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Center for Veterinary Biologics  
Testing Protocol  

SAM 118  

Supplemental Assay Method for Titration of Pseudorabies Virus in Vaccines  

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* test method which utilizes viral plaque forming units (PFU) in a cell culture system to titer pseudorabies virus (PRV) in modified-live vaccines.

2. Materials

2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 36°± 2°C, 5% ± 1% CO₂, high humidity incubator (Model 3158, Forma Scientific Inc.)

2.1.2 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries Inc.)

2.1.3 Blender

2.1.4 Micropipettors: 200-µL and 1000-µL single channel

2.1.5 Water bath

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.2.1 PRV Reference Virus, Shope strain

2.2.2 Madin-Darby bovine kidney (MDBK) or other permissive cells

2.2.3 Minimum Essential Medium (MEM)

1. 9.61 g MEM with Earles salts without bicarbonate

2. 1.1 g sodium bicarbonate (NaHCO₃)

3. Q.S. to 1000 mL with deionized water (DI), adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

4. Sterilize through 0.22-µm filter.
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5. Aseptically add:
   a. 10 mL L-glutamine (200 mM)
   b. 5 mL lactalbumin hydrolysate or edamine
   c. 50 μg/mL gentamicin sulfate

6. Store at 2°-7°C.

2.2.4 Growth Medium

1. 900 mL of MEM
2. Aseptically add 100 mL gamma-irradiated fetal bovine serum (FBS).
3. Store at 2°-7°C.

2.2.5 2X Medium

1. 100 mL 10X MEM with Earles salts without bicarbonate
2. 2.2 g sodium bicarbonate
3. 340 mL DI
4. Sterilize through 0.22-μm filter.
5. Aseptically add:
   a. 50 μg/mL gentamicin sulfate
   b. 50 mL FBS
6. Store at 2°-7°C.

2.2.6 2% Tragacanth Gum (Trag)

1. 20 g Trag
2. 1000 mL DI
3. Mix vigorously small amounts at a time with a blender set on high.
4. Pour 500 mL each into 1000-mL media bottles.
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5. Sterilize by autoclaving at 15 psi for 30 minutes.

6. Store at 2°C - 7°C.

2.2.7 Overlay Medium

1. Mix equal volumes of 2X Medium and 2% Trag.

2. Store at 2°C - 7°C.

2.2.8 70% Ethyl Alcohol

1. 74 mL ethyl alcohol

2. 26 mL DI

3. Store at room temperature.

2.2.9 Crystal Violet Stain

1. 7.5 g crystal violet

2. 50 mL 70% ethyl alcohol

3. Dissolve crystal violet in alcohol.

4. 250 mL formaldehyde

5. Q.S. to 1000 mL with DI, filter through filter paper.

6. Store at room temperature.

2.2.10 Tissue culture plates, 6-well

2.2.11 12 x 75-mm polystyrene tubes

2.2.12 Graduated cylinders, 25-, 50-, 100-, and 250-mL, sterile

2.2.13 Serological pipette, 10-mL
3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have training in the immunologic basis of virus titration assays, cell culture maintenance, and in the principles of aseptic techniques.

3.2 Preparation of equipment/instrumentation

Set the water bath at 36°± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 Two days prior to test performance

Seed 6-well tissue culture plates with MDBK cells, in Growth Medium, at a cell count that will produce a monolayer after 2 days of incubation at 36°± 2°C.

These become the MDBK plates. Growth Medium is changed if excess acidity of the medium is observed as indicated by a change from red to yellow of Growth Medium.

3.3.2 On day of test performance

Rapidly thaw a vial of PRV Reference Virus in a water bath at 36°± 2°C. Dilute the virus in 4.5 mL MEM to contain 15-40 PFU/100 µL.

3.4 Preparation of the sample (on day of test performance)

3.4.1 Rehydrate a vial of the Test Vaccine by removing the seal and stopper from both the Test Vaccine bottle and the bottle containing the accompanying diluent. Measure the diluent into a sterile graduated cylinder according to the number of doses indicated on the manufacturer’s instructions (e.g., for a 50-dose container of 2-mL-per-dose, reconstitute with 100 ml of diluent), and aseptically pour the diluent into the lyophilized bottle of vaccine. Mix by vortexing. Allow to sit for 15 ± 5 minutes at room temperature.

3.4.2 Prepare serial tenfold dilutions of test vaccine. Serial tenfold dilutions may be made as follows:

1. Place 4.5 mL of MEM with the pipette into labeled 12 x 75-mm polystyrene tubes.

2. Pipette 500 µL of test vaccine to the 10⁻¹ tube, mix by vortexing. Discard pipette tip.
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3. Repeat **Step 2** to the remaining tubes. Continue as needed (10^2 to 10^5), transferring 500 µL from previous tube to the next dilution.

4. **Performance of the Test**

4.1 Decant the Growth Media from MDBK plates.

4.2 Pipette 100 µL/well from each dilution of test vaccine to 2 wells of a MDBK plate. Mix by gentle swirling.

4.3 Pipette 100 µL/well of the diluted Reference Virus Control to 2 wells of 1 MDBK plate. Mix by gentle swirling.

4.4 Maintain 2 or more wells as uninoculated cell culture controls.

4.5 Incubate inoculated plates at 36°± 2°C in a CO₂ atmosphere for 60 ± 10 minutes for virus adsorption.

4.6 Add 3.0 mL/well of warmed (36°) Overlay Medium (see Section 2.2.7) to the plates. Discard any unused, warmed Overlay Medium.

4.7 Incubate the MDBK plates undisturbed at 36°± 2°C in a CO₂ atmosphere for 4 days.

4.8 At the end of incubation, decant Overlay Medium. Pipette 2 mL of the Crystal Violet Stain (see Section 2.2.9) into each well of the plates using the repetitive syringe.

4.9 Allow plates to stand at room temperature for 15 ± 5 minutes.

4.10 Discard the crystal violet stain down a sink. Wash the cell monolayers by dipping each plate several times in a container of running water from the cold faucet. Allow to air dry.

4.11 **PFU counting**

4.11.1 The PFU are visible as clear, circular areas in the cell monolayer where the cells have been destroyed by the virus.

4.11.2 Count the number of PFU for each well.

1. Average the number of PFU between the duplicate wells for each test vaccine dilution.
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2. Average the number of PFU between the 2 wells of the Reference Virus Control wells.

3. Determine the virus titers and express as PFU/dose. Take the dilution of a test vaccine that contains an average of at least 30 PFUs.

Example:

\[
\log_{10} \text{ of plaque count (30)} = 1.48 \\
\log_{10} \text{ of dilution counted (10}^{-3}) = 3.00 \\
\log_{10} \text{ of 2-mL dose factor (20)} = 1.30
\]

Virus titer/dose (total) = 5.78

The test vaccine contains \(10^{5.78}\) PFU/2-mL-dose.

5. Interpretation of the Test Results

5.1 The test is invalid if visible contamination is observed in all dilutions of a Test Vaccine.

5.2 For a valid assay, the Reference Virus Control must have an average PFU count between 15-40.

5.3 Any test not meeting the criteria of Sections 5.1 and 5.2 is considered a NO TEST and may be repeated without prejudice.

5.4 One plaque represents a single infective unit whereas the 50% endpoint infective dose (one ID\(_{50}\)) is statistically equivalent to a theoretical 0.69 of an infective unit. Fifty percent endpoints will be 1.44 times those expressed as PFU/unit of inoculation. Therefore, to express PFU titer as 50% tissue culture infective dose (TCID\(_{50}\)) titer, multiply the PFU by 1.44 or add 0.16 to the \(\log_{10}\) value of the PFU titer. In the example above, the TCID\(_{50}\) would be \(5.78 + 0.16 = 5.94\) or \(10^{5.94}\) TCID\(_{50}\)/2-mL-dose.

5.5 If the validity requirements are met and the titer of the vaccine is greater than or equal to the titer contained in the filed outline of production for the product under test, the product is considered SATISFACTORY.

5.6 If the validity requirements are met but the titer of the Test Vaccine is lower than the required minimum, it must be retested according to 9 CFR 113.8.

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6. **Report of Test Results**

6.1 Test results are reported as the virus titer in TCID\textsubscript{50}/dose.

6.2 Record all test results on the test record.

7. **References**


8. **Summary of Revisions**

**Version .05**

- The Contact information has been updated; however, the Virology Section has elected to keep the same next review date for the document.

**Version .04**

- The phrase "available from the Center for Veterinary Biologics/CVB" has been removed from the document as these reagents are no longer supplied by the CVB.

**Version .03**

- The Contact information has been updated.

- The document number has been changed from VIRSAM0118 to SAM 118.

**Version .02**

This document was revised to clarify the practices currently in use at the Center for Veterinary Biologics and to provide additional detail. While no significant changes were made that impact the outcome of the test, the following changes were made to the document:

- **2.1.7** “Self-refilling syringe” has been removed.

- **2.2.3.2** The amount of sodium bicarbonate (NaHCO\textsubscript{3}) has been changed from 2.2 g to 1.1 g.
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- **2.2.3/2.2.5.5** Penicillin and streptomycin have been deleted.

- **2.2.12** “10 ml syringe and needles” has been replaced with graduated cylinders.

- **3.4.1** “10 ml syringe and needle” has been deleted. Instructions have been added for reconstituting the test vaccine with accompanying diluent by graduated cylinders.

- The refrigeration temperatures have been changed from 4° ± 2°C to 2°- 7°C. This reflects the parameters established and monitored by the Rees system.