

United States Department of Agriculture  
Center for Veterinary Biologics  
Testing Protocol

SAM 115

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

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Supplemental Assay Method for Titration of Bovine Virus Diarrhea Neutralizing Antibody  
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**Supplemental Assay Method for Titration of Bovine Virus Diarrhea Neutralizing Antibody  
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**1. Introduction**

This Supplemental Assay Method (SAM) describes an *in vitro* serum neutralization (SN) assay to determine the neutralizing activity against Bovine Virus Diarrhea (BVD) type 1 and 2 virus.

**2. Materials**

**2.1 Equipment/instrumentation**

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

**2.1.1** Incubator,  $36^{\circ} \pm 2^{\circ}\text{C}$ ,  $5\% \pm 1\% \text{CO}_2$ , high humidity

**2.1.2** Vortex mixer

**2.1.3** Microscope, inverted light

**2.1.4** Microscope, fluorescent

**2.1.5** Micropipettor, 200- $\mu\text{L}$ , 1000- $\mu\text{L}$  single channel, 5- to 50- $\mu\text{L}$  x 12-channel, and tips

**2.1.6** Water bath

**2.1.7** Centrifuge and rotor

**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 Indicator Virus**

1. BVD type 1

2. BVD type 2

**2.2.2** Bovine Turbinate (BT) cell cultures free of extraneous agents as tested by title 9, *Code of Federal Regulations* (9 CFR).

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**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. 2.2 g sodium bicarbonate (NaHCO<sub>3</sub>)
3. Dissolve **Steps 1 and 2** with 900 mL deionized water (DI).
4. Add 5 g lactalbumin hydrolysate or edamine to 10 mL DI. Heat to 60°± 2°C until dissolved. Add to **Step 3** with constant stirring.
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
  - b. 50 µg/mL gentamicin sulfate
8. Store at 4°± 2°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 4°± 2°C.

**2.2.5 Monoclonal antibodies (MAb)**

1. Anti-BVD type 1 MAb
2. Anti-BVD type 2 MAb

**2.2.6 Anti-mouse fluorescein isothiocyanate labeled conjugate (Anti-Mouse Conjugate)**

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**2.2.7 80% Acetone**

1. 80 mL acetone
2. 20 mL DI
3. Store at room temperature ( $23^{\circ}\pm 2^{\circ}\text{C}$ ).

**2.2.8 0.01 M Phosphate buffered saline (PBS) (NCAH Media #30054)**

1. 1.33 g sodium phosphate, dibasic, anhydrous ( $\text{Na}_2\text{HPO}_4$ )
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 ( $\text{NaCl}$ )
4. Q.S. to 1000 mL with DI.
5. Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide ( $\text{NaOH}$ ) or 2N HCl.
6. Sterilize by autoclaving at 15 psi,  $121^{\circ}\pm 2^{\circ}\text{C}$  for  $35 \pm 5$  minutes.
7. Store at  $4^{\circ}\pm 2^{\circ}\text{C}$ .

**2.2.9 FBS negative for BVD antibodies**

**2.2.10 Cell culture plate, 96-well**

**2.2.11 Polystyrene tube, 17 x 100-mm**

**2.2.12 Serological pipette, 10-mL**

**2.2.13 Plastic wash bottle, 500-mL**

**3. Preparation for the Test**

**3.1 Personnel qualifications/training**

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

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**3.2 Preparation of equipment/instrumentation**

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

**3.2.2** On day of test initiation, set a water bath at  $56^{\circ} \pm 2^{\circ}\text{C}$ .

**3.3 Preparation of reagents/control procedures**

**3.3.1** One day prior to test initiation, seed 96-well cell culture plates with BT cells, in Growth Medium, at a cell count that will produce a confluent monolayer after 1 day of incubation at  $36^{\circ} \pm 2^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator. These become the BT Test Plates.

**3.3.2** On day of test initiation, rapidly thaw a vial of an appropriate Indicator Virus in a  $36^{\circ} \pm 2^{\circ}\text{C}$  water bath. The number of dilutions depends upon the predetermined titer of the Indicator Virus. Prepare serial tenfold dilutions as follows:

- 1.** Pipette 9.0 mL of MEM with a serological pipette into 8, 17 x 100-mm polystyrene tubes labeled  $10^{-1}$  to  $10^{-6}$ .
- 2.** Transfer 1.0 mL of an Indicator Virus to the  $10^{-1}$  tube; mix by vortexing. Discard the pipette.
- 3.** Transfer 1.0 mL from the  $10^{-1}$  tube to the  $10^{-2}$  tube; mix by vortexing. Discard the pipette.
- 4.** Repeat **Step 3** for each of the subsequent dilutions, transferring 1.0 mL from the previous dilution to the next dilution.

**3.3.3** On the day of BT Test Plate examination, dilute Anti-BVD MAb and Anti-Mouse Conjugate in PBS according to the supplied Center for Veterinary Biologics (CVB) Reference and Reagent Data Sheet.

**3.4 Preparation of the sample**

On day of, or prior to test initiation, heat inactivate all Test Sera in a  $56^{\circ} \pm 2^{\circ}\text{C}$  water bath for  $30 \pm 5$  minutes.

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**4. Performance of the Test**

- 4.1** Add 150  $\mu$ L/well of an undiluted Test Serum into a column of a 96-well cell culture plate, which becomes the Dilution Plate (**Appendix I**).
- 4.2.** Add 150  $\mu$ L/well of Reference Serum (if used) into a column of a Dilution Plate.
- 4.3** Add 150  $\mu$ L/well of the last 4 dilutions of the Indicator Virus to a row of the Dilution Plate.
- 4.4** Mix by tapping the edge of the Dilution Plate with fingers. Incubate for  $60 \pm 10$  minutes at  $36^\circ \pm 2^\circ\text{C}$  to allow for neutralization of the Indicator Virus.
- 4.5** Inoculate 50  $\mu$ L/well of each Virus-serum mixture into 5 wells/dilution of the appropriate Test Plate (**Appendix II**).

**Note: The Growth Media is not removed from the BT Test Plates prior to inoculation.**

- 4.6** Maintain 5 or more wells on each Test Plate as uninoculated cell controls.
- 4.7** Inoculate 25  $\mu$ L/well of the last 4 dilutions of the Indicator Virus to 5 wells/dilution as an endpoint titration.
- 4.8** Incubate the BT Test Plates for 4 days  $\pm$  12 hours postinoculation at  $36^\circ \pm 2^\circ\text{C}$  in a  $\text{CO}_2$  incubator.
- 4.9** At the end of the incubation period, examine the wells with an inverted light microscope. Record the number of wells/dilution showing any characteristic CPE for each Test Sera and for the Reference Serum.
- 4.10** Alternatively, an indirect fluorescent antibody technique (IFA) may be conducted to determine the SN titer against BVD as follows:
- 4.10.1** Decant media from the BT Test Plates.
  - 4.10.2** Fill wells with 80% acetone.
  - 4.10.3** Incubate at room temperature for  $15 \pm 5$  minutes.
  - 4.10.4** Decant the 80% acetone from the BT Test Plate; air dry at room temperature.

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**4.10.5** Pipette 50  $\mu\text{L}$ /well of a diluted Anti-BVD MAb into a BT Test Plate; incubate for  $45 \pm 15$  minutes at  $36^\circ \pm 2^\circ\text{C}$ .

**4.10.6** Rinse by filling the wells completely with PBS; decant the liquid.

**4.10.7** Repeat **Step 6** for a total of 2 washes.

**4.10.8** Pipette 50  $\mu\text{L}$ /well of the diluted Anti-mouse Conjugate into the BT Test Plates; incubate for  $45 \pm 15$  minutes at  $36^\circ \pm 2^\circ\text{C}$ .

**4.10.9** Repeat **Step 6** for a total of 2 washes.

**4.10.10** Dip the plate in DI, decant; allow to air dry or dry at  $36^\circ \pm 2^\circ\text{C}$ .

**4.10.11** Examine wells with a fluorescent microscope.

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

**4.10.13** Record the number of wells/dilution showing any characteristic fluorescence for each Test Sera and the Reference Serum.

**4.11** Calculate the  $\text{TCID}_{50}$  of the Test Sera and the virus alone using the Spearman-Kärber method as commonly modified.

**4.12** The neutralizing ability is determined by subtracting the log of the titer obtained with the Test serum from the titer obtained with the virus alone.

|          |  |              |
|----------|--|--------------|
| Example: | $\text{Log}_{10} \text{TCID}_{50} \text{ Indicator Virus titer}$ | 6.0          |
|          | $\text{Log}_{10} \text{TCID}_{50} \text{ titer with Test Serum}$ | <u>- 2.7</u> |
|          | Neutralizing ability   | = 3.3 logs   |

**5. Interpretation of the Test Results**

**5.1** No visible contamination or serum toxicity should be observed in any well or any dilution of a Test Sera or the Reference Serum.

**5.2** The titer of the Reference Serum should be negative for neutralizing antibodies against BVD.



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**5.3** The endpoint titration of the Indicator Virus should fall within 2 standard deviations of its mean titer as determined by a minimum of 10 previous titrations.

**6. Report of Test Results**

Record the neutralizing activity titers for the Test Serum on the test record.

**7. References**

**7.1** Title 9, *Code of Federal Regulations*, parts 113.215 and 113.216, U.S. Government Printing Office, Washington, DC.

**7.2** Finney DJ. *Statistical Method in Biological Assay*. 3<sup>rd</sup> ed. 1978. Charles Griffin and Co., London.

**7.3** Rose NR, H Friedman, JL Fahey, eds. *Manual of Clinical Laboratory Immunology*. Chapter 11: Neutralization Assays. 1986. ASM, Washington, D.C.

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

- The Contact information has been updated.
- Test incubation conditions have been changed from room temperature to  $36^{\circ}\pm 2^{\circ}\text{C}$  to reflect commonly accepted procedures.

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**Appendix I**

Minimum Essential Medium (MEM)

|                                       |                    |
|---------------------------------------|--------------------|
| MEM (Eagle) with Earles salts q.s. ad | 100.0%             |
| Edamin                                | 0.5%               |
| L-Glutamine                           | 1.0%               |
| Gentamicin                            | 50.0 mcg per mL    |
| Penicillin                            | 100.0 units per mL |
| Streptomycin                          | 100.0 mcg per mL   |
| Amphotericin B                        | 2.5 mcg per mL     |
| Fetal Bovine Serum                    | 10.0%              |

**Appendix II**

Phosphate Buffered Saline (PBS-Dulbecco)

|                                     |        |
|-------------------------------------|--------|
| NaCl                                | 0.8%   |
| KCl                                 | 0.02%  |
| Na <sub>2</sub> HPO <sub>4</sub>    | 0.115% |
| KH <sub>2</sub> PO <sub>4</sub>     | 0.02%  |
| CaCl <sub>2</sub> (anhy)            | 0.01%  |
| Mgcl <sub>2</sub> 6H <sub>2</sub> O | 0.01%  |
| Distilled H <sub>2</sub> O q.s. ad  | 100.0% |

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