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Supplemental Assay Method for Titration of Porcine Rotavirus in Modified-live Vaccines

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Supplemental Assay Method for the Titration of Porcine Rotavirus in Modified-live Vaccines

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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* assay method which utilizes cytopathic effect (CPE) in a cell culture system or an indirect fluorescent antibody (IFA) technique for determining the Group A porcine rotavirus (PROTA) content of modified-live vaccines.

Note: For this SAM, the dilution terminology of 1:10, 1:20, etc., specifies 1 part plus 9 parts (liquid), 1 part plus 19 parts, etc.

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% CO_2, high-humidity incubator (Model 3158, Forma Scientific Inc.)

2.1.2 Water bath

2.1.3 Microscope, inverted light (Model CK, Olympus America Inc.)

2.1.4 Microscope, ultraviolet (UV) light (Model BH2, Olympus America Inc.)

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

2.1.6 Micropipettors: 200-µL and 1000-µL single channel; 300-µL x 12-channel

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 PROTA Reference Viruses

1. Serotype 4 (Gottfried strain)

2. Serotype 5 (OSU strain)

2.2.2 Rhesus monkey kidney cells (MA-104) free of extraneous agents as tested by title 9, *Code of Federal Regulations* (9 CFR) (available from the Center for Veterinary Biologics (CVB)).

2.2.3 Minimum essential medium (MEM) (National Centers for Animal Health (NCAH) Media #20030)

1. 9.61 g MEM with Earles salts without bicarbonate

2. 1.1 g sodium bicarbonate ($NaHCO_3$)

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 a 0.22-µm filter.

5. Aseptically add:

a. 10 mL L-glutamine (200 mM)

b. 50 µg/mL gentamicin sulfate

6. Store at 2°- 7°C.

2.2.4 Growth Medium

1. 930 mL of MEM

2. Aseptically add 70 mL of gamma-irradiated fetal bovine serum (FBS).

3. Store at 2°- 7°C.

2.2.5 Diluent Medium

1. 100 mL MEM

2. 83.3 µL pancreatin (4XNF 10X)

3. Store at 2°- 7°C.

2.2.6 Anti-PROTA monoclonal antibody (MAb)

1. MAb against Serotype 4 (Gottfried strain)

2. MAb against Serotype 5 (OSU strain)

2.2.7 Rabbit anti-mouse fluorescein isothiocyanate-labeled conjugate (Rabbit Anti-mouse Conjugate)

2.2.8 Anti-PROTA serum against OSU strain

2.2.9 Anti-PROTA serum against Gottfried strains

2.2.10 0.01 M Phosphate buffered saline (PBS) (NCAH Media #30054)

1. 1.19 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)

2. 0.22 g sodium phosphate, monobasic, monohydrate ($NaH_2PO_4 \cdot H_2O$)

3. 8.5 g sodium chloride (NaCl)

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

5. Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH) or 2.0 N HCl.

6. Sterilize by autoclaving at 15 psi, 121°± 2°C for 35 ± 5 minutes.

7. Store at 2°- 7°C.

2.2.11 80% Acetone

1. 80 mL acetone

2. 20 mL DI

3. Store at room temperature.

2.2.12 Cell culture plates, 96-well

2.2.13 Polystyrene tubes, 12 x 75-mm

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

2.2.15 Pipette

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have training in the immunological basis of cell culture techniques, the principles of IFA, and aseptic techniques.

3.2 Preparation of equipment/instrumentation

On the day of test initiation, set a water bath at 36°± 2°C.

3.3 Preparation of reagents/control procedures

3.3.1 MA-104 Plates

Two days prior to test initiation, seed 96-well cell culture plates with MA-104 cells, in Growth Medium, at a cell count that will produce a monolayer after 2 days of incubation at 36°± 2°C in a CO_2 incubator. These become the MA-104 Plates. One test vaccine or 1 of the 2 PROTA Reference Viruses can be tested on each MA-104 Plate. Growth Medium is changed if excess acidity of the medium is observed or cells are not confluent after incubation.

3.3.2 Pipette 2.0 mL of Dilution Medium into 2, 12 x 75-mm polystyrene tubes. Label each tube with the appropriate Reference Virus.

1. Transfer 500 µL of each Reference Virus to the corresponding labeled tube; mix by vortexing. Discard pipette tip. This is a 1:5 dilution of the Reference Virus.

2. Transfer 1.0 mL of each 1:5 Reference Virus to 2, 12 x 75-mm polystyrene tubes labeled 10^{-1} for each Reference Virus (total of 4 tubes).

3. Pipette 1.0 mL/tube of a specific Anti-PROTA serum to separate 10^{-1} labeled tubes of each Reference Virus. This becomes the Reference Virus-Specific Antiserum Mixture. Repeat for the other specific Anti-PROTA serum (see **Appendix**).

4. Mix with the vortex mixer; incubate for 2 hours ± 10 minutes at room temperature.

5. Pipette 1.8 mL/tube of Diluent Medium into 2 sets of 12 x 75-mm polystyrene tubes labeled 10^{-2} through 10^{-8} for each Reference Virus-Specific Antiserum Mixture.

6. Prepare vaccine tenfold dilutions of each PROTA Reference Virus-Specific Antiserum Mixture. Transfer 200 µL from each Reference Virus-Specific Antiserum Mixture tube to the corresponding labeled 10^{-2} tube. Mix with the vortex mixer. Discard pipette tip.

7. Repeat **Step 6** for the remaining tubes, transferring 200 µL sequentially from the previous dilution to the next dilution, until the dilution sequence is completed.

3.3.3 Working Anti-PROTA MAb

On the day of MA-104 plate examination, if an IFA test is to be conducted, dilute the appropriate Anti-PROTA MAb in PBS, according to the CVB supplied Reagent Data Sheet or as determined for that specific MAb.

3.3.4 Working Rabbit Anti-mouse Conjugate

On the day of MA-104 plate examination, if an IFA test is to be conducted, dilute the Rabbit Anti-mouse Conjugate in PBS, according to the manufacturer's recommendations.

3.4 Preparation of the sample

3.4.1 Conduct the initial test of a Test Vaccine with a single vial (a single sample from 1 vial). On the day of test initiation, remove 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.

3.4.2 Neutralize Test Vaccines containing multiple PROTA and make vaccine tenfold dilutions similar to the methods described in **Section 3.3.2** (see **Appendix**).

4. Performance of the Test

4.1 On the day of test initiation, decant Growth Medium from the MA-104 Plates.

4.2 Add 200 µL/well Diluent Medium to the MA-104 Plates. Decant the Diluent Medium.

4.3 Again, add 200 µL/well Diluent Medium to the MA-104 Plates and incubate for 60 ± 10 minutes at 36°± 2°C in a CO_2 incubator. Decant the Diluent Medium.

4.4 Inoculate 200 µL/well of each dilution of the Test Vaccine-Specific Antiserum Mixture and each Reference Virus-Specific Antiserum Mixture into a minimum of 5 wells/dilution of an MA-104 Plate.

4.5 Add 200 µL/well Diluent Medium to a minimum of 5 wells on each MA-104 plate, to serve as uninoculated cell controls.

4.6 Incubate the MA-104 Plates for 5 days ± 1 day postinoculation at 36°± 2°C in a CO_2 incubator.

4.7 CPE counting is the primary method of determining the $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$).

4.7.1 After 5 days ± 1 day postinoculation, examine the wells with an inverted light microscope. The CPE of PROTA is visible as cell death in the cell monolayer.

4.7.2 Record the number of wells/dilution showing any characteristic PROTA CPE for each Test Vaccine-Specific Antiserum Mixture and Reference Virus-Specific Antiserum Mixture.

4.7.3 Calculate the $TCID_{50}$ of the Test Vaccine-Specific Antiserum Mixture and each Reference Virus-Specific Antiserum Mixture using the method of Spearman-Kärber as modified by Finney.

Example:

10^0 dilution of the Test Vaccine = 5 of 5 wells CPE Pos
10^{-1} dilution of the Test Vaccine = 5 of 5 wells CPE Pos
10^{-2} dilution of the Test Vaccine = 3 of 5 wells CPE Pos
10^{-3} dilution of the Test Vaccine = 0 of 5 wells CPE Pos

Test Vaccine titer = (X - d/2 + [d • S]) where:
X = Log_{10} of lowest dilution (=0)
d = Log_{10} of dilution factor (=1)
S = Sum of proportion of CPE positive

$$\frac{5}{5} = 1.0 + \frac{5}{5} = 1.0 + \frac{3}{5} = 0.6 + \frac{0}{5} = 0 \quad = 2.6$$

Titer = (0 – 0.5 + [1 • 2.6]) = 2.1

Adjust the titer to the recommended Test Vaccine dose as follows:

1. Divide the **Test Vaccine Dose** by the **Inoculation Dose**

Test Vaccine Dose = manufacturer's recommended vaccination dose (for this test vaccine, the recommended dose is 2 mL)

Inoculation Dose = amount of diluted Test Vaccine added to each well of the Test Plate (for this test vaccine, the inoculation dose is 0.2 mL)

$$\frac{2 \text{ mL dose}}{0.2 \text{ mL}} = 10$$

2. Calculate $\log_{10}$ of value in **A** and add it to the **Test Vaccine titer** as illustrated below:

Log of 10 = 1.0

Test Vaccine titer = 2.1 + 1.0 = 3.1

Therefore the titer of the **Test Vaccine** is $10^{3.1}$ $TCID_{50}$/1 mL.

4.8 Certain strains of PROTA may not exhibit pronounced CPE. An IFA may be conducted to determine the titer:

4.8.1 Decant Growth Media from the MA-104 Plates.

4.8.2 Rinse the MA-104 Plates with PBS and incubate at room temperature for 5 ± 2 minutes. Decant the PBS.

4.8.3 Fill the wells with 80% acetone and incubate at room temperature for 15 ± 5 minutes.

4.8.4 Decant the 80% acetone from the MA-104 Plates and air dry at room temperature.

4.8.5 Pipette 35 µL of the Working Anti-PROTA MAb into all wells and incubate for 45 ± 15 minutes at room temperature.

4.8.6 Decant the Working Anti-PROTA MAb. Fill the wells completely with PBS and incubate at room temperature for 5 ± 2 minutes. Decant the PBS.

4.8.7 Repeat for a total of 2 washes.

4.8.8 Gently tap the MA-104 Plates onto paper towels to remove excess liquid.

4.8.9 Pipette 35 µL of the Working Rabbit Anti-mouse Conjugate into all wells and incubate for 40 ± 10 minutes at room temperature.

4.8.10 Repeat **Steps 6 through 8.**

4.8.11 Fill the wells completely with distilled water; decant. Allow to air dry or dry at 36°± 2°C.

4.8.12 Examine the MA-104 Plates with a UV-light microscope at 100 to 200X magnification.

4.8.13 A well is considered positive if typical cytoplasmic, apple-green fluorescence is observed.

4.8.14 Record and calculate as in **Sections 4.7.2** and **4.7.3.**

5. Interpretation of the Test Results

5.1 The test is not a valid test if CPE, fluorescence, or bacterial/fungal contamination is observed in any of the uninoculated control wells.

5.2 An Anti-PROTA serum must neutralize the corresponding homologous Reference Virus.

5.3 The calculated titer of each Reference Virus with heterologous antiserum must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.4 If the validity requirements are not met, then the assay is considered a **NO TEST** and can be retested without prejudice.

5.5 If the validity requirements are met and the titer of the Test Vaccine is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production or Special Outline, the Test Vaccine is considered **SATISFACTORY**.

5.6 If the validity requirements are met but the titer of the Test Vaccine is lower than the required minimum contained in the APHIS filed Outline of Production or Special Outline, the Test Vaccine may be retested in accordance with the 9 CFR 113.8.

6. Report of Test Results

Record all test results on the test record. The titer of the test vaccine is reported as the $\log_{10}$ $TCID_{50}$ per dose.

7. References

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

7.2 Conrath TB. Handbook of Microtiter Procedures. *Clinical and Research Applications Laboratory,* Alexandria, Virginia: Cooke Engineering Co., 1972.

7.3 Finney DJ. *Statistical Method in Biological Assay*, 3rd ed. London: Charles Griffin and Co., 1978, pp. 394-401.

7.4 Rose NR, Friedman H, Fahey JL, eds. Neutralization Assays. In: *Manual of Clinical Laboratory Immunology*. Washington, DC: ASM 1986.

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 document number has been changed from VIRSAM0121 to SAM 121.
- The Contact information has been updated.

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** has been deleted.
- **2.2.3.2** The amount of sodium bicarbonate ($NaHCO_3$) has been changed from 2.2 g to 1.1 g.
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- **2.2.3.5** Penicillin, streptomycin and amphotericin B have been removed.
- **2.8** Anti-PROTA serums have been separated.
- **2.2.15** Pipette has been added.
- **3.3.2.2** “Repetitive syringe” has been deleted and “pipette” has been added.

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- **4.4** "Specific Antiserum Mixture" has been added to Test Vaccine.
- **4.7.3** Additional steps have been added to clarify the titer calculations by Spearman-Kärber formula.
- **4.8.6** "Decant the working Anti-PROTA(MAb)" has been added.
- **4.8.11** "Fill the wells completely with DI" has been added.
- **5.3** "With heterologous antiserum" has been added after Reference Virus.
- 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.
- "Test Serial" has been changed to "Test Vaccine" throughout the document.
- "Reference and Reagent Sheet" has been changed to "Reagent Data Sheet" throughout the document.

Supplemental Assay Method for the Titration of Porcine Rotavirus in Modified-live Vaccines

Appendix

Gottfried RV* (1:5): 1 mL + 1 mL Anti-Gottfried AS
1 mL + 1 mL Anti-OSU AS

OSU RV* (1:5): 1 mL + 1 mL Anti-Gottfried AS
1 mL + 1 mL Anti-OSU AS

Test Vaccine (1:5): 1 mL + 1 mL Anti-Gottfried AS
1 mL + 1 mL Anti-OSU AS

* RV = Reference Virus
AS = Anti-PROTA serum