

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

SAM 101

**Supplemental Assay Method for the Titration of Bovine Viral Diarrhea Virus
in Vaccines**

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Supplemental Assay Method for the Titration of Bovine Viral Diarrhea Virus in Vaccines

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Supplemental Assay Method for the Titration of Bovine Viral Diarrhea Virus in Vaccines

1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* assay method which employs a cell culture system utilizing cytopathic effect (CPE), direct fluorescence antibody (FA), and/or indirect FA (IFA) staining to determine the bovine viral diarrhea virus (BVDV) content of modified-live veterinary 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 Incubator, $36^{\circ} \pm 2^{\circ}\text{C}$, high humidity, $5\% \pm 1\%$ CO_2 (Model 3336, Forma Scientific Inc.)

2.1.2 Water bath, $37^{\circ} \pm 1^{\circ}\text{C}$

2.1.3 Pipettors, 25- μL , 500- μL , and tips

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

2.1.5 Multichannel pipettor, 50- to 300- μL x 8- or 12-channel

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

2.1.7 Ultraviolet light microscope (Model BH2, Olympus America Inc.)

2.2 Reagent/supplies

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

2.2.1 BVDV Positive Control (available from the Center for Veterinary Biologics [CVB])

1. NADL strain (cytopathic type 1)

2. 890 strain (noncytopathic type 2)

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3. 125 strain (cytopathic type 2)

2.2.2 Embryonic bovine kidney primary cells (EBKp) or bovine turbinate (BOTURs) cells found to be free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR).

2.2.3 Diluent Medium

1. 9.61 g minimum essential medium (MEM) with Earles salts without bicarbonate

2. 1.1 g sodium bicarbonate (NaHCO₃)

3. Dissolve with 900 mL deionized water (DI).

4. Add 5.0 g lactalbumin hydrolysate or edamine to 10 mL DI. Heat to 60°± 2°C until dissolved. Add to ingredients in **Step 3** with constant mixing.

5. Q.S. to 1000 mL with DI; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

6. Sterilize through a 0.22-µm filter.

7. Aseptically add:

a. 10 mL L-glutamine (200 mM)

b. 50 µg/mL gentamicin sulfate

8. Store at 2°- 7°C.

2.2.4 Growth Medium

1. 900 mL of Diluent Medium

2. Aseptically add 100 mL gamma-irradiated fetal bovine serum (FBS)

3. Store at 2°- 7°C.

2.2.5 Anti-positive control BVDV (Anti-BVDV) fluorescein isothiocyanate labeled conjugate (available from the CVB)

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2.2.6 Monoclonal antibody (MAb) against BVDV type 1 or 2 (available from the CVB)

2.2.7 Anti-mouse fluorescein isothiocyanate labeled conjugate (Anti-mouse Conjugate).

2.2.8 0.01 M Phosphate buffered saline (PBS) (National Veterinary Services Laboratories [NVSL] Media #30054)

1. 1.19 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)
2. 0.22 g sodium phosphate, monobasic, monohydrate($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)
3. 8.5 g sodium chloride (NaCl)
4. Q.S. to 1000 mL with DW
5. Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide(NaOH) or 2N HCl
6. Sterilize by autoclaving at 15 psi, $121^\circ \pm 2^\circ\text{C}$ for 35 ± 5 minutes; store at $2^\circ - 7^\circ\text{C}$.

2.2.9 Trypsin versene (TV) Solution (NVSL Media #20005)

1. 8.0 g NaCl
2. 0.40 g KCl
3. 0.58 g NaHCO_3
4. 0.50 g irradiated trypsin
5. 0.20 g versene or disodium salt ethylenediaminetetraacetic acid (EDTA)
6. 1.0 g dextrose
7. 0.4 mL 0.5% phenol red
8. Q.S. with DI to 1000 mL.
9. pH to 7.3 with NaHCO_3 .
10. Filter through a 0.22- μm filter.

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11. Store at $-20^{\circ}\pm 2^{\circ}\text{C}$.

2.2.10 80% acetone

1. 80 mL acetone
2. 20 mL distilled water
3. Store at room temperature.

2.2.11 Tissue culture plates, 96-well

2.2.12 Polystyrene tubes, 17 x 100-mm

2.2.13 Polystyrene tubes, 12 x 75-mm

2.2.14 Serological pipette, 10-mL

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

2.2.16 Infectious bovine rhinotracheitis monospecific antiserum (IBR AS)
(available from the CVB)

2.2.17 Parainfluenza 3 virus monospecific antiserum (PI₃V AS) (available the
from CVB)

2.2.18 Bovine respiratory syncytial virus monospecific antiserum (BRSV AS)
(available from the CVB)

2.2.19 Plastic squirt bottle, 500-mL

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have training in cell culture technique, the principles of aseptic technique, and virus titration assays.

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3.2 Preparation of equipment/instrumentation

On the day of test initiation, set the water bath at $36^{\circ} \pm 2^{\circ}\text{C}$.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of EBKp/BOTUR Plates

Cells are prepared from healthy, confluent EBKp (pass 3 or lower) cells or BOTURs (passage 20 or lower), that are maintained by passing every 5 ± 2 days. One day prior to test initiation, cells are removed from the growth containers by using TV Solution. Using a multichannel pipettor, add $200 \mu\text{L}/\text{well}$ of $10^{5.4}$ to $10^{5.6}$ cells/mL cells suspended in Growth Medium into all wells of a 96-well cell culture plate.

Prepare 1 plate of cells for the controls and the first Test Vaccine. Each additional plate allows testing of 2 Test Vaccines. Incubate at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO_2 incubator for 24 ± 12 hours. Growth Medium is not changed unless excess acidity occurs or cells are not confluent in 24 hours.

3.3.2 Preparation of BVDV Positive Control

On the day of test initiation, the BVDV Positive Control is selected based on the genotype (type 1 or 2) and the phenotype (cytopathic or noncytopathic) of the vaccine virus to be titered. Once selected, a vial of the BVDV Positive Control is rapidly thawed in a $36^{\circ} \pm 2^{\circ}\text{C}$ water bath and tenfold dilutions made:

1. Place 4.5 mL of Diluent Medium into 6, 17 x 100-mm polystyrene tubes labeled 10^{-1} to 10^{-6} respectively, using a 10-mL serological pipette.
2. Using a 500- μL pipettor, transfer 500 μL of BVDV Positive Control to the 10^{-1} tube; mix by vortexing. Discard pipette tip.
3. Using a new pipette tip, transfer 500 μL from the 10^{-1} labeled tube to the 10^{-2} tube; mix by vortexing.
4. Repeat **Step 3** for each of the subsequent dilutions, transferring 500 μL from the previous dilution to the next dilution tube until the dilution series is completed.

3.3.3 Working Anti-BVDV Conjugate. On the day of examination, if an FA test is to be conducted, dilute the appropriate Anti-BVDV Conjugate in PBS, according to the CVB supplied Reagent Data Sheet or as determined for that specific conjugate.

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

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

3.4 Preparation of the sample

3.4.1 The initial test of a Test Vaccine will be 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 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 Viral neutralization. In order to determine the BVDV titer in a multifraction product, on the day of test initiation, neutralize the IBR, PI₃V, and/or BRSV fractions with monospecific antiserum.

1. IBR/BVDV Vaccine

- a.** 1.0 mL of the reconstituted Test Vaccine is diluted with 4.0 mL of Diluent Medium in a 17 x 100-mm polystyrene tube; mix by vortexing.
- b.** Mix 500 μ L of **Step 1a** dilution of the Test Vaccine with 500 μ L IBR AS in a 12 x 75-mm polystyrene tube, labeled 10⁻¹. Mix by vortexing.
- c.** Incubate at 36 \pm 2 $^{\circ}$ C for 60 \pm 15 minutes.
- d.** The mixture constitutes a 10⁻¹ dilution of the Test Vaccine.

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2. IBR/PI₃V/BVDV Vaccine

- a.** 1.0 mL of the reconstituted Test Vaccine is diluted with 4.0 mL of Diluent Medium in a 17 x 100-mm polystyrene tube; mix by vortexing.
- b.** 1.0 mL of **Step 2a** dilution of the Test Vaccine is mixed with 500 µL of IBR AS and 500 µL of PI₃V AS in a 12 x 75-mm polystyrene tube, labeled 10⁻¹. Mix by vortexing
- c.** Incubate at 36°± 2°C for 60 ± 15 minutes.
- d.** The mixture constitutes a 10⁻¹ dilution of the Test Vaccine.

3. IBR/PI₃V/BVD/BRSV Vaccine

- a.** 2.0 mL of the reconstituted Test Vaccine is diluted with 8.0 mL of Diluent Medium in a 17 x 100-mm polystyrene tube; mix by vortexing.
- b.** 1.5 mL of **Step 3a** dilution of the Test Vaccine is mixed with 500 µL of IBR AS, 500 µL of PI₃V AS and 500 µL of BRSV AS in a 12 x 75-mm polystyrene tube, labeled 10⁻¹. Mix by vortexing.
- c.** Incubate at 36°± 2°C for 60 ± 15 minutes.
- d.** The mixture constitutes a 10⁻¹ dilution of the Test Vaccine.

4. BVDV Monovalent Vaccine

- a.** 500 µL of the reconstituted Test Vaccine is diluted with 4.5 mL of Diluent Medium in a 17 x 100-mm polystyrene tube, labeled 10⁻¹. Mix by vortexing.
- b.** The mixture constitutes a 10⁻¹ dilution of the Test Vaccine.

3.4.3 Sample dilutions. Five, tenfold dilutions are made from the 10⁻¹ dilution of the Test Vaccine using Diluent Medium.

- 1.** Place 4.5 mL of Diluent Medium into each of five 17 x 100-mm polystyrene tubes labeled 10⁻² through 10⁻⁶, using a 10-mL serological pipette.

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2. Pipette 500 μL of the Test Vaccine from the 10^{-1} tube into the 10^{-2} tube, using a 500- μL pipettor; mix by vortexing. Discard pipette tip.
3. Using a new tip each time, repeat **Step 2** to the remaining tubes transferring 500 μL from the previous dilution tube to the next tube until the final dilution is made (10^{-6}); mix by vortexing between each dilution.

4. Performance of the Test

- 4.1 On the day of test initiation, inoculate 5 wells/dilution with 50 μL /well of the diluted Test Vaccine and the BVDV Positive Control (10^{-3} through 10^{-6}). Change tips between each unique sample (e.g., each Test Vaccine and the BVDV Positive Control), but tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g. 10^{-6} through 10^{-3}).
- 4.2 Maintain 5 wells as uninoculated cell culture controls on each plate.
- 4.3 Incubate the plates undisturbed at $36^{\circ}\pm 2^{\circ}\text{C}$ in a CO_2 incubator for 4 days \pm 1 day.
- 4.4 At the end of incubation, examine the plates at 100X magnification on an inverted light microscope for CPE characterized by cell fusion.
 - 4.4.1 Wells displaying 1 or more CPE foci, are considered to be positive for BVDV.
 - 4.4.2 Results are recorded as the number of CPE positive wells versus the total number of wells examined for each dilution of the Test Vaccine and the BVDV Positive Control.
- 4.5 If CPE is not detected by microscopic examination, the plates may be read by FA or IFA (FA used for most type 1 BVDV; IFA used for type 2 BVDV):
 - 4.5.1 FA method:
 1. Growth Medium is decanted from the plates into a suitable autoclavable container and cells are rinsed once with room temperature PBS using a plastic wash bottle or by immersion into a pan filled with room temperature PBS. PBS is decanted immediately after filling.
 2. Fill each well of the plates with 80% acetone using a plastic squirt bottle.

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3. The plates are allowed to incubate for 15 ± 5 minutes at room temperature.
4. Decant the 80% acetone into a suitable container. Allow to air dry.
5. Using a multichannel pipettor, add $50 \mu\text{L}/\text{well}$ of the Anti-BVDV Conjugate to each well of the plates.
6. The plates are incubated for 30 ± 15 minutes at $36^\circ \pm 2^\circ\text{C}$.
7. Decant the conjugate from all wells into a suitable container and rinse twice with PBS as in **Step 1**. The plates may be read immediately or air dried.
8. The monolayers are examined for specific cytoplasmic fluorescence using an ultraviolet light microscope at 100X magnification. Wells containing one or more FA foci clusters adhering to the cell monolayer are considered to be positive for BVDV.
9. Record the number of FA positive wells versus the total number of wells examined for each dilution of the Test Vaccine and the BVDV Positive Control.

4.5.2 IFA method:

1. Proceed as for FA procedure in **Section 4.5.1, Steps 1 through 4**.
2. Using a multichannel pipettor, add $50 \mu\text{L}/\text{well}$ of the MAAb against BVDV type 1 or 2, depending on which type is to be titrated, to all wells of the plates.
3. The plates are incubated for 30 ± 15 minutes at $36^\circ \pm 2^\circ\text{C}$.
4. Decant the monoclonal antibody from wells and rinse 2 times with PBS. Remove as much PBS as possible by blotting on an absorbent surface.
5. Using a multichannel pipettor add $50 \mu\text{L}/\text{well}$ of Anti-mouse Conjugate to all wells of the plates.
6. The plates are incubated for 30 ± 15 minutes at $36^\circ \pm 2^\circ\text{C}$.
7. Proceed as in **Section 4.5.1, Steps 7 through 9**.

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4.6 Calculate the BVDV endpoints of the Test Vaccine and the BVDV Positive Control using the method of Spearman-Kärber as modified by Finney. The titers are expressed as \log_{10} 50% tissue culture infective dose (TCID₅₀) of the test wells.

Example:

10^{-3} dilution of Test Vaccine = 5/5 wells CPE/FA positive
 10^{-4} dilution of Test Vaccine = 5/5 wells CPE/FA positive
 10^{-5} dilution of Test Vaccine = 2/5 wells CPE/FA positive
 10^{-6} dilution of Test Vaccine = 0/5 wells CPE/FA positive

Test Vaccine titer = $(X - d/2 + [d \cdot S])$ where:

X = \log_{10} of lowest dilution (3)
d = \log_{10} of dilution factor (1)
S = sum of proportion of CPE/FA positive

$$\frac{5}{5} + \frac{5}{5} + \frac{2}{5} + \frac{0}{5} = \frac{12}{5} = 2.4$$

Test Vaccine titer = $(3 - 1/2 + (1 \cdot 2.4)) = 4.9$

Adjust the titer to the Test Vaccine dose size by adding the \log_{10} of the reciprocal of the Inoculation Dose divided by the Test Vaccine dose where:

Inoculation Dose = amount of diluted Test Vaccine added to each well of the Test Plate

Test Vaccine Dose = manufacturer's recommended vaccination dose

Example:

$$\begin{aligned} \text{BVDV endpoint} &= 4.9 \\ \frac{2 \text{ mL dose}}{0.050 \text{ mL inoculum}} &= 40 \\ &= \underline{1.6 \log} \\ \text{Total} &= 6.5 \log \end{aligned}$$

Titer of the Test Vaccine is $10^{6.5}$ TCID₅₀.

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5. Interpretation of the Test Results

5.1 For a valid assay

5.1.1 The calculated TCID₅₀ titer of the BVDV Positive Control 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.

5.1.2 The uninoculated cell controls can not exhibit any CPE or cloudy media that would indicate contamination.

5.1.3 The lowest dilution of the BVDV Positive Control must exhibit a 100% positive CPE/FA (5/5), and the highest (most dilute) must exhibit no positive CPE/FA (0/5).

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

5.3 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, the Test Vaccine is considered **SATISFACTORY**.

5.4 If the validity requirements are met but the titer of the Test Vaccine is less than the titer contained in the APHIS filed Outline of Production, the Test Vaccine is retested according to 9 CFR, Part 113.8.b.

6. Report of Test Results

Results are reported as TCID₅₀ per dose. Record all test results on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.309, U.S. Government Printing Office, Washington, DC.

7.2 Cottral GE, (Ed.), 1978, *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY, pg.731.

7.3 Finney, DJ, 1978, *Statistical method in biological assay*. Griffin, London. 3rd edition, pp. 394-401.

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8. Summary of Revisions

Version .03

- The Contact has been changed from Kenneth Eernisse to Joseph Hermann.
- The term “Reference” has been changed to “Positive Control” throughout 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:

- **2.2.2** The use of Bovine Turbinate cells has been added.
- **2.2.3** The formulation for the Diluent Medium has been changed and penicillin, streptomycin and amphotericin B have been removed.
- **4.6** Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.
- The refrigeration temperatures have been changed from $4^{\circ} \pm 2^{\circ}\text{C}$ to $2^{\circ} - 7^{\circ}\text{C}$. 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.