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

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

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

1. Introduction

This supplemental assay method (SAM) describes an *in vitro* assay method that determines the serum neutralizing (SN) antibody titer to bovine viral diarrhea virus (BVDV) in Test Sera as part of the potency requirements for veterinary vaccines. This test applies to BVDV cytopathic strains of either genotype 1 or genotype 2. The assay uses Bovine Turbinate (BT) or other permissive cells and inhibition of viral cytopathic effect as an indicator of the specific SN activity.

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°± 2°C, high humidity, 5% ± 1% CO_2 (Model 3336, Forma Scientific Inc.)

2.1.2 Water bath, 37°± 1°C; 56°± 1°C

2.1.3 Pipettors, 50-µL and 500-µL and tips

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

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

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

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 Reference

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

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2.2.3 Diluent Medium (National Centers for Animal Health (NCAH) Media #20030)

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

2. 1.1 g sodium bicarbonate ($NaHCO_3$)

3. Dissolve with 900 mL deionized water (DI)

4. Add 5.0 g lactalbumin hydrolysate or edamine to 10 mL of DI. Heat to 60°± 2°C until dissolved. Add to **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 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 of gamma-irradiated fetal bovine serum (FBS).

3. Store at 2°- 7°C.

2.2.5 Trypsin Versene (TV) Solution (NCAH Media #20005)

1. 8.0 g NaCl

2. 0.40 g KCL

3. 0.58 g $NaHCO_3$

4. 0.50 g irradiated trypsin

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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 7.5% $NaHCO_3$.

10. Filter through a 0.22-µm filter.

11. Store at -20°± 2°C.

2.2.6 Tissue culture plates, 96-well

2.2.7 Polystyrene tubes, 17 x 100-mm

2.2.8 Serological pipette, 10-mL

2.2.9 A negative serum control (NSC)

2.2.10 A positive serum control (PSC) for BVDV, genotype 1

2.2.11 A PSC for BVDV, genotype 2

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have training in cell culture techniques and the propagation and maintenance of animal viruses. Personnel shall have an understanding of the immunological basis of SN assays and the principles of aseptic techniques.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of test initiation, set a water bath at 56°± 2°C.

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

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3.3 Preparation of reagents/control procedures

3.3.1 Preparation of BT Cell Plates

Cells are prepared from healthy, confluent BT cells that are maintained by passing every 7 ± 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 µL/well of 10^5 to $10^{5.3}$ cells/mL cells suspended in Growth Medium into all wells of a 96-well cell culture plate.

These become the BT Test Plates. Incubate at 36°± 2°C in a 5 ± 1% CO_2 incubator for 24 ± 12 hours. The cell layer should be at least 70% confluent at 24 hours, and the Growth Medium is not changed unless excess acidity occurs or cells are not 70% confluent in 24 hours. Up to 4 Test Sera may be tested per plate.

3.3.2 Preparation of the BVDV Reference Working Dilution

1. On the day of test initiation, a vial of Reference BVDV is rapidly thawed in a 36°± 2°C water bath and diluted in Diluent Medium to contain 50-300 50% tissue culture infectious doses ($TCID_{50}$) per 25 µL. This chosen virus dose is in agreement with the requirements specified in 9 CFR, Parts 113.215 and 113.311. The procedure for determining the Reference BVDV dilution containing 50-300 $TCID_{50}$ is described in **Appendix III.**

2. Working BVDV back titration

The BVDV Working Dilution is back titrated by preparing serial tenfold dilutions (10^{-1}, 10^{-2}, and 10^{-3}) in the following manner.

a. Place 4.5 mL of Diluent Medium into 3 sterile, 17 x 100-mm tubes labeled 10^{-1}, 10^{-2}, and 10^{-3} using a 10-mL serological pipette.

b. Using a 500-µL pipettor, transfer 500 µL of BVDV Working Dilution to the 10^{-1} tube; mix by vortexing. Discard pipette tip.

c. Using a new pipette tip, transfer 500 µL from the 10^{-1} labeled tube to the 10^{-2} tube; mix by vortexing. Discard pipette tip.

d. Repeat **Step c** to the remaining tube 10^{-3} by transferring 500 µL from the 10^{-2} dilution and mix the 10^{-3} tube by vortexing.

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3.3.3 Preparation of PSC and NSC

On day of test initiation, serial twofold dilutions of heat-inactivated (56º± 2ºC for 30 ± 5 minutes) PSC and NSC are made from the original whole sera in a 96-well tissue culture plate labeled as the Dilution/Transfer Plate (**Appendix I**). The NSC final dilutions range from 1:2 to 1:16 (rows A - D). The range of the PSC twofold final dilutions will depend on its SN titer which has been previously determined. **A sufficient number of dilutions are performed in order to ensure reaching an endpoint for the PSC.**

1. With a multichannel pipettor, dispense 150 µL of Diluent Medium/well into rows B through H.

2. With a 200-µL pipettor, add 150 µL of specific genotype 1 or 2 undiluted PSC to wells A11 and B11, and 150 µL of undiluted NSC to wells A12 and B12 on the Transfer Plate. Use a new tip for each serum control.

3. With a multichannel pipettor, mix the samples by aspirating and expelling 7 ± 2 times in the wells of row B11 and B12, then transfer 150 µL of the mixture to the corresponding wells of row C. Discard tips after use.

4. Using new pipettor tips, repeat **Step 3** mixing row C and transferring to row D. Continue in this manner for the remaining wells in each column changing tips between dilutions until twofold serum dilutions are completed. Change tips, mix and discard 150 µL from the highest dilution of both the PSC and NSC.

3.4 Preparation of Test Serum

3.4.1 Test Serum samples are heat inactivated at 56°± 2°C for 30 ± 5 minutes on day of test initiation or prior to testing.

3.4.2 On day of test initiation, the Test Sera are diluted in the Dilution/Transfer Plate from 1:2 to 1:256 in rows A - H (**Appendix I**) as described for the PSC and NSC in **Sections 3.3.3 (Steps 1 through 4)**.

4. Performance of the Test

4.1 On day of test initiation with new pipette tips, dispense 150 µL of the BVDV Working Dilution to each well of the Dilution/Transfer Plate. Mix by gently tapping the

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sides of the Transfer Plate. The addition of the BVDV Working Dilution to the wells results in an additional twofold serum dilution, which becomes the final serum dilution.

4.2 Incubate the Dilution/Transfer Plate at 36°± 2°C for 60 ± 10 minutes in a CO_2 incubator.

4.3 Using an 8-channel multipipettor, inoculate 5 wells/dilution/Test Serum with 50 µL/well of the virus-serum mixture from the Dilution/Transfer plate into the BT Test Plate (**Appendix II**). All 8 dilutions of 1 Test Serum will be inoculated on cells at once. Replace tips for each unique Test Serum column being tested. This 96-well plate becomes the BVDV Test Plate.

4.4 Inoculate 5 wells/dilution with 50 µL/well of the PSC and another set of 5 wells/dilution of the NSC.

4.5 Inoculate 5 wells/dilution with 25 µL/well of the BVDV Working Dilution and Back Titration dilutions.

4.6 Dispense 25 µL of diluent to all wells of the back titration.

4.7 Maintain 5 or more wells as uninoculated cell culture controls per plate.

4.8 Incubate the BVDV Test Plates undisturbed at 36°± 2°C in a CO_2 incubator for 5 ± 1 days.

4.9 At the end of incubation, read the BVDV Test Plates at 100X magnification on an inverted light microscope and examine for BVDV cytopathic effect (CPE).

4.9.1 Results of the Test Serum, PSC, and NSC are recorded as the number of CPE negative wells versus total number of wells examined for each dilution of that serum.

4.9.2 Results of the BVDV Working Dilution and Back Titration are recorded as the number of CPE positive wells versus total number of wells examined for each dilution.

4.10 Calculate the endpoint for each Test Serum, PSC, and NSC using the Spearman-Kärber method as modified by Finney. The endpoints of the Test Serum, PSC, and NSC are reported as SN titer which corresponds to the reciprocal of the highest serum dilution that neutralizes the reference BVDV.

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Example:

1:2 dilution of Test Serum = 5 of 5 wells CPE Negative
1:4 dilution of Test Serum = 5 of 5 wells CPE Negative
1:8 dilution of Test Serum = 3 of 5 wells CPE Negative
1:16 dilution of Test Serum = 0 of 5 wells CPE Negative

Titer = (**X - d/2** + [**d • S**]) where:

X = Log_{10} of lowest dilution (=0.3)
d = Log_{10} of dilution factor (=0.3)
S = Sum of proportion of CPE negative

$$\frac{5}{5} = 1 + \frac{5}{5} = 1 + \frac{3}{5} = 0.6 = 2.6$$

Titer = (0.3 - 0.3/2 + [0.3 • 2.6]) = 0.93
antilog of 0.93 = 8.5

Titer of the Test Serum is 1:9

4.11 By the same Spearman-Kärber method, calculate the endpoint of the BVDV Back Titration. The titer is expressed as $\log_{10}$ tissue culture infective doses fifty ($TCID_{50}$) per 25 µL dose.

Example:

10^{0} dilution of the Working BVDV = 5 of 5 wells CPE Positive
10^{-1} dilution of the Working BVDV = 5 of 5 wells CPE Positive
10^{-2} dilution of the Working BVDV = 3 of 5 wells CPE Positive
10^{-3} dilution of the Working BVDV = 0 of 5 wells CPE Positive

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

$$\frac{5}{5} = 1 + \frac{5}{5} = 1 + \frac{3}{5} = 0.6 = 2.6$$

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Titer = (0 - d/2 + [1 • 2.6])= 2.1
antilog of 2.1 = 125.9

Titer of the Working BVDV is 126 $TCID_{50}$/25 µL dose in the test.

5. Interpretation of the Test Results

5.1 For a valid assay the following criteria must be met; otherwise the test is considered a **NO TEST** and is repeated.

5.1.1 The NSC must be negative at the 1:2 dilution.

5.1.2 The endpoint SN titer of the PSC should vary by no more than twofold from its previous determined mean titer as established from a minimum of 10 SN titrations.

5.1.3 The uninoculated cell controls cannot exhibit any CPE or cloudy media that would indicate contamination.

5.1.4 The BVDV Back Titration titer must be between 100-300 $TCID_{50}$/25 µL (**Section 4.11**).

5.2 For a **SATISFACTORY TEST**, the postvaccination SN titers shall meet the requirements as stated in an Animal and Plant Inspection Service (APHIS) approved Outline of Production.

5.3 If postvaccination SN titers are less than the requirements in an APHIS approved Outline of Production, the sera will be retested (first retest).

5.3.1 If the SN titers of the Test Serum from the first valid retest are less than the required titers in an APHIS approved Outline of Production, the serial is **UNSATISFACTORY**.

5.3.2 If the SN titers of the Test Serum from the first valid retest are greater than or equal to the titer in an APHIS approved Outline of Production, the serum will be retested (second retest).

5.3.3 If the SN titers of the Test Serum from the second valid retest are greater than or equal to the titer in an APHIS approved Outline of Production, the serial is **SATISFACTORY**.

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5.3.4 If the SN titers of the Test Serum from the second valid retest are less than the titers in an APHIS approved Outline of Production, the serial is **UNSATISFACTORY**.

6. Report of Test Results

6.1 Results are reported as SN titers.

6.2 Record all test results on the test record.

7. References

7.1 Title 9, *Code of Federal Regulations*, part 113.6, U.S. Government Printing Office, Washington, DC.

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

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

8. Summary of Revisions

<u>Version .04</u>

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

<u>Version .03</u>

- 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.

<u>Version .02</u>

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:

- The document number has been changed from MVSAM0112 to SAM 112.

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- The Contact information has been updated.

- References to embryonic bovine kidney primary (EBKp) cells have been changed to Bovine Turbinate (BT) cells and the fluorescence antibody (FA or IFA) reference has been removed.

- **2.2:** Phosphate Buffered Saline; Monoclonal antibody to BVDV type 1 or 2; and Anti-Reference Virus Fluorescein Isothiocyanate Labeled Conjugates have been removed from the document. The use of a 500-mL plastic wash bottle and acetone has also been removed from the document.

- **2.2.3:** The formulation for the Diluent Medium has been changed and penicillin, streptomycin and amphotericin B have been removed.

- **3.3.2:** Reference to the 9 CFR, Parts 113.215 and 113.311 has been added for clarification of the procedure.

- **4.12:** The processes of fluorescent staining of plate cultures have been deleted.

- Virus titrations and dilutions calculations have been added to the Appendices.

- The recommended cell culture of EBKp has been changed to BT throughout the document.

- The refrigeration temperatures have been changed from 4°± 2°C to 2°- 7°C. This reflects the parameters established and monitored by the Amega system.

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Appendix I
Dilution/Transfer Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	**TS1** dilute **1:2**	**TS2** dilute	**TS3** dilute	**TS4** dilute	**TS5** dilute	**TS6** dilute	**TS7** dilute	**TS8** dilute	**TS9** dilute	**TS 10** dilute	**TS 11** dilute	**TS 12** dilute
B	**4**											
C	**8**											
D	**16**											
E	**32**											
F	**64**											
G	**128**											
H	**256**											

TS= Test Serum

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Appendix II

BVDV Test Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A 1:2	TS1	TS1	TS1	TS1	TS1	CC	TS2	TS2	TS2	TS2	TS2	CC
B 4												
C 8												
D 16												
E 32												
F 64												
G 128												
H 256												

TS= Test Serum
CC= Cell Control

BVDV Control Serum Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A 1:2	PSC	PSC	PSC	PSC	PSC	CC	PSC	PSC	PSC	PSC	PSC	CC
B 4												
C 8												
D 16												
E 32												
F 64												
G 128												
H 256												

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Appendix III

Titration of BVDV and Determination of the Dilution Containing the Optimal Virus Dose for the Beta SN Test

1. Label eight 17 x 100-mm plastic tubes by identifying one tube as 10^{-1}, one tube for 10^{-2}, etc. through 10^{-8}. The number of tubes required will depend on the endpoint of the reference virus.

2. Dispense 4.5 mL of cell culture medium in each of the tubes.

3. Rapidly thaw, using a 36ºC water bath, one vial of Reference cytopathic BVD virus, and using a pipette or pipettor and tip, transfer 0.5 mL of virus to the first tube (10^{-1}).

4. Discard pipette or pipettor tip, and vortex the 10^{-1} virus dilution tube.

5. Using a new pipette or pipettor tip, transfer 0.5 mL of the 10^{-1} dilution of virus to the 10^{-2} tube, discard pipette or pipettor tip, and vortex the 10^{-2} dilution. Continue diluting in a similar fashion through the last tube, using a new pipette or pipettor tip for each transfer.

6. Transfer 0.025 mL of each virus dilution to each of 5 wells of a BT cell plate as prepared in **3.3.1**. The plate is then incubated in a 36º± 2ºC, 5% CO_2 incubator for 4 to 5 days.

7. The titration plate is read for CPE, and the results are recorded as number of wells exhibiting CPE per number of wells inoculated with each virus dilution.

8. Using the Spearman-Kärber method, calculate the endpoint of the BVDV virus titration. The titer is expressed as $\log_{10}$ tissue culture infective doses fifty ($TCID_{50}$) per 25 µL dose.

Example:

10^{-1} dilution of the Working BVDV = 5 of 5 wells CPE Positive
10^{-2} dilution of the Working BVDV = 5 of 5 wells CPE Positive
10^{-3} dilution of the Working BVDV = 5 of 5 wells CPE Positive
10^{-4} dilution of the Working BVDV = 5 of 5 wells CPE Positive
10^{-5} dilution of the Working BVDV = 3 of 5 wells CPE Positive 10^{-6} dilution of the Working BVDV = 0 of 5 wells CPE Positive

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Titer = (**X - d/2** + [**d • S**]) where:

X = Log_{10} of lowest dilution (=1)
d = Log_{10} of dilution factor (=1)
S = Sum of proportion of CPE Positive

$$\frac{5}{5} = 1 + \frac{5}{5} = 1 + \frac{5}{5} = 1 + \frac{5}{5} = 1 + \frac{3}{5} = 0.6 = 4.6$$

Titer = (1 - 1/2 + [1 • 4.6]) = 5.1
antilog of 5.1 = 125893

Therefore, a 1:125,893 dilution of the virus should yield a virus concentration of 1 TCID_{50} per 25 µL. Assuming a 200 TCID_{50} per 25 µL is the optimal dosage of virus to use in the SN test, divide the 125,893 by 200 to obtain 629.46. The dilution factor to obtain 200 TCID_{50} per 25 µL would then be 1:629. This Working Reference BVDV dilution must be confirmed by performing a back titration. A series of three to five dilutions of virus bracketing the expected dilution may be tested with back titrations of each. Adjustments from the original calculated dilution may need to be made to obtain the correct working virus dose.

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