure times are requested, the cytotoxicity control will be performed at the longest requested exposure time. The cytotoxicity of the cell cultures is scored at the same time as virus-test substance and virus control cultures. Cytotoxicity is graded on the basis of cell viability as determined microscopically. Cellular alterations due to toxicity will be graded and reported as toxic (T) if greater than or equal to 50% of the monolayer is affected.

Neutralization Control
Each cytotoxicity control mixture (above) will be challenged with low titer stock virus to determine the dilution(s) of test substance at which virucidal activity, if any, is retained. Dilutions that show virucidal activity will not be considered in determining reduction of the virus by the test substance.

Using the cytotoxicity control dilutions prepared above, an additional set of indicator cell cultures will be inoculated with each dilution in quadruplicate. A 100 µL aliquot of a low titer stock virus will be inoculated into each cell culture well and the indicator cell cultures will be incubated along with the test and virus control plates.

Infectivity Assay
The RMK cell line, which exhibits cytopathic effect (CPE) in the presence of Influenza A virus, will be used as the indicator cell line in the infectivity assays. Cells in multiwell culture dishes will be inoculated in quadruplicate with 100 µL of the dilutions prepared from test and control groups. Uninfected indicator cell cultures (cell controls) will be inoculated with test medium alone. The cultures are incubated at 36-38°C in a humidified atmosphere of 5-7% CO_2 in sterile disposable cell culture labware. The cultures will be scored periodically for approximately seven days for the absence or presence of CPE, cytotoxicity and for viability.

Test Criteria
A valid test will require 1) that stock virus be recovered from the virus control and 2) that the cell controls be negative for virus. If any of the previous requirements are not met, the test may be repeated under the current protocol number.

Calculations
Viral and cytotoxicity titers will be 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 Percent (%) Reduction

$$\% \text{ Reduction} = 1 - \left[\frac{TCID_{50} \text{ test}}{TCID_{50} \text{ virus control}}\right] \times 100$$

Calculation of Log Reduction

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

*Note: If multiple replicates are performed the average 50 percent endpoints will be calculated and used to determine the average percent and log reductions.

PROCEDURE FOR IDENTIFICATION OF THE TEST SYSTEM
The specialized virucidal testing section of ATS Labs maintains Standard Operating Procedures (SOPs) relative to virucidal efficacy testing studies. Virucidal efficacy testing is performed in strict adherence to these SOPs which have been constructed to cover all aspects of the work including, but not limited to, receipt, log-in, and tracking of biological reagents including virus and cell stocks for purposes of identification, receipt and use of chemical reagents including cell culture medium components, etc. These procedures are designed to document each step of virucidal efficacy testing studies. Appropriate references to medium, batch number, etc. are documented in the raw data collected during the course of each study.

Additionally, each virucidal efficacy test is assigned a unique Project Number when the Study Director initiates the protocol for the study. This number is used for identification of the test culture plates, etc. during the course of the test. Test culture plates are also labeled with reference to the test virus, experimental start date, and test product. These measures are designed to document the identity of the test system.

METHOD FOR CONTROL OF BIAS: N/A

FINAL REPORT
The report will include, but not be limited to, identification of the sample and date received, dates on which the test was initiated and completed, identification of the virus strain used and composition of the inoculum, description of cells, medium and reagents, description of the methods employed, tabulated results, calculated titers for infectivity and cytotoxicity, and a conclusion as it relates to the purpose of the test.

PROTOCOL CHANGES
If it becomes necessary to make changes in the approved protocol, the revision and reasons for change will be documented, reported to the Sponsor and will become a part of the permanent file for that study. Similarly, the Sponsor will be notified as soon as possible whenever an event occurs that may have an effect on the validity of the study.

PRODUCT DISPOSITION
Test substance retention shall be the responsibility of the Sponsor. Unused test substance will be **discarded** following study completion unless otherwise requested.

RECORD RETENTION

Study Specific Documents

All of the original raw data developed exclusively for this study shall be archived at ATS Labs. 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 the final study report.
7. Study-specific SOP deviations made during the study.

Facility Specific Documents

The following records shall also be archived at ATS Labs. These documents include, but are not limited to, the following:

1. SOPs which pertain to the study conducted.
2. Non study-specific SOP deviations made during the course of this study, which may affect the results obtained during this study.
3. Methods which were used or referenced in the study conducted.
4. QA reports for each QA inspection with comments.
5. Facility Records: Temperature Logs (ambient, incubator, etc.), Instrument Logs, Calibration and Maintenance Records.
6. Current curriculum vitae, training records, and job descriptions for all personnel involved in the study.

PROPOSED STATISTICAL METHODS: N/A

REFERENCES

1. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E 1052 (current version).
2. Annual Book of ASTM Standards, Section 11 Water and Environmental Technology Volume 11.05 Pesticides; Environmental Assessment; Hazardous Substances and Oil Spill Response, E 1482 (current version).
3. Diagnostic Procedures for Viral, Rickettsial, and Chlamydial Infections. Lennette, E. H, Lennette, D.A., and Lennette, E.T. editors. Seventh edition, 1995.

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STUDY INFORMATION

(All sections must be completed prior to submitting protocol)

Test Substance (Name and Batch Number - exactly as it should appear on final report):

_____

Expiration Date: _____

Product Description

- ❑ Quaternary ammonia
- ❑ Iodophor
- ❑ Sodium hypochlorite
- ❑ Peracetic acid
- ❑ Peroxide
- ❑ Other _____

Test Substance Active Concentration (upon submission to ATS Labs): _____

Storage Conditions

- ❑ Room Temperature
- ❑ 2-8°C
- ❑ Other _____

Hazards

- ❑ None known: Use Standard Precautions
- ❑ Material Safety Data Sheet, Attached for each product
- ❑ As Follows: _____

Product Preparation

- ❑ No dilution required, Use as received (RTU)
- ❑ *Dilution(s) to be tested:

_____ defined as _____ + _____
(example: 1 oz/gallon) (amount of test substance) (amount of diluent)

- ❑ Deionized Water (Filter or Autoclave Sterilized)
- ❑ Tap Water (Filter or Autoclave Sterilized)
- ❑ AOAC Synthetic Hard Water: _____PPM
- ❑ Other _____

Note: An equivalent dilution may be made unless otherwise requested by the Sponsor.

Test Virus: Influenza A virus **Indicator Cell Line:** Rhesus monkey kidney (RMK)

Exposure Time(s): 1)_____ 2) NA 3) NA

Exposure Temperature:

- ❑ Room temperature
- ❑ Other:_____°C (please specify range)

Organic Soil Load

- ❑ 1% fetal bovine serum (lowest level that can be tested)
- ❑ 5% fetal bovine serum
- ❑ Other _____

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TEST SUBSTANCE SHIPMENT STATUS

- ☐ Has been used in one or more previous studies at ATS Labs.
- ☐ Has been shipped to ATS Labs (but has not been used in a previous study).
  Date shipped to ATS Labs: _____ Sent via *overnight* delivery? ☐ Yes ☐ No
- ☑ Will be shipped to ATS Labs.
  Date of expected receipt at ATS Labs: _____
- ☐ Sender (if other than Sponsor): _____

COMPLIANCE

This study will be conducted in compliance with the FDA (21 CFR Part 58) and in accordance to standard operating procedures.

☑ Yes
☐ No (Non-GLP Study)

PROTOCOL MODIFICATIONS

☑ Approved without modification
☐ Approved with modification

_____

_____

PROTOCOL ATTACHMENTS

Supplemental Information Form Attached - ☐ Yes ☑ No

APPROVAL SIGNATURES

SPONSOR:

NAME: Mr. Kevin Intrieri TITLE: _____

SIGNATURE: _____ DATE: _____

PHONE: (612) 989 - 6515 FAX: (612) 586 - 8147 EMAIL: kevin@trevitechnology.com

For confidentiality purposes, study information will be released only to the sponsor/representative signing the protocol (above) unless other individuals are specifically authorized in writing to receive study information.

Other individuals authorized to receive information regarding this study: ☐ See Attached

_____

ATS LABS:

NAME: _____
Study Director

SIGNATURE: _____ DATE: _____
Study Director