Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for the Titration of Infectious Bovine Rhinotracheitis Virus in Vaccines

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Supplemental Assay Method for the Titration of Infectious Bovine Rhinotracheitis Virus in Vaccines

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Supplemental Assay Method for the Titration of Infectious Bovine Rhinotracheitis Virus in Vaccines

1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes an *in vitro* test method which utilizes viral plaque-forming units (PFU) in a cell culture system to titer infectious bovine rhinotracheitis virus (IBR) in modified-live veterinary vaccines.

Note: For this SAM, the dilution terminology of 1:5 specifies 1 part plus 4 parts (liquid).

1.2 Keywords

Infectious bovine rhinotracheitis virus, IBR, *in vitro*, potency test, PFU, plaque

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator,[1] 36° ± 2°C, 5% ± 1% CO_2, high humidity

2.1.2 Vortex mixer[2]

2.1.3 Blender[3]

2.1.4 Micropipettor, 200 µl, 1000 µl,[4] and tips[5]

2.1.5 Water bath[6]

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[1] Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent
[2] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent
[3] Waring blender, Cat. No. 14-509-35, Fisher Scientific Co., 2000 Park Ln., Pittsburgh, PA 15275 or equivalent
[4] Pipetman®, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
[5] Cat. No. YE-3R, Analytic Lab Accessories, P.O. Box 345, Rockville Center, NY 11571 or equivalent
[6] Cat. No. 15-461-10, Fisher Scientific Co. or equivalent

2.1.6 Self-refilling, repetitive syringe, 2 ml[7]

2.1.7 Media bottle, borosilicate glass with screw-top lid, 1000 ml[8]

2.2 Reagents/supplies

2.2.1 IBR Reference,[9] Cooper strain

2.2.2 Madin-Darby bovine kidney (MDBK) cells[10]

2.2.3 Minimum essential medium (MEM)

2.2.3.1 9.61 g MEM with Earle's salts without bicarbonate[11]

2.2.3.2 2.2 g sodium bicarbonate ($NaHCO_3$)[12]

2.2.3.3 Dissolve **Sections 2.2.3.1 and 2.2.3.2** with 900 ml deionized water (DW).

2.2.3.4 Add 5 g lactalbumin hydrolysate or edamine[13] to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to **Section 2.2.3.3** with constant stirring.

2.2.3.5 Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[14]

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[7] Wheaton®, Cat. No. 13-689-50C, Fisher Scientific Co. or equivalent
[8] Wheaton®, Cat. No. 219760, Fisher Scientific Co. or equivalent
[9] Reference quantities are available upon request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent
[10] Cat. No. ATCC CCL-22, American Type Culture Collection, 12301 Parklawn Dr., Rockville, MD 20852-1776
[11] Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
[12] Cat. No. S-5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[13] Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent
[14] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

2.2.3.6 Sterilize through a 0.22-µm filter.[15]

2.2.3.7 Aseptically add:

1. 10 ml L-glutamine[16]

2. 25 units/ml penicillin[17]

3. 50 µg/ml gentamicin sulfate[18]

4. 100 µg/ml streptomycin[19]

5. 2.5 µg/ml amphotericin B[20]

2.2.3.8 Store at 4° ± 2°C.

2.2.4 Growth Medium

2.2.4.1 900 ml of MEM

2.2.4.2 Aseptically add 100 ml gamma-irradiated fetal bovine serum (FBS)

2.2.4.3 Store at 4° ± 2°C.

2.2.5 2X Medium

2.2.5.1 19.22 g MEM with Earle's salts without bicarbonate

2.2.5.2 2.2 g $NaHCO_3$

2.2.5.3 Dissolve with 900 ml DW.

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[15] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent
[16] L-glutamine-200 mm (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. Or equivalent
[17] Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth,NJ 07033 or equivalent
[18] Cat. No. 0061-0464-04, Schering Laboratories or equivalent
[19] Cat. No. S-9137, Sigma Chemical Co. or equivalent
[20] Cat. No. A-4888, Sigma Chemical Co. or equivalent

2.2.5.4 Add 5 g lactalbumin hydrolysate or edamin to 10 ml DW. Heat to 60° ± 2°C until dissolved. Add to **Section 2.2.5.3** with constant stirring.

2.2.5.5 Q.S. to 1000 ml with DW and adjust to pH 6.8-6.9 with 2N HCl.

2.2.5.6 Sterilize through a 0.22-µm filter.

2.2.5.7 Store at 4° ± 2°C.

2.2.6 2% Tragacanth Gum (Trag)

2.2.6.1 20 g Trag[21]

2.2.6.2 1000 ml DW

2.2.6.3 Using a blender, vigorously mix small amounts of Trag with DW.

2.2.6.4 Pour 500 ml each into 1000-ml media bottles.

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

2.2.6.6 Store at 4° ± 2°C.

2.2.7 7.5% Sodium Bicarbonate

2.2.7.1 7.5 g $NaHCO_3$

2.2.7.2 Q.S. to 100 ml with DW.

2.2.7.3 Sterilize through a 0.22-µm filter.

2.2.7.4 Store at room temperature (RT) (23° ± 2°C).

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[21] Acros AC42138-5000, Fisher Scientific Co. or equivalent

2.2.8 70% Ethyl Alcohol

2.2.8.1 73 ml ethyl alcohol,[22] denatured, 190 proof

2.2.8.2 27 ml DW

2.2.8.3 Store at RT.

2.2.9 Crystal Violet Stain

2.2.9.1 7.5 g crystal violet[23]

2.2.9.2 50 ml 70% Ethyl Alcohol

2.2.9.3 Dissolve crystal violet in alcohol.

2.2.9.4 Add 250 ml formaldehyde.[24]

2.2.9.5 Q.S. to 1000 ml with DW.

2.2.9.6 Filter through a Whatman #1 filter paper.[25]

2.2.9.7 Store at RT.

2.2.10 Tissue culture plates, 6 well[26]

2.2.11 Polystyrene tubes, 12 x 75 mm[27]

2.2.12 Polystyrene tubes, 17 x 100 mm[28]

2.2.13 Pipette,[29] 25 ml

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[22] Cat. No. 7018, J.T. Baker, Inc. or equivalent
[23] Cat. No. C 0775, Sigma Chemical Co. or equivalent
[24] 37% by weight, Cat. No. F79, Fisher Scientific Co. or equivalent
[25] Cat. No. 1001, Fisher Scientific Co. or equivalent
[26] Falcon 3046, Becton Dickinson Labware, Becton Dickinson & Co., 2 Oak Park, Bedford, MA 01730 or equivalent
[27] Falcon 2058, Becton Dickinson Labware or equivalent
[28] Falcon 2057, Becton Dickinson Labware or equivalent
[29] Cat. No. 13-675-30, Fisher Scientific Co. or equivalent

2.2.14 Graduated cylinders, 25, 50, 100, and 250 ml[30], sterile

2.2.15 Parainfluenza-3 antiserum (PI3 AS)[9]

2.2.16 Bovine viral diarrhea antiserum (BVD AS)[9]

2.2.17 Bovine respiratory syncytial virus antiserum (BRSV AS)[9]

3. Preparation for the test

3.1 Personnel qualifications/training

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

3.2 Preparation of equipment/instrumentation

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

3.3 Preparation of reagents/control procedures

3.3.1 MDBK Plates. Two days prior to test performance, seed 1, 6-well tissue culture plate per Test Serial and 3, 6-well tissue culture plates for the controls with MDBK cells, in Growth Medium, at a cell count that will produce a monolayer after 48 ± 6 hr of incubation at 36° ± 2°C. These become the MDBK Plates.

3.3.2 IBR Reference Control. On day of test performance, rapidly thaw a vial of IBR Reference in a water bath at 36° ± 2°C. Tenfold dilutions are made as follows:

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[30] Cat. No. P34546-02, P34546-03, P34546-04, and P34546-05, Cole-Parmer Instrument Co., 625 Bunker Court, Vernon Hills, IL 60061-9872 or equivalent

3.3.2.1 Add 4.5 ml of MEM into 6, 17 x 100-mm polystyrene tubes labeled 10^{-1} to 10^{-6} respectively.

3.3.2.2 Using a micropipettor, transfer 500 µl of IBR Reference to the 10^{-1} tube; mix by vortexing. Discard pipette tip.

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

3.3.2.4 Repeat **Section 3.3.2.3** for each of the subsequent dilutions, transferring 500 µl from the previous dilution to the next dilution tube until the dilution sequence is completed.

3.3.3 On the day of test initiation, prepare Overlay Medium. Approximately 20 ml per plate is required.

3.3.3.1 Aseptically add to 1 volume of 2X Medium:

1. 10% gamma-irradiated FBS

2. 2% of 7.5% Sodium Bicarbonate

3. 25 units/ml penicillin

4. 50 µg/ml gentamicin sulfate

5. 100 µg/ml streptomycin

6. 2.5 µg/ml amphotericin B

7. 1 volume of 2% Trag

3.3.3.2 Mix and warm the Overlay Media in a 36° ± 2°C water bath for 60 ± 10 min prior to performing the procedure in **Section 4.5**.

3.4 Preparation of the Test Serial

3.4.1 The initial test of a Test Serial 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 Serial bottle and the bottle containing the accompanying diluent. Measure the diluent into a sterile graduated cylinder according to the Test Serial's total volume indicated on the manufacturer's label (e.g., for a 100-dose container of 2 ml per dose, reconstitute with 200 ml of diluent). Aseptically pour the diluent into the lyophilized bottle of vaccine. Mix by vortexing.

3.4.2 10^{-1} dilution of the Test Sample. Polyvalent vaccines require neutralization of the BVD, PI3, and BRSV fractions with antiserum prior to titering for IBR.

3.4.2.1 IBR monovalent vaccine

1. Mix 1.0 ml of the rehydrated Test Serial with 9.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube labeled 10^{-1}; mix by vortexing.

3.4.2.2 IBR/PI3 polyvalent vaccine

1. Mix 1.0 ml of the rehydrated Test Serial with 4.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube labeled 1:5; mix by vortexing.

2. Mix 500 µl from **Section 3.4.2.2.1** with 500 µl PI3 AS in a 12 x 75-mm polystyrene tube labeled 10^{-1}; mix by vortexing.

3. Incubate at RT for 45 ± 15 min.

3.4.2.3 IBR/BVD/PI3 polyvalent vaccine

1. Mix 1.0 ml of the rehydrated Test Serial with 4.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube labeled 1:5; mix by vortexing.

2. Mix 1.0 ml from **Section 3.4.2.3.1** with 500 µl of BVD AS and 500 µl of PI3 AS in a 12 x 75-mm polystyrene tube labeled 10^{-1}; mix by vortexing.

3. Incubate at RT for 45 ± 15 min.

3.4.2.4 IBR/BVD/PI3/BRSV polyvalent vaccine

1. Mix 1.0 ml of the rehydrated Test Serial with 4.0 ml of Diluent Medium in a 17 x 100-mm polystyrene tube labeled 1:5; mix by vortexing.

2. Mix 1.5 ml from **Section 3.4.2.4.1** with 500 µl of BVD AS, 500 µl of PI3 AS, and 500 µl of BRSV AS in a 12 x 75-mm polystyrene tube labeled 10^{-1}; mix by vortexing.

3. Incubate at RT for 45 ± 15 min.

3.4.3 Three tenfold dilutions are made from the 10^{-1} dilution of the Test Serial using MEM.

3.4.3.1 Pipette 4.5 ml of MEM into each of 3, 17 x 100-mm polystyrene tubes labeled 10^{-2} through 10^{-4} respectively.

3.4.3.2 Pipette 500 µl of the Test Serial from the 10^{-1} tube into the 10^{-2} tube; mix by vortexing. Discard pipette tip.

3.4.3.3 Repeat **Section 3.4.2.2** for each of the subsequent dilutions, transferring 500 µl from the previous dilution to the next dilution tube until the dilution sequence is completed.

4. Performance of the test

4.1 On the day of test initiation, decant the Growth Media from the MDBK Plates.

4.2 Add 100 µl/well from each dilution of the Test Serial to 2 wells of an MDBK Plate; mix by gentle swirling. Pipette tip changes are not necessary between each dilution in a series when pipetting from the most dilute to the most concentrated (e.g., 10^{-4} through 10^{-2}).

4.3 Add 100 µl/well from each dilution of the IBR Reference Control to 2 wells of an MDBK Plate; mix by gentle swirling.

4.4 Maintain 2 wells as uninoculated cell culture controls.

4.5 Incubate inoculated MDBK Plates for virus adsorption at 36° ± 2°C in a CO_2 incubator for 60 ± 10 min.

4.6 Add 3 ml/well of Overlay Medium (**Section 3.3.3**) to the MDBK Plates. Discard any unused, warmed Overlay Medium.

4.7 Incubate the MDBK Plates undisturbed at 36° ± 2°C in a CO_2 incubator for 72 ± 8 hr.

4.8 Following incubation, decant Overlay Medium. Pipette
2 ml of the Crystal Violet Stain (**Section 2.2.9**) into each well of an MDBK Plate, using the repetitive syringe.

4.9 Allow the MDBK Plates to incubate at RT for 15 ± 5 min.

4.10 Wash the Crystal Violet Stain from the cell monolayers by dipping each MDBK Plate several times in a container of running cold tap water. Allow to air dry.

4.11 The plaques are visible as clear, circular areas in the cell monolayer where the cells have been destroyed by the virus.

4.11.1 Count the number of plaques for each well.

4.11.2 Average the number of plaques between the duplicate wells for each Test Serial and IBR Reference Control dilution.

4.12 To determine the virus titer for each Test Serial and IBR Reference Control and express as PFU/dose, use the average from the dilution that contains between 10 and
100 PFU. Example:

Log_{10} of plaque count	(30)	1.48
Log_{10} of dilution counted	(10^{-3})	3.00
Log_{10} of 2-ml-dose factor $\frac{100\ \mu l\ inoculum}{2\ ml\text{-}dose}$	(20)	1.30
Virus titer (total)		5.78

The Test Serial contains $10^{5.78}$ PFU per dose.

4.13 One plaque represents a single infective unit (IU), whereas 1, 50% tissue culture infective dose ($TCID_{50}$) is statistically equivalent to a theoretical 0.69 IU. A $TCID_{50}$ endpoint will be 1.44 times those expressed as PFU per unit of inoculation. Therefore, to express PFU titer as $TCID_{50}$ titer, multiply the PFU titer by 1.44 or add 0.16 (the log of 1.44) to the log_{10} value of the PFU titer. In the example above, the $TCID_{50}$ would be 5.78 + 0.16 = 5.94 or $10^{5.94}$ $TCID_{50}$ per 2-ml dose.

5. Interpretation of the test results

5.1 For a valid test:

5.1.1 No visible contamination shall be observed in all dilutions of a Test Serial.

5.1.2 The Test Serial and the IBR Reference Control must have dilutions in which 10 to 100 PFU are counted.

5.1.3 The calculated titer of the IBR Reference 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.

5.1.4 The Negative Cell Control shall be negative for plaques.

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

5.2 If the titer of the Test Serial in a valid test is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production (OP) for the product under test, the Test Serial is **SATISFACTORY**.

5.3 If the validity requirements are met but the titer of the Test Serial is less than the required minimum titer contained in the APHIS filed Outline of Production for the product under test, the Test Serial may be retested in accordance with the Code of Federal Regulations, Title 9, Part 113.8.b.

6. Report of test results

Report test results as the virus titer in PFU per dose or $TCID_{50}$ per dose.

7. References

7.1 Code of Federal Regulations, Title 9, Part 113.310, U.S. Government Printing Office, Washington, DC, 2000.

7.2 Conrath TB. *Handbook of Microtiter Procedures*. Clinical and Research Applications Laboratory, Cooke Engineering Company, Alexandria, VA, 1972.

7.3 Davis, Dulbecco, Eisen, and Ginsberg, ed., *Microbiology including Immunology and Molecular Genetics,* 3rd ed. Harper and Row, Hagerstown, MD, pg 880.

8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.