

United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

SAM 107

**Supplemental Assay Method for the Titration of Neutralizing Antibody
against Selected Bovine Viruses (Constant Serum-Varying Virus Method)**

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Supplemental Assay Method for the Titration of Neutralizing Antibody against Selected Bovine Viruses
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**Supplemental Assay Method for the Titration of Neutralizing Antibody against Selected Bovine Viruses
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1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* serum neutralization (SN) assay test method to determine the neutralization index (NI) against bovine rhinotracheitis virus (IBR), bovine virus diarrhea (BVD) Type 1 and Type 2, parainfluenza (PI₃), or bovine respiratory syncytial virus (BRSV).

2. Materials

2.1 Equipment/instrumentation

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

- 2.1.1 Incubator, 36° ± 2°C, 5% ± 1% CO₂, high humidity
- 2.1.2 Vortex mixer
- 2.1.3 Microscope, inverted light
- 2.1.4 Microscope, fluorescent
- 2.1.5 Micropipettor, 200-µL, 1000-µL single channel, 5- to 50-µL 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
 - IBR
 - BVD Type 1
 - BVD Type 2
 - PI₃
 - BRSV

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2.2.2 Cell cultures free of extraneous agents as tested by title 9, *Code of Federal Regulations*, (9 CFR):

1. Madin-Darby bovine kidney (MDBK) cells used for IBR and PI₃ SN testing
2. Bovine Turbinate (BT) used for BVD Type 1 and Type 2 testing
3. Embryonic bovine lung (EBL) cells used for BRSV testing

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 (NaHCO₃)
3. Dissolve **Sections 2.2.3(1) and 2.2.3(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 **Section 2.2.3(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.

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2.2.5 Maintenance Media

1. 980 mL of MEM
2. Aseptically add 20 mL gamma-irradiated FBS.
3. Store at $4^{\circ}\pm 2^{\circ}\text{C}$.

2.2.6 Monoclonal antibodies (MAb)

1. Anti-BVD Type 1 MAb
2. Anti-BVD Type 2 MAb

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

2.2.8 80% Acetone

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

2.2.9 0.01 M Phosphate buffered saline (PBS)

1. 1.33 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 DI.
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.
7. Store at $4^{\circ}\pm 2^{\circ}\text{C}$.

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2.2.10 FBS negative for IBR, BVD, PI₃, and BRSV antibodies

2.2.11 Cell culture plate, 96-well

2.2.12 Polystyrene tube, 17 x 100-mm

2.2.13 Serological pipette, 10-mL

2.2.14 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.

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 Two days prior to test initiation, seed 96-well cell culture plates with MDBK cells, in Growth Medium, at a cell count that will produce a confluent monolayer after 2 days of incubation at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO₂ incubator. These become the MDBK Test Plates.

3.3.2 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 CO₂ incubator. These become the BT Test Plates.

3.3.3 On the day of the test initiation, seed 96-well cell culture plates with EBL cells, in Growth Medium. These become the EBL Test Plates.

3.3.4 On the 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

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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^{-8} .
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 **Section 3.3.4(3)** for each of the subsequent dilutions, transferring 1.0 mL from the previous dilution to the next dilution.

3.3.5 On the day of BT Test Plate examination, dilute Anti-BVD MAb and Anti-Mouse Conjugate in PBS according to the manufacturer's recommendations.

3.4 Preparation of the Test Serum Samples

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

4. Performance of the Test

4.1 Add 150 μL /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 μL /well of FBS into a column of a Dilution Plate.

4.3 Add 150 μL /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 ± 10 minutes at room temperature to allow for neutralization of the Indicator Virus.

4.5 At the end of the incubation period, decant Growth Medium from the MDBK Test Plates.

Note: The Growth Media is not removed from the BT and EBL Test Plates.

4.6 Inoculate 50 μL /well of each Virus-serum mixture into 5 wells/dilution of the appropriate Test Plate (**Appendix II**).

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Note: MDBK Test Plates are used to determine the SN titer against IBR and PI₃. BT Test Plates are used for BVD. EBL Test Plates are used for BRSV.

- 4.7 Maintain 5 or more wells on each Test Plate as uninoculated cell controls.
- 4.8 Inoculate 25 µL/well of the last 4 dilutions of the appropriate Indicator Virus to 5 wells/dilution as an endpoint titration.
- 4.9 Incubate MDBK Test Plates for 60 ± 10 minutes at 36° ± 2°C in a CO₂ incubator.
- 4.10 Add 200 µL/well of Maintenance Medium to all wells (**do not remove virus-serum inoculum**) of the MDBK Test Plate.
- 4.11 Incubate the MDBK and BT Test Plates for 4 days ± 12 hours postinoculation at 36° ± 2°C in a CO₂ incubator.
- 4.12 Incubate the EBL Test Plate for 6 days ± 12 hours postinoculation at 36° ± 2°C in a CO₂ incubator.
- 4.13 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 IBR, PI₃, or BRSV for Test Serum, FBS, and Indicator Viruses.
- 4.14 An indirect fluorescent antibody technique (IFA) is conducted to determine the SN titer against BVD as follows:
 - 4.14.1 Decant media from the BT Test Plates.
 - 4.14.2 Fill wells with 80% Acetone.
 - 4.14.3 Incubate at room temperature for 15 ± 5 minutes.
 - 4.14.4 Decant the 80% Acetone from the BT Test Plate; air dry at room temperature.
 - 4.14.5 Pipette 50 µL/well of a diluted Anti-BVD MAb into a BT Test Plate; incubate for 45 ± 15 minutes at room temperature.
 - 4.14.6 Rinse by filling the wells completely with PBS; decant the liquid.
 - 4.14.7 Repeat **Section 4.14.6** for a total of 2 washes.

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4.14.8 Pipette 50 μ L/well of the diluted Anti-mouse Conjugate into the BT Test Plates; incubate for 45 ± 15 minutes at room temperature.

4.14.9 Repeat **Section 4.14.6** for a total of 2 washes.

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

4.14.11 Examine wells with a fluorescent microscope.

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

4.14.13 Record the number of wells/dilution showing any characteristic fluorescence for the Test Serum, FBS, and Indicator Virus.

4.15 Calculate the TCID_{50} of the Test Sera, FBS, and Indicator Virus using the Spearman-Kärber method as commonly modified.

4.16 The neutralizing ability is determined by subtracting the log of the titer obtained with the Test serum from the titer obtained with the Indicator Virus.

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

5. Interpretation of the Test Results

For a valid test:

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

5.2 The titer of the FBS should be negative for neutralizing antibody against IBR, BVD, PI₃, or BRSV.

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 ability titers for the Test Serum on the test record.

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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*. 3rd 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 .04

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

Version .03

- The Contact information has been updated.
- References to NVSL have been changed to NCAH 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 impacting the test were made from the previous protocol, the following changes were made to the document:

- The Contact has been changed from Larry Ludemann to Joseph Hermann.
- The testing format has been changed from roller tubes and Leighton tubes to a 96-well plate format.
- The terminology of identifying types of BVD has been changed from BVD Type I to BVD Type 1 and from BVD Type II to BVD Type 2, to conform to a common format.
- **2.2:** Bovine Turbinate cells have been substituted for embryonic bovine kidney cells for BVD Type 1 and Type 2 testing to conform to cells commonly used for BVD testing.

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- **2.2.3:** The description of antibiotics added to the Diluent Medium has been changed to reflect the antibiotics used. Penicillin and streptomycin have been replaced by gentamicin sulfate, and Amphotericin B has been eliminated from the formulation.
- **4.13:** The provision for conducting a hemadsorption test for detecting antibody against PI₃ has been replaced by detecting antibody by CPE.
- **4.14:** The fluorescent antibody test for detecting antibody against BVD has been replaced with an IFA to allow the differentiation between Type 1 and Type 2 BVD antibodies. Indicator Virus has been added for clarification purposes.
- **4.15:** Indicator Virus has been added for clarification purposes.
- **4.16:** “The neutralizing ability is determined by subtracting the log of the titer obtained with the Test serum from the titer obtained with the FBS” has been changed to “The neutralizing ability is determined by subtracting the log of the titer obtained with the Test serum from the titer obtained with the Indicator Virus”.

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**Appendix I
Dilution Plate**

	1	2	3	4	5	6	7	8	9	10	11	12
A IV 10 ⁻³	TS1	TS2	TS3	TS4	TS5	TS6	TS7	TS8	TS9	TS10	TS11	FBS
B IV 10 ⁻⁴												
C IV □10 ⁻⁵												
D IV 10 ⁻⁶												
E IV 10 ⁻³	TS12	TS13	TS14	TS15	TS16	TS17	TS18	TS19	TS20	TS21	TS22	TS23
F IV 10 ⁻⁴												
G IV 10 ⁻⁵												
H IV 10 ⁻⁶												

IV = Indicator Virus dilution, TS = Test Serum, NC = FBS

**Appendix II
Test Plate**

	1	2	3	4	5	6	7	8	9	10	11	12
A 10 ⁻³	TS1	TS1	TS1	TS1	TS1	CC	TS2	TS2	TS2	TS2	TS2	CC
B 10 ⁻⁴												
C 10 ⁻⁵												
D 10 ⁻⁶												
E 10 ⁻³	TS3	TS3	TS3	TS3	TS3	CC	TS4	TS4	TS4	TS4	TS4	CC
F 10 ⁻⁴												
G 10 ⁻⁵												
H 10 ⁻⁶												

TS= Test Serum, CC= Cell Control