

殺ウイルス効果試験 まとめ

試験の表題 豚インフルエンザウイルスに対する殺ウイルス効果試験

試験目的 豚インフルエンザウイルスに対する殺ウイルス効果の有無を判定
(EPA米環境保護庁ガイドライン810.2100に基づく)

試験終了日 2009年10月7日

試験実施者 マイクロバイオテスト
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供試ウイルス 豚インフルエンザウイルス(H1N1型) 米環境保護庁指定
Swine Influenza Virus (H1N1), A/Swine/1976/31, ATCC VR-99

供試液剤 機能性アロマ

接触時間 10分

接触温度 21度

希釈倍率 50倍、100倍

試験方法 供試ウイルス0.4mlをシャーレ(2X2インチ四方)に薄く広げ、乾燥後、それぞれ50倍と100倍に希釈した供試液剤2.0mlを供試ウイルスに満遍なく噴霧した後、10分間接触させた。
10分後に中和剤2.0mlを足し、シャーレからスクレーパで掻き採った。
さらに採取した供試液を宿主培養細胞と混ぜ、ウイルス細胞変性効果方により顕微鏡下で観察(4~6日間)。
対照は、(1)中和剤効果、(2)細胞毒性、(3)プレート回復、(4)カラム力価、(5)ウイルス備蓄力価、(6)細胞の制御能力、の6通りで試験を行った。
試験は、1サンプル、規定温度、規定接触時間に基づき、それぞれの希釈倍率で1回反復のみ行われた。

試験結果

希釈 (\log_{10})	機能性アロマ		プレート回復対照	
	50倍	100倍		
-2	C/4	C/4	4/4 - ND	細胞毒性 —
-3	C/4	0/4	4/4 - ND	
-4	0/4	0/4	4/4 - 4/4	
-5	0/4	0/4	4/4 - 4/4	対数減少率 ≥4.00 / ≥5.00
-6	0/4	0/4	4/4 - 4/4	
-7	0/4	0/4	4/4 - 4/4	
TCID ₅₀ /ml (\log_{10})	≤3.50	≤2.50	≥7.50 / 8.0	

上記の表の通り、豚インフルエンザウイルス(H1N1型)に対する殺ウイルス試験では、供試ウイルスに対して完全な不活化が見られたので、当該試験に合格した。

なお、計算方法については、スピアマン・カルベル方式によるTCID₅₀/ml (平均半数感染量)を使って測定した(細胞毒性が検出された場合、対数減少率3.0以上必要)。



MICROBIOTEST, INC

*The Microbiology and
Virology Laboratory*

FINAL REPORT

**VIRUCIDAL EFFICACY TEST
SWINE INFLUENZA VIRUS (H1N1) R&D**

Test Agent: Functional Aroma

**Data Requirements
EPA Guidelines 810.2100**

**Study Director
Tien V. Mai**

**Study Completion Date
October 7, 2009**

**Performing Laboratory
MICROBIOTEST
105 Carpenter Drive
Sterling, Virginia 20164**

**Sponsor
Japan Ecologia
c/o Pyxis Regulatory Consulting, Inc.
4110 136th St. NW
Gig Harbor, WA 98332**

**Laboratory Project Identification Number
666-124**

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STATEMENT OF 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).

SPONSOR: Japan Ecologia Co., Ltd.

SPONSOR'S REPRESENTATIVE: Ann M. Tillman

SPONSOR'S REP. TITLE: Consultant - Agent for Japan Ecologia Co., Ltd.

SIGNATURE: 

DATE: 10/13/2009

GOOD LABORATORY PRACTICE STATEMENT

This study was not conducted according to 40 CFR § 160.

Sponsor:



Ann M. Tillman
Consultant – Agent for Japan Ecologia Co., Ltd.

10/13/2009
Date

Submitter:



Ann M. Tillman
Consultant – Agent for Japan Ecologia Co., Ltd.

10/13/2009
Date

QUALITY ASSURANCE STATEMENT

The material presented in this report has been audited by the Quality Assurance Unit of MICROBIOTEST.

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TEST SUMMARY

TITLE: VIRUCIDAL EFFICACY TEST –SWINE INFLUENZA VIRUS (H1N1) R&D

STUDY DESIGN: This study was performed according to the signed protocol and project sheet(s) issued by the Study Director (See Appendix).

TEST MATERIALS SUPPLIED BY THE SPONSOR OF THE STUDY:

1. Functional Aroma, Lot No. AOP 090907, received at MICROBIOTEST 09/14/09 and assigned DS No. 10312.

SPONSOR: Japan Ecologia
c/o Pyxis Regulatory Consulting, Inc.
4110 136th St. NW
Gig Harbor, WA 98332

CONFIDENTIAL FINAL REPORT SENT VIA E: MAIL**SPONSOR:** Japan Ecologia c/o Pyxis Regulatory Consulting, Inc.**SPONSOR'S REPRESENTATIVE:** Ann M. Tillman**STUDY TITLE:** Virucidal Effectiveness Test – Swine Influenza Virus (H1N1) R&D**STUDY IDENTIFICATION:** MICROBIOTEST Project No. 666-124 (refer to signed protocol)

TEST AGENT NAME	LOT NO.	DATE RECEIVED	DS NO.
FUNCTIONAL AROMA	AOP 090907	09/14/09	10312

NEUTRALIZER: Minimum Essential Medium (MEM) + 1% Fetal Bovine Serum + 1% Polysorbate 80
+ 1% HEPES + 1% NaHCO₃**DILUTION MEDIUM:** MEM + 1 µg/mL Trypsin**CHALLENGE VIRUS:** Swine Influenza Virus (H1N1), A/Swine/1976/31, ATCC VR-99**HOST CELL LINE:** MDCK cells, ATCC CCL-34**CONTACT TIME:** 10 minutes**ORGANIC LOAD:** ≥5% serum**CONTACT TEMPERATURE:** 20±1C (actual temperature was 21C)**DILUTION:** 1:49 [1 part test agent + 49 parts diluent]
1:99 [1 part test agent + 99 parts diluent]**DILUENT:** 400 ppm ± 2.9% AOAC hard water**TEST AGENT APPLICATION:** Inoculated carriers were sprayed until thoroughly wet at a distance of
6 inches**INCUBATION TEMPERATURE:** 36±2C with 5±1% CO₂

INCUBATION TIME: 4-6 days

NUMBER OF REPLICATES: 4 wells per dilution

MEDIA AND REAGENTS: MEM + 1 µg/mL Trypsin
 400 ppm ± 2.9% AOAC hard water
 MEM + 1% Fetal Bovine Serum + 1% Polysorbate 80 + 1% Hepes
 + 1% NaHCO₃
 Phosphate Buffered Saline
 Fetal Bovine Serum

CALCULATION OF TITER AND 95% CONFIDENCE INTERVAL:

The 50% tissue culture infectious dose per mL (TCID₅₀/mL) was determined using the Spearman-Kärber method using the following formula:

$$m = x_k + \left(\frac{d}{2}\right) - d \sum p_i$$

where:

- m = the logarithm of the titer relative to the test volume
- x_k = the logarithm of the smallest dosage which induces infection in all cultures
- d = the logarithm of the dilution factor
- p_i = the proportion of positive results at dilution i

The values were converted to TCID₅₀/mL using a sample inoculum of 1.0 mL.

RESULTS:

Results are presented in Tables 1-4.

The Log₁₀ Reduction Factor (LRF) was calculated in the following manner:

$$\text{Log}_{10} \text{Reduction} = \text{Log}_{10} \text{TCID}_{50} (\text{Plate Recovery Control}) - \text{Log}_{10} \text{TCID}_{50} (\text{Test})$$

The Load (Log₁₀ TCID₅₀) per carrier was calculated in the following manner:

$$\text{Load (Log}_{10} \text{TCID}_{50}) = \text{Titer (Log}_{10} \text{TCID}_{50}/\text{mL}) + \text{Log}_{10} [\text{volume per carrier (mL)}]$$

RESULTS (continued)

For all tables:

C/y = Cytotoxicity observed in y wells inoculated; viral cytopathic effects (CPE) could not be determined

X/y = X wells out of y wells inoculated exhibited positive viral cytopathic effect

0/y = 0 wells out of y wells inoculated exhibited positive viral CPE; no cytotoxicity or bacterial contamination was observed in any of the wells inoculated

Table 1
Test Agent Results

Dilution*	FUNCTIONAL AROMA (Lot No. AOP 090907)	
	1:49 Dilution	1:99 Dilution
10 ⁻²	C/4	C/4
10 ⁻³	C/4	0/4
10 ⁻⁴	0/4	0/4
10 ⁻⁵	0/4	0/4
10 ⁻⁶	0/4	0/4
10 ⁻⁷	0/4	0/4
Titer (Log₁₀ TCID₅₀/mL)	≤ 3.50	≤ 2.50
Load (Log₁₀ TCID₅₀) per carrier (0.4 mL challenge)	≤ 3.10	≤ 2.10
Log₁₀ Reduction	≥ 4.00	≥ 5.00

*Dilution refers to the fold of dilution from virus inoculum.

Table 2
Neutralizer Effectiveness and Cytotoxicity Related Controls

Dilution*	FUNCTIONAL AROMA (Lot No. AOP 090907) 1:49 Dilution	
	Neutralizer Effectiveness Control	Cytotoxicity Control
10 ⁻²	C/4	C/4
10 ⁻³	C/4	C/4
10 ⁻⁴	4/4	0/4

*Dilution refers to the fold of dilution from mock inoculum.

RESULTS (continued)

Table 3
Viability Control Results

Cell Viability Control
0/4
Cells were viable; media were sterile

Table 4
Virus Recovery Controls

Dilution*	Plate Recovery Control	Virus Stock Titer Control
10 ⁻²	4/4	Not Determined
10 ⁻³	4/4	Not Determined
10 ⁻⁴	4/4	4/4
10 ⁻⁵	4/4	4/4
10 ⁻⁶	4/4	4/4
10 ⁻⁷	4/4	4/4
10 ⁻⁸	Not Determined	2/4
10 ⁻⁹	Not Determined	0/4
Titer (Log ₁₀ TCID ₅₀ /mL)	≥ 7.50	8.0
Load (Log₁₀ TCID₅₀) per carrier (0.4 mL challenge)	≥ 7.10	NA

* Dilution refers to the fold of dilution from virus inoculum.

NA = Not Applicable.

CONCLUSIONS

When tested as described, FUNCTIONAL AROMA (Lot No. AOP 090907), diluted 1:49 and 1:99, passed the Virucidal Effectiveness Test when Swine Influenza Virus (H1N1), containing at least 5% organic soil, was exposed to the test agent for 10 minutes at 21C. All of the controls met the criteria for a valid test. These conclusions are based on observed data.

Study director: 
_____ Tien V. Mai


_____ Date

APPENDIX 1

MICROBIOTEST PROTOCOL

VIRUCIDAL EFFECTIVENESS TEST

Swine Influenza Virus (H1N1)

R&D

Prepared for
Japan Ecologia
c/o Pyxis Regulatory Consulting, Inc.
4110 136th St. NW
Gig Harbor, WA 98332

September 21 2009

MICROBIOTEST Protocol: 666.2.09.21.09

MICROBIOTEST Project: 666-124

OBJECTIVE:

This test is designed to substantiate virucidal effectiveness claims for a product to be labeled as a virucide. It determines the potential of the test agent to disinfect hard surfaces contaminated with virus. The test is designed to simulate consumer use and conforms to EPA Guidelines DIS/TSS-7, November 1981, and follows the procedure outlined in the American Society for Test Materials (ASTM) test method designated E 1053-97.

TESTING CONDITIONS:

Virus will be dried on a sterile glass Petri dish at room temperature. One lot of one type of test agent, at two dilutions, will be used to treat the dried virus according to the label claims. One replicate will be performed for each dilution of the product. After a defined exposure period, as specified by the sponsor in the miscellaneous section of the protocol, the neutralized test agent-virus mixture will be scraped from the surface, serially diluted and assayed for the presence of infectious virus. One exposure (contact) time point will be evaluated.

MATERIALS:

- A. Test, control and reference substances will be supplied by the sponsor of the study (see last page).

The test agent will be tested as supplied by the sponsor unless directed otherwise. All operations performed on the test agent such as dilution or specialized storage conditions must be specified by the sponsor before initiation of testing.

The sponsor assures MICROBIOTEST testing facility management that the test agent has been appropriately tested for identity, strength, purity, stability, and uniformity as applicable.

MICROBIOTEST will retain all unused test agents for a period of at least three months after completion of the test, and then discard them in a manner that meets the approval of the safety officer.

B. Materials supplied by MICROBIOTEST, including, but not limited to:

1. Challenge virus requested by the sponsor of the study: Swine Influenza Virus (H1N1), A/Swine/1976/31 (ATCC VR-99)
2. Host cell lines: MDCK cells
3. Laboratory equipment and supplies.
4. Media and reagents:

Media and reagents relevant to the virus-host system and test agent being tested will be documented in the first project sheet and data pack.

TEST SYSTEM IDENTIFICATION:

All Petri dishes, dilution tube racks, and host-containing apparatus will be labeled with the following information: virus and project number.

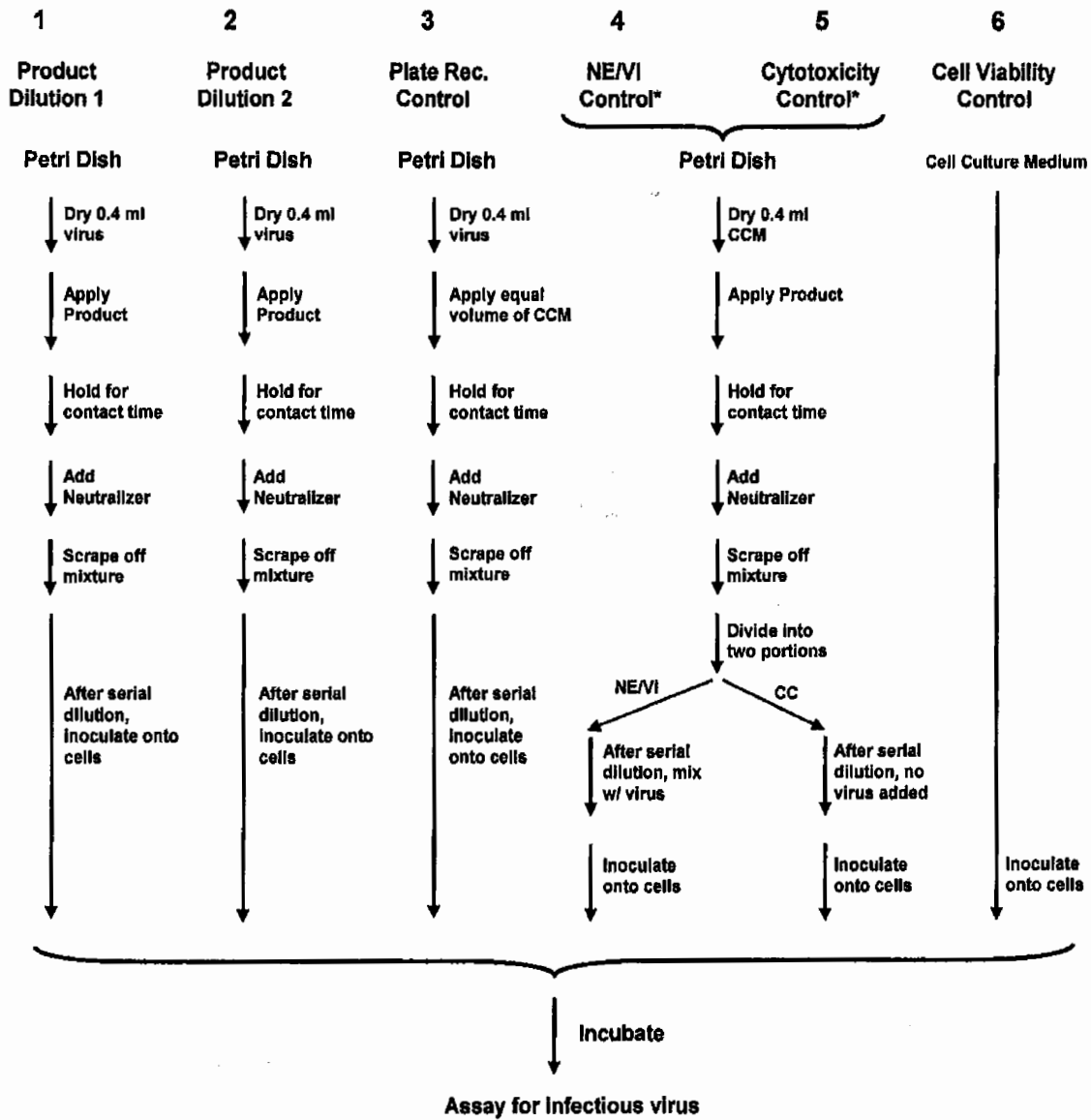
EXPERIMENTAL DESIGN:

All of the procedures involved in performance of this study are described in a detailed series of SOPs that are maintained at MICROBIOTEST. SOPs and Logs are referred to in the raw data.

The study flow diagram is shown in Figure 1, with details described below.

FIGURE 1

Title: Virucidal Effectiveness Test - Swine Influenza Virus (H1N1)



CCM: Cell Culture Medium

NEVI: Neutralizer Effectiveness/Viral Interference control

CC: Cytotoxicity Control

* The NEVI and CC controls will be performed at the highest concentration product only as a worst case.

Note: The volume of virus inoculum per carrier may be changed depending on the titer of the virus. This volume will remain consistent among all test and control runs.

Protocol: 666.2.09.21.09

A. Inoculum preparation:

Viral stocks are purchased from reputable sources that identify them by scientifically accepted methods and may have been further propagated at MICROBIOTEST. Records are maintained that demonstrate the origin of the virus. The virus stocks are stored at an ultra-low temperature.

Frozen viral stocks will be thawed on the day of the test (fresh stock cultures may be used at the discretion of the Study Director). The organic soil concentration will be adjusted to at least 5% for the virus (if not already 5% or above) unless otherwise specified by the Sponsor.

B. Carrier preparation:

One lot of the test agent will be tested at two dilutions and one contact time. One replicate will be performed for each condition.

For each run, an aliquot of 0.4 mL of stock virus will be spread, with the cell scraper, over an area of approximately 4 in² that has been marked on the underside of pre-sterilized Petri dishes. Then the virus will be allowed to dry at ambient temperature. The drying time and temperature will be recorded. One carrier will be prepared for each contact time of the test product and one carrier will be prepared for the plate recovery control.

Additionally, one carrier will be prepared for the neutralizer effectiveness control using cell culture medium (CCM) in place of stock virus.

C. Test agent preparation:

The agent will be prepared according to the sponsor's directions or proposed label claims.

D. Test:

One lot of the test product, two dilutions, will be evaluated at one exposure (contact) time. One replicate run will be performed for each test condition.

For each replicate run, after the inoculum has dried, 2.0 mL of the test product will be applied. The plates will remain at the temperature and for the time specified by the sponsor.

After the contact period, the test agent will be neutralized with 2.0 mL of appropriate neutralizer and the mixture will be scraped from the surface of the dish with a cell scraper. This will be considered approximately one log₁₀ dilution.

For spray type agents, the test agent will be sprayed at the time and distance as directed by the sponsor or the label instructions after the inoculum has dried. The dried virus film should be completely covered. The plates will remain at the temperature and for the exposure time as specified by the sponsor. During the contact period, the volume dispensed will be measured. Post the contact time, an equal volume of neutralizer will be used. The mixture will be scraped from the surface of the dish with a cell scraper. This will be considered approximately a one log₁₀ dilution.

If columns are utilized, an aliquot of each sample will be loaded into individual pre-spun Sephacryl columns. The columns will be spun for 4 minutes at 1000 rpm. Following passage through the columns, the eluates will be aseptically collected and tenfold serially diluted in CCM. If columns are not used, serial tenfold dilutions of neutralized inoculum/test agent mixture(s) virus will be prepared in CCM.

E. Infectivity assay:

The residual infectious virus in both test and controls will be detected by viral-induced cytopathic effect (CPE).

Selected dilutions of the neutralized inoculum/disinfectant mixture will be added to cultured host cells (at least four wells per dilution, per reaction mixture) and incubated at 36±2C with 5±1% CO₂ for a period of 4-6 days. The cell plates may be washed twice with phosphate buffered saline (PBS) before inoculation. The host cell cultures will be observed and re-fed as necessary, during the incubation period. These activities, if applicable, will be recorded. The host cells will be examined microscopically for presence of infectious virions upon completion of incubation. The resulting virus-specific cytopathic effects and test agent-specific cytotoxic effects will be scored by examining both test and controls. These observations will be recorded.

F. Controls:

All controls will be performed at the same time as the test, incubated under the same conditions and assayed in the same manner as the test (see above).

1. Cell viability control:

This control will demonstrate that cells remain viable throughout the course of the assay period. In addition, it will confirm the sterility of the CCM employed throughout the assay period. At least four wells will be inoculated with an appropriate CCM during the incubation phase of the study.

2. Plate recovery control (PRC):

One replicate will be performed for the PRC.

For each replicate of PRC, an equal amount of CCM as is used for the test agent analysis will be added to the dried virus. Post-contact time, the virus/CCM mixture will be subjected to the identical neutralization procedure as the test agent. If columns are used, a portion of the virus/CCM/neutralizer mixture will be used for the column titer control (see section F3).

The results from the PRC will be compared with the test results to confirm recovery of at least four log₁₀ of infectious virus following drying and neutralization. Its titer will be used to compare with the titers of the test results to reach the acceptable test criteria (see below).

3. Column titer control:

This control will be performed only if Sephacryl columns are used. It is performed to determine any effects of Sephacryl columns on infectious virus titer while passing through the columns.

The sample for this control will be acquired from a portion of the Plate recovery control prior to passing through the columns. This sample is used to make direct ten-fold serial dilutions in CCM. Then it will be processed in the same manner as the rest of the test and controls.

4. Neutralizer effectiveness/viral interference control:

This control will determine if residual active ingredient is present after neutralization and if the neutralized test agent interferes with virus infectivity. It will be performed at the highest concentration test product only as a worst case.

This control will be processed exactly as the test procedure but instead of viral inoculum, dried CCM will be exposed to the test agent and assayed as previously described. After treatment and neutralization, this control will be divided into two portions, one for cytotoxicity control, the other for neutralizer effectiveness/viral interference control, and processed as the test.

If columns are used, each portion will be passed through individual columns and the eluate will be serially diluted ten-fold in CCM. If columns are not used, the neutralizer effectiveness sample will be diluted using serial ten-fold dilutions in CCM.

Following serial dilution of the reaction mixture in CCM, 100 μ L of a low titered virus stock will be added to 4.5 mL of each dilution and held for a period greater than or equal to the contact time. Then these selected dilutions will be used to inoculate host cells as described for the test procedure.

5. Cytotoxicity Control:

This control will be performed at the highest concentration test product only as a worst case.

The cytotoxicity sample, acquired from the neutralizer effectiveness control, will be diluted and have no virus added. Selected dilutions will be inoculated and incubated in the same manner as the rest of the test and control samples. These effects are distinct from virus-specific cytopathic effects, which will be evident in the stock titer and plate recovery control cultures.

6. Virus Stock Titer control (VST)

An aliquot of the virus used in the study will be directly serially diluted and inoculated onto the host cells to confirm the titer of the stock virus. This control will demonstrate that the titer of the stock virus is appropriate for use and that the viral infectivity assay is performed appropriately.

G. Calculation:

The 50% tissue culture infectious dose per mL (TCID₅₀/mL) will be determined using the method of Spearman-Kärber, or other appropriate methods. The test results will be reported as the reduction of the virus titer due to treatment with test agent expressed as log₁₀.

TEST ACCEPTANCE CRITERIA:

The test will be acceptable for evaluation of the test results if the criteria listed below are satisfied. The study director may consider other causes that may affect test reliability and acceptance.

- The infectious virus recovered from the PRC control must be $\geq 4\text{-log}_{10}$.
- Viral-induced cytopathic effect must be distinguishable from test agent-induced cytotoxic effects.
- Virus must be recovered from the neutralizer effectiveness/viral interference controls (not exhibiting cytotoxicity).
- Virus must not be detected in the cell viability control.

PRODUCT EVALUATION CRITERIA:

According to the regulatory agencies, the test agent passes the test if there is complete inactivation of the virus at all dilutions. When cytotoxicity is evident, at least a three-log reduction in titer must be demonstrated beyond the cytotoxic level.

PERSONNEL AND TESTING FACILITIES:

A study director will be assigned prior to initiation of the test. Resumes are maintained and are available on request. This study will be conducted at MICROBIOTEST, 105 Carpenter Drive, Sterling, Virginia 20164.

REPORT FORMAT:

MICROBIOTEST employs a standard report format for each test design. Each final report will provide the following information:

- Sponsor identification
- Test agent identification
- Type of assay and project number
- Dates of study initiation and completion
- Interpretation of results and conclusions
- Test results presented in tabular form
- Methods and evaluation criteria, if applicable
- Dates of study initiation and completion (GLP studies only)
- Signed Quality Assurance and Compliance Statements (GLP studies only)

RECORDS TO BE MAINTAINED:

All raw data, protocol, protocol modifications, test agent records, final report, and correspondence between MICROBIOTEST and the sponsor will be stored in the archives at MICROBIOTEST, 105 Carpenter Drive, Sterling, Virginia 20164 or in a controlled facility off site.

All changes or revisions to this approved protocol will be documented, signed by the study director, dated and maintained with this protocol. The sponsor will be notified of any change, resolution, and impact on the study as soon as practical.

The proposed experimental start and termination dates; additional information about the test agent; challenge virus and host cell line monolayers used; media and reagent identified; and the type of neutralizers employed in the test will be addressed in a project sheet issued separately for each study. The date the study director signs project sheet number one will be the initiation date. All project sheets will be forwarded to the study sponsor.