# United States Department of Agriculture
# Center for Veterinary Biologics
# Testing Protocol

## SAM 110

## Supplemental Assay Method for Titration of Eastern, Western, and Venezuelan Equine Encephalomyelitis Virus Neutralizing Antibodies

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**Supplemental Assay Method for Titration of Eastern, Western, and Venezuelan Equine Encephalomyelitis Virus Neutralizing Antibodies**

## 1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* assay method, employing a cell culture system, for determining the neutralizing antibody titers of sera from guinea pigs vaccinated against Eastern (EEE), Western (WEE), and/or Venezuelan (VEE) Equine Encephalomyelitis viruses.

## 2. Materials

### 2.1 Equipment/instrumentation

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

**2.1.1** Pipetters: 20-µL and 200-µL

**2.1.2** Blender

**2.1.3** 1000-mL borosilicate glass media bottle with screw-top lid

**2.1.4** 36°± 2°C, 5% ± 1% $CO_2$, 70-80% humidity incubator

**2.1.5** Water bath

**2.1.6** Vortex mixer

### 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** Vero 76 cell culture free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

**2.2.2** Growth Medium

**1.** 1000 mL MEM

**2.** Sterilize through a 0.22-µm filter.

**3.** Aseptically add:

**a.** 10 mL L-glutamine

**b.** 5 mL lactalbumin hydrolysate or edamine

**c.** 100 units/mL penicillin

**d.** 50 µg/mL gentamicin sulfate

**e.** 100 µg/mL streptomycin

**f.** 2.5 µg/mL amphotericin B

**g.** 100 mL gamma-irradiated fetal bovine serum (FBS)

**4.** Store at 2°- 7°C.

**2.2.3** Diluent Medium

**1.** 1000 mL MEM

**2.** 2.2 g sodium bicarbonate

**3.** Sterilize through a 0.22-µm filter.

**4.** Aseptically add:

**a.** 10 mL L-glutamine

**b.** 5 mL lactalbumin hydrolysate or edamine

**c.** 100 µg/mL streptomycin

**d.** 100 units/mL penicillin

**e.** 50 µg/mL gentamicin sulfate

**f.** 2.5 µg/mL amphotericin B

**5.** Store at 2°- 7°C

**2.2.4** 2% Diluent Medium

**1.** 100 mL Diluent Medium

**2.** 2 mL FBS

**3.** Store at 2°- 7°C.

**2.2.5** 2X Medium

**1.** 100 mL 10X MEM

**2.** 2.2 g sodium bicarbonate

**3.** 340 mL deionized water (DI)

**4.** Sterilize through a 0.22-µm filter.

**5.** Aseptically add:

**a.** 2% of 7.5% sodium bicarbonate

**b.** 5 mL lactalbumin hydrolysate or edamine

**c.** 100 units/mL penicillin

**d.** 50 µg/mL gentamicin sulfate

**e.** 100 µg/mL streptomycin

**f.** 2.5 µg/mL amphotericin B

**g.** 50 mL gamma-irradiated FBS

**6.** Store at 2°- 7°C.

**2.2.6** 2% Tragacanth Gum (Trag)

**1.** 20 g Trag

**2.** 1000 mL DI

**3.** Mix small amounts at a time vigorously with a blender set on high.

**4.** Pour 500 mL each into 1000-mL media bottles.

**5.** Sterilize by autoclaving for 30 minutes.

**6.** Store at 2°- 7°C

**2.2.7** 7.5% Sodium Bicarbonate

**1.** 7.5 g $NaHCO_3$

**2.** Q.S. to 100 mL with DI.

**3.** Sterilize through a 0.22-µm filter.

**4.** Store at room temperature.

**2.2.8** Overlay Medium

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

**2.** Store at 2°- 7°C.

**2.2.9** 70% Ethyl Alcohol

**1.** 74 mL ethyl alcohol

**2.** 26 mL DI

**3.** Store at room temperature.

**2.2.10** Crystal Violet Stain

**1.** 7.5 g crystal violet

**2.** 50 mL 70% ethyl alcohol

**3.** 250 mL formaldehyde

**4.** Q.S. to 1000 mL with DI.

**5.** Dissolve crystal violet in alcohol, add remaining ingredients, filter through filter paper.

**6.** Store at room temperature.

**2.2.11** EEE, WEE, and VEE Indicator Viruses

**2.2.12** Tissue culture plates, 6-well

**2.2.13** 12 x 75-mm polystyrene tubes

**2.2.14** Pipette

**2.2.15** Bleach

## 3. Preparation for the test

### 3.1 Personnel qualifications/training

Personnel shall have received the required number of vaccinations against EEE, WEE and VEE, and have evidence of serological protection. Personnel shall have sufficient training in standard laboratory procedures.

**3.1.1** All work shall be performed in a Biosafety Level 3 room when live indicator virus is present.

**3.1.2** Live Indicator Virus shall be collected in suitable containers for inactivation by either adding bleach and allowing to sit overnight then pour down the sink to catch tank to be heated before being released, or by autoclaving. Fluids from plate cultures are treated with crystal violet/formalin stain, and should be disposed of in accordance with Biosafety Level 3 agent disposal procedures.

### 3.2 Preparation of equipment/instrumentation

**3.2.1** Set the water bath at 56°± 2°C for serum inactivation.

**3.2.2** Set the water bath at 36°± 2°C to warm the Overlay Medium.

## 3.3 Preparation of reagents/control procedures

**3.3.1** Preparation of Vero 76 cell cultures

Multiple 6-well plates are seeded with Vero 76 cells, in Growth Medium, at a cell count that will produce a monolayer after one day of incubation at 36°± 2°C. (Five plates are needed for each virus, 1 plate for Trag control, and 1 plate for cell control.) Cells older than three days should not be used in the test. Growth Medium is changed if excess acidity of the medium is observed as indicated by a change from red to yellow of Growth Medium or cells are not confluent two days after seeding.

**3.3.2** Indicator Virus Dilution

A vial of Indicator Virus is thawed with warm tap water and diluted in 2% Diluent Medium to contain 60-200 plaque forming units (PFU) per 0.1 mL. This is the Working Dilution of the Indicator Virus.

An Indicator Virus Control is run at the time of the plaque reduction assay to determine the plaque count.

The Working Dilution of each Indicator Virus is mixed with an equal volume of Diluent Medium to represent the same dilution used with the guinea pig/virus mixture.

## 3.4 Preparation of the sample

**3.4.1** Dilution of vaccinated guinea pig sera (VGPS) for EEE and WEE serum neutralization (SN).

**1.** Heat inactivate VGPS in a 56°± 2°C water bath for 30 minutes ± 5 minutes.

**2.** Pipette 190 µL of Diluent Medium into ten labeled tubes.

**3.** Pipette 10 µL of serum from each VGPS into the tubes and vortex. This is a 1:20 dilution of the sera.

**3.4.2** Dilution of control guinea pig sera (CGPS) and VGPS for VEE SN.

**1.** Heat inactivate sera in a 56°± 2°C water bath for 30 ± 5 minutes.

**2.** Pipette 100 µL of Diluent Medium into two labeled tubes for CGPS dilutions or ten labeled tubes for VGPS dilutions.

**3.** Pipette 100 µL of serum from each CGPS or VGPS into the tubes and vortex. This is a 1:2 dilution of the sera.

## 4. Performance of the test

**4.1** Add 200 µL of the Working Dilution of an Indicator Virus (**Section 3.3.2**) to each labeled tube of diluted sera and vortex. This increases the serum dilution to 1:40 for the EEE and WEE SN and 1:4 for the VEE SN.

**4.2** Incubate at 36°± 2°C for 60 ± 10 minutes.

**4.3** Pour off the medium from the plates containing Vero 76 cells.

**4.4** Inoculate 2 wells/sample with 100 µL/well of each virus-serum mixture.

**4.5** Inoculate 100 µL/well of the Indicator Virus Control mixture (**Section 3.3.2[1]**) into each of 6 wells/Indicator Virus.

**4.6** Maintain 2 or more wells as uninoculated cell culture controls.

**4.7** Incubate inoculated plates at 36°± 2°C for 60 ± 10 minutes to allow for virus adsorption.

**4.8** Add 3.0 mL/well of Overlay Medium (**Section 2.2.7**) to the plates. Discard any unused, warmed Overlay Medium.

**4.9** Incubate the WEE plates undisturbed at 36°± 2°C for 2 days. Incubate the EEE and VEE plates undisturbed at 36°± 2°C for 3 days.

**4.10** At the end of incubation, without removing Overlay Medium, pipette 3 mL of the Crystal Violet Stain (**Section 2.2.9**) into each well of the plates using the repetitive syringe.

**4.11** Allow plates to stand at room temperature overnight in a biological safety cabinet.

**4.12** Wash the Crystal Violet Stain from the cell monolayers by dipping each plate several times in a container of running cold tap water. Allow to air dry.

**4.13** PFU counting

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

**4.13.2** Count the number of PFU for each well.

**1.** Average the number of PFU between the duplicate wells for each VGPS and CGPS sample on each Indicator Virus.

**2.** Average the number of PFU between the 6 wells of the Indicator Virus Control wells.

## 5. Interpretation of the test results

**5.1** The average number of PFU from the 6 wells of the Indicator Virus Control is divided by 2 to obtain the 50% plaque reduction count. For example, if the average PFU count for EEE Indicator Virus is 60, the 50% plaque reduction count for EEE would be 30.

**5.2** For a valid assay, the Indicator Virus Control must have an average PFU count between 30-100.

**5.3** For a valid assay, the CGPS must have a titer <1:4 against each Indicator Virus. CGPS samples with PFU above the 50% plaque reduction count have a titer <1:4 for that Indicator Virus.

**5.4** Compare the average PFU for each VGPS to the 50% plaque reduction count. VGPS samples with PFU below the 50% plaque reduction count have a titer ≥1:40 for EEE or WEE or a titer ≥1:4 for VEE.

## 6. Report of test results

**6.1** Test results are reported as the number of VGPS having a titer ≥1:40 for EEE or WEE or a titer ≥1:4 for VEE (e.g. 9/10 ≥1:40). CGPS are reported as the number having a titer <1:4 (e.g. 2/2 <1:4).

**6.2** Record all test results on the test record (worksheet).

## 7. References

**7.1** Code of Federal Regulations, Title 9, Part 113.207.

**7.2** Katz JB, SK Hanson. Encephalomyelitis vaccines: a Vero-derived cell culture alternative to primary duck embryonic cell cultures. 1988. Vaccine 6:6.

**7.3** Richmond JY, RW McKinney, ed. Biosafety in Microbiological and Biomedical Laboratories. Centers for Disease Control and Prevention. Third edition. 1993. U.S. Government Printing Office.

## 8. Summary of revisions

**Version .03**

- The Contact has been changed from Kenneth Eernisse to Joseph Hermann.
- **2.2:** Bleach has been added to the list of reagents/supplies used in this test.
- **3.1:** The choice to use of bleach as a deactivator for live virus has been added to the procedure.
- **4.11:** The use of a biological safety cabinet has been added.

**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 impacting the test were made from the previous protocol, the following changes were made to the document:

- **2.1.1** Deleted self-refilling syringe
- **2.2.2.3/2.2.5.5** Changed heat-inactivated FBS to gamma-irradiated serum
- **3.1** Added WEE and need to have evidence of serological protection.
- **3.1.2** Changed statement for disposal of live virus to conform to Biosafety Level 3 guidelines
- **3.3.1** Added amount of plates needed
- **3.3.2.1** Added to represent the same dilution as the guinea pig/virus mixture

- **4.9** Changed 48 hours to 2 days and 72 hours to 3 days
- **5.1** Expressed that the PFU average comes from the 6 wells of the Indicator virus.
- **6.1** Added examples
- 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.