

United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 119

Supplemental Assay Method for Titration of Pseudorabies Virus Neutralizing
Antibody (Constant Virus-Varying Serum Method)

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Supplemental Assay Method for Titration of Pseudorabies Virus Neutralizing Antibody
(Constant Virus-Varying Serum Method)

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* assay method which uses plaque reduction in a cell culture system to determine the antibody titer of serum against Pseudorabies virus.

2. Materials

2.1 Cell cultures

Multiple 6-well disposable plastic plates (35-mm well diameter, Linbro Multi-Dish Dispo-Trays) are seeded with Madin-Darby Bovine Kidney (MDBK) cells free of extraneous agents, at a cell count that will produce a monolayer after 2 days of incubation, e.g., 3 mL/well of cells split 1 to 4 in 10% fetal bovine serum (FBS)-supplemented growth medium.

2.2 Growth medium

The cells are grown in Minimum Essential Medium (MEM) with additives (**Appendix I**) at a temperature of 35°- 37°C in an incubator containing an atmosphere of 5% carbon dioxide (CO₂) and a relative humidity of 70% to 80%. Growth medium is not changed unless acidity occurs or cells are not growing well.

2.3 Indicator virus

National Veterinary Services Laboratories (NVSL) reference PRV virus is used as the indicator virus.

2.4 Diluent

Diluent (**Appendix II**), without serum, is used to make dilutions.

3. Preparation for the Test

3.1 Preparation of reagents/control procedures

3.1.1 Dilution of test serum

The serum is heat-treated at 56°C for 30 minutes. Serial twofold dilutions are made in sterile tubes containing diluent and mixed using a Vortex or similar type

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mixer. For example, twofold dilutions can be made as follows:

1. 0.5 mL diluent is added to tubes 2, 3, 4, and 5.
2. 0.5 mL serum is added to tubes 1 and 2. The pipette is discarded and tube 2 is mixed. Tube 1 contains 0.5 mL of the undiluted serum, and tube 2 contains a 1:2 dilution of serum.
3. 0.5 mL from tube 2 is transferred to tube 3. The pipette is discarded and tube 3 is mixed. Serum in tube 3 is a 1:4 dilution.
4. This process is continued until the desired number of serum dilutions are made. 0.5 mL is discarded from the last dilution tube.

3.1.2 Dilution of indicator virus

A vial of NVSL PRV reference virus is thawed, mixed, and diluted to contain 30 to 70 plaque forming units (PFU) per 0.1 mL. This dilution is determined by previous titrations and is designated the “stock virus.”

3.2 Preparation of the sample

Serum neutralization of virus and virus control.

3.2.1 An equal volume of stock virus (0.5 mL) is added to each serum dilution tube, mixed, and incubated at 37°C for 1 hour to allow for neutralization of virus. This mixing of equal volumes of serum dilution and virus results in a further twofold dilution of serum. Thus, the undiluted serum (tube 1) becomes a 1:2 final dilution, the initial 1:2 becomes 1:4, etc.

3.2.2 Virus controls are prepared by making serial tenfold dilutions of stock virus in diluent. Dilutions of 10^0 , 10^{-1} , and 10^{-2} are incubated at 37°C for 1 hour, in the same way as the virus-serum mixture.

4. Performance of the Test

4.1 Inoculation of cells and virus adsorption

Before inoculation of the MDBK cell monolayers, the growth medium is removed by aspiration with a sterile Pasteur pipette attached to a vacuum tube, or by decantation.

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Two wells are inoculated with 0.2 mL of each virus-serum mixture. Four wells are inoculated with 0.1 mL of the virus-diluent mixture.

In each series of tests, 2 or more wells containing cell monolayers are maintained as uninoculated controls.

Inoculated cells are placed in a 3-5% CO₂ incubator at 37°C for 1 hour to allow for virus adsorption. During the incubation, plates are gently rocked and rotated every 20 minutes to redistribute inoculum.

4.2 Overlay and incubation

Following incubation, the plates are ready for the overlay, which serves as maintenance media for the cells. Three mL of the overlay medium (**Appendix III**) at room temperature are added to each well and the plates are returned to the CO₂ incubator. The plates remain in the incubator undisturbed for 4 days.

4.3 Plaque counting

The cultures are prepared and counted as follows.

4.3.1 Overlay medium is poured off.

4.3.2 One or 2 mL crystal violet solution are added to each well and allowed to spread evenly over the monolayers. The plates are allowed to stand at room temperature for a minimum of 10 minutes.

4.3.3 The crystal violet solution is washed from the cell monolayers by dipping each plate several times in a container of running cold tap water. The plates are dried.

4.3.4 Plaques are counted and recorded. The plaques are visible as circular areas in the monolayer where cells have been destroyed by the virus and fail to retain the dye as the uninfected cells do.

5. Interpretation of the Test Results

The plaque reduction titer is the highest serum dilution which causes a 50% reduction in the virus plaque count as compared to the average plaque count of the virus-diluent mixture.

Example: Stock virus plaque count is 50. The serum-virus dilution with half the plaques (24) is the titer of the serum.

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6. Report of Test Results

Record all test results on the test record.

7. References

Title 9, *Code of Federal Regulations*, part 113.333, U.S. Government Printing Office, Washington, DC.

8. Summary of Revisions

Version .03

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

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:

- The Contact information has been updated.

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Appendices

Appendix I

Growth Medium

Lactalbumin hydrolysate or Edamin	0.5%
MEM (Eagle) with Earles salts q.s. ad	100.00%
Sodium bicarbonate	2.2 g
Gentamicin sulfate	50 mcg per mL
Penicillin	25 U per mL
Streptomycin	100 mcg per mL
Heat-inactivated or irradiated fetal bovine serum	10.0%
L-Glutamine	1.0%

Appendix II

Diluent

Lactalbumin hydrolysate or Edamin	0.5%
MEM (Eagle) with Earles salts q.s. ad	100.0%
Sodium bicarbonate	2.2 g
Gentamicin sulfate	50 mcg per mL
Amphotericin B	5.0 mcg per mL
Penicillin	100 U per mL
Streptomycin	100 mg per mL
L-Glutamine	1.0%

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Appendix III

Overlay (gum tragacanth)

Preparation of 2% gum tragacanth:

Tragacanth is added (2.0 g/100 mL for 2%), a little at a time, to the desired volume of deionized water and mixed vigorously with a blender.

This is sterilized by autoclaving at 15 lbs/psi for 15 minutes. It is then stored at 4°C.

The viscosity of tragacanth does not seem to vary significantly with temperature change. The 2% tragacanth can be made up in large volumes and stored at 4°C until used.

Use of 2% gum tragacanth:

The needed volume of 2% tragacanth is added to an equal volume of 2X medium. This mixture is warmed to room temperature before adding to cell cultures.