

**Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol**

**Supplemental Assay Method for the Detection of
Extraneous Bovine Viral Diarrhea Virus in Modified-Live
Vaccines**

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1. Introduction

1.1 Background

This Supplemental Assay Method (SAM) describes an *in vitro* test method using a cell culture procedure and a direct or an indirect fluorescent antibody technique (DFAT, IFAT) for detecting extraneous bovine viral diarrhea virus (BVDV) in modified-live vaccines (MLV) produced in cell cultures which support the growth of BVDV.

1.2 Key Words

Extraneous virus; bovine viral diarrhea virus; BVDV; *in vitro* test; fluorescent antibody technique; DFAT; IFAT

2. Materials

2.1 Equipment/instrumentation

2.1.1 Incubator,¹ 36° ± 2°C, high humidity, 5 ± 1% CO₂

2.1.2 Incubator,² 36° ± 2°C, aerobic incubator

2.1.3 Microscope,³ inverted light

2.1.4 Microscope,⁴ ultraviolet light (UV-light microscope)

2.1.5 Microscope slide, glass staining dish with rack⁵ (glass staining dish)

2.1.6 Laboratory stirrer/hot plate and magnetic stir bar⁶

¹ Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent

² Model 2, Precision Scientific, 3737 West Cortland St., Chicago, IL 60647 or equivalent

³ Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747 or equivalent

⁴ Model BH2, Olympus America, Inc. or equivalent

⁵ Cat. No. 121, Shandon Lipshaw, 171 Industry Dr., Pittsburg, PA 15275 or equivalent

⁶ Model PC-320, Corning Costar Corporation, One Alewife Center, Cambridge, MA 02140 or equivalent

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2.2 Reagents/supplies

2.2.1 Monospecific Antiserum⁷ that is neutralizing, nontoxic to cell cultures, free of BVDV and BVDV antibodies, and specific to the agent(s) in the Test Serial.

2.2.2 Bovine turbinate secondary cell culture⁵ (BT) free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR).

2.2.3 Minimum essential medium (MEM)

2.2.3.1 9.61 g MEM with Earle's salts without bicarbonate⁸

2.2.3.2 2.2 g sodium bicarbonate (NaHCO₃)⁹

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 edamine¹⁰ 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).¹¹

2.2.3.6 Sterilize through a 0.22-µm filter.¹²

2.2.3.7 Aseptically add:

1. 10 ml L-glutamine¹³

⁷ Reference quantities available on request from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent

⁸ Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent

⁹ Cat. No. S 5761, Sigma Chemical Co., P.O. Box 14508, St. Louis, MO 63178 or equivalent

¹⁰ Edamine, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent

¹¹ Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent

¹² Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent

¹³ L-glutamine-200 mm (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc. or equivalent

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2. 25 units/ml penicillin¹⁴
3. 50 µg/ml gentamicin sulfate¹⁵
4. 100 µg/ml streptomycin¹⁶
5. 2.5 µg/ml amphotericin B¹⁷

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 Maintenance Medium

2.2.5.1 980 ml of MEM

2.2.5.2 Aseptically add 20 ml gamma-irradiated fetal bovine serum (FBS).

2.2.5.3 Store at 4° ± 2°C.

2.2.6 BVDV Reference Viruses⁷

2.2.6.1 Noncytopathic BVDV type I, e.g., New York-1 strain

2.2.6.2 Noncytopathic BVDV type II, e.g., 890 strain

2.2.6.3 Other BVDV strains (optional), e.g., Singer strain (cytopathic type I), 125 Strain (cytopathic type II)

¹⁴ Cat. No. 0049-0530-28, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent

¹⁵ Cat. No. 0061-0464-04, Schering Laboratories or equivalent

¹⁶ Cat. No. S 9137, Sigma Chemical Co. or equivalent

¹⁷ Cat. No. A 4888, Sigma Chemical Co. or equivalent

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2.2.7 For DFAT, anti-BVDV fluorescein isothiocyanate labeled conjugate (Anti-BVDV FITC Conjugate),⁵ reactive to all type I and type II strains of BVDV

2.2.8 For IFAT, monoclonal antibodies (MAb) or antisera,⁵ reactive to all type I and type II strains of BVDV (BVDV types I and II MAb or Antiserum)

2.2.9 For IFAT, appropriate anti-species IgG (H+L) fluorescein isothiocyanate labeled conjugate (Anti-Species FITC Conjugate)¹⁸

2.2.10 0.01 M Phosphate buffered saline (PBS)

2.2.10.1 1.33 g sodium phosphate, dibasic, anhydrous (Na_2HPO_4)¹⁹

2.2.10.2 0.22 g sodium phosphate, monobasic, monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$)²⁰

2.2.10.3 8.5 g sodium chloride (NaCl)²¹

2.2.10.4 Q.S. to 1000 ml with DW.

2.2.10.5 Adjust pH to 7.2-7.6 with 0.1 N sodium hydroxide (NaOH)²² or 2N HCl.

2.2.10.6 Sterilize by autoclaving at 15 psi, $121^\circ \pm 2^\circ\text{C}$ for 35 ± 5 min.

2.2.10.7 Store at $4^\circ \pm 2^\circ\text{C}$.

2.2.11 Acetone,²³ 99.3%

2.2.12 Cell culture flask,²⁴ 25 cm^2

¹⁸ Cat. Nos. 81-6511, 81-6111, etc., appropriate antispecies FITC IgG (H&L), Zymed Laboratories Inc., 458 Carlton Court, South San Francisco, CA 94080 or equivalent

¹⁹ Cat. No. S 0876, Sigma Chemical Co. or equivalent

²⁰ Cat. No. S 9638, Sigma Chemical Co. or equivalent

²¹ Cat. No. S 9625, Sigma Chemical Co. or equivalent

²² Cat. No. 925-30, Sigma Chemical Co. or equivalent

²³ Cat. No. A 4206, Sigma Chemical Co. or equivalent

²⁴ Falcon® 3824, Becton Dickinson Labware, Becton Dickinson & Co., 2 Bridgewater Lane, Lincoln Park, NJ 07035 or equivalent

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2.2.13 Cell culture slides,²⁵ 8 chamber (Lab-Tek®
Slide)

2.2.14 Polystyrene tubes,²⁶ 12 x 75 mm

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have experience in aseptic techniques, ultraviolet light microscopy, and cell culture techniques.

3.2 Preparation of equipment/instrumentation

On the day of the DFAT or IFAT, prepare a humidity chamber in the aerobic incubator by filling a pan in the bottom with DW.

3.3 Preparation of reagents/control procedures

3.3.1 One day prior to test initiation (first passage) and one day prior to second passage, seed 1 25-cm² cell culture flask per Test Serial, 1 25-cm² cell culture flask for a Media Control, and 1 25-cm² cell culture flask for an uninoculated Cell Control, using 10 ml of approximately 10^{5.2} to 10^{5.5} BT cells/ml in Growth Medium. Incubate at 36° ± 2°C in a CO₂ incubator, to 50 to 75% confluency. These become the BT Flasks.

3.3.2 One day prior to inoculation of the Lab-Tek® Slides, seed 1 Lab-Tek® Slide per Test Serial with 0.3 to 0.4 ml of approximately 10^{5.2} to 10^{5.5} BT cells/ml in Growth Medium. Incubate at 36° ± 2°C in a CO₂ incubator, to 50 to 75% confluency.

²⁵ Cat. No. 177402, Nunc, Inc., 2000 N. Aurora Rd., Naperville, IL 60563 or equivalent

²⁶ Falcon® 2058, Becton Dickinson Labware or equivalent

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3.3.3 Preparation of BVDV Positive Controls.

On the day of inoculation of the Lab-Tek® Slides, dilute the BVDV type I and type II Reference Viruses in MEM, according to the Center for Veterinary Biologics-Laboratory (CVB-L) Reference and Reagent data sheet or the manufacturer's recommendations.

3.3.4 Preparation of Working Anti-BVDV FITC Conjugate for DFAT

On the day of the DFAT, dilute Anti-BVDV FITC Conjugate in PBS to the working dilution according to the CVB-L Reference and Reagent data sheet or the manufacturer's recommendations.

3.3.5 Preparation of Working BVDV type I and II MAb or Antiserum for IFAT

On the day of the IFAT, dilute the BVDV types I and II MAb or Antiserum in PBS to the IFA working dilution as determined for that specific MAb or Antiserum according to the CVB-L Reference and Reagent data sheet or the manufacturer's recommendations.

3.3.6 Preparation of Working Anti-Species FITC Conjugate for IFAT

On the day of the IFAT, dilute the Anti-Species FITC Conjugate in PBS to the working dilution according to the manufacturer's recommendations.

3.4 Preparation of the sample

3.4.1 The initial test of a Test Serial will be with a single vial (a single sample from 1 vial).

3.4.2 On the day of test initiation, rehydrate the Test Serial according to the manufacturer's instructions. If the Test Serial contains a bacterin for the diluent, sterile DW is used to replace the diluent. Mix by vortexing.

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3.4.3 Test Serials may require neutralization (see **Appendix 1** for listing of viruses able to replicate in BT)

3.4.3.1 Monovalent Test Serial: 0.5 ml of rehydrated Test Serial and 0.7 ml of Monospecific Antiserum against virus able to replicate in BT that is a fraction in the Test Serial are mixed in a labeled 12 x 75-mm polystyrene tube. Depending on the titer of the virus to be neutralized and the antiserum neutralization titer, it may be necessary to increase the amount of antiserum used for neutralization of a given virus, as determined empirically. Incubate for 60 ± 10 min at room temperature (RT) ($23^{\circ} \pm 2^{\circ}\text{C}$).

3.4.3.2 Bivalent Test Serial: 0.5 ml of rehydrated Test Serial and 0.5 ml of each Monospecific Antisera against viruses able to replicate in BT that are a fraction in the Test Serial are mixed in a labeled 12 x 75-mm polystyrene tube. Depending on titer of the virus to be neutralized and the antiserum neutralization titer, it may be necessary to increase the amount of antiserum used for neutralization of a given virus, as determined empirically. Incubate for 60 ± 10 min at RT.

3.4.3.3 Test Serial containing 3 or more fractions: 0.5 ml of the rehydrated Test Serial and 0.3 ml of each Monospecific Antisera against viruses able to replicate in BT that are a fraction in the Test Serial are mixed in a labeled 12 x 75-mm polystyrene tube. Depending on the titer of the virus to be neutralized and the antiserum neutralization titer, it may be necessary to increase the amount of antiserum used for neutralization of a given virus, as determined empirically. Incubate for 60 ± 10 min at RT.

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4. Performance of the test

4.1 Just prior to test initiation, decant the Growth Medium from all but one of the BT Flasks.

4.2 Inoculation

4.2.1 Inoculate 0.5 ml of Test Serial not requiring neutralization into a BT Flask; q.s. to 2.5 ml with Maintenance Medium. This becomes the Test Flask.

4.2.2 When neutralization is required, inoculate the entire volume of the neutralized Test Serial (**Section 3.4.3**) after the incubation period into a BT Flask; q.s. to 2.5 ml with Maintenance Medium. This becomes the Test Flask.

4.3 Maintain 1 BT Flask per test as an unopened, uninoculated Cell Control.

4.4 Maintain 1 BT Flask per test as a Media Control. Inoculate the Media Control with 2.5 ml Maintenance Medium.

4.5 Allow each Test Flask and Media Control to absorb at $36^{\circ} \pm 2^{\circ}\text{C}$ for 60 ± 10 min.

4.6 Add 7.5 ml Maintenance Medium to each Test Flask and the Media Control (i.e., q.s. to 10 ml).

4.7 Incubate the flasks at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO_2 incubator. Observe each Test Flask, Media Control, and Cell Control not less often than every 3 days with an inverted light microscope for any signs of cytopathic effect (CPE) or contamination.

4.8 Ninety-six \pm 24 hr post inoculation (HPI). Observe each Test Flask, Media Control, and Cell Control for any signs of CPE. Discard the Cell Control if no CPE is evident. Freeze the remaining flasks at $-70^{\circ} \pm 5^{\circ}\text{C}$ for a minimum of 30 min. Flasks may be maintained at $-70^{\circ} \pm 5^{\circ}\text{C}$ until the next passage. Thaw each flask at RT and shake contents. This is considered a passage of the Test Serial and the Media Control.

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4.9 Decant Growth Medium from all but one of the new BT Flasks seeded one day previously, as described in **Section 3.3.1**.

4.10 Pipette 1.0 ml of the cell suspension from the first passage (see **Section 4.8**) of a Test Serial into a new labeled BT Flask from which the Growth Medium has been removed; q.s. to 2.5 ml with Maintenance Medium. Repeat for the Media Control. These are the new Test Flask and Media Control. A new BT Flask is maintained as an unopened, uninoculated Cell Control.

4.10.1 Additional neutralizing antisera may be necessary to suppress replication of specific viral fractions in the vaccine. If so, the cell suspension neutralization may be performed according to **Sections 3.4.3.1, 3.4.3.2, or 3.4.3.3**.

4.10.2 Inoculate the entire volume of the neutralized cell suspension after the incubation period into a BT Flask; q.s. to 2.5 ml with Maintenance Medium. This becomes the Test Flask.

4.11 Allow each Test Flask and Media Control to absorb at $36^{\circ} \pm 2^{\circ}\text{C}$ for 60 ± 10 min, then q.s. to 10 ml with 7.5 ml Maintenance Medium, as in **Sections 4.5 and 4.6**.

4.12 Incubate flasks at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO_2 incubator. Observe all flasks not less than every 3 days for signs of CPE or contamination.

4.13 Ninety-six \pm 24 hr after the second inoculations, repeat **Section 4.8**. This completes the second passage of the Test Serial.

4.14 Inoculate 0.1 ml/well of the cell suspension from each Test Flask into at least 4 wells of a Lab-Tek® Slide, seeded one day previously, as described in **Section 3.3.2**. For every assay, at least 4 wells serve as uninoculated Cell Control wells. Incubate at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO_2 incubator for 96 ± 24 HPI.

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- 4.14.1** Additional neutralizing antisera may be necessary to suppress replication of specific viral fractions in the vaccine. If so, add antiserum directly to the Lab-Tek® Slide.
- 4.14.2** Alternately, the cell suspension neutralization may be performed similar to **Sections 3.4.3.1, 3.4.3.2, or 3.4.3.3.**
- 4.15** Add 0.1 ml/well of each BVDV Positive Control to at least 4 wells of a Lab-Tek® Slide, seeded 1 day previously, as described in **Section 3.3.2.** Incubate at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO₂ incubator for 96 ± 24 HPI.
- 4.16** Add 0.1 ml/well of the cell suspension from the Media Control Flask to at least 4 wells of a Lab-Tek® Slide, seeded one day previously, as described in **Section 3.3.2.** Incubate at $36^{\circ} \pm 2^{\circ}\text{C}$ in a CO₂ incubator for 96 ± 24 HPI.
- 4.17** Following incubation, decant the media from all Lab-Tek® Slides. Remove the plastic walls by twisting them away from the Lab-Tek® Slides, leaving the gasket attached.
- 4.18** Place the Lab-Tek® Slides in a slide rack; place the rack in a glass staining dish filled with PBS (Optional: a magnetic stir bar can be placed to rotate slowly in the dish, which is set on a laboratory stir plate). Let stand 15 ± 5 min at RT. This step may be omitted in the event cells are in danger of sloughing from the Lab-Tek® Slides. In that case, cells are immediately fixed in Acetone, without a PBS rinse (see **Section 4.19**).
- 4.19** Discard the PBS, replace it with Acetone, and fix the Lab-Tek® Slides for 15 ± 5 min at RT. Remove and allow to air dry.
- 4.20** Either DFAT or IFAT may be used to detect possible extraneous BVDV. A suitable Anti-BVDV FITC Conjugate used as a DFAT offers the advantage of less steps in processing the Lab-Tek® Slides. IFAT is more time-consuming, but use of type I- and type II-specific antibodies provides information on the genotype of BVDV strains.

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4.20.1 For DFAT, pipette $75 \pm 25 \mu\text{l}$ of a Working Anti-BVDV FITC Conjugate into each well of the Lab-Tek® Slides. Incubate in an aerobic incubator at $36^\circ \pm 2^\circ\text{C}$ for 30 ± 5 min.

4.20.2 For IFAT, pipette $75 \pm 25 \mu\text{l}$ of a Working BVDV type I MAb or Antiserum into at least 4 wells of a Lab-Tek® Slide inoculated with a given Test Serial. Pipette $75 \pm 25 \mu\text{l}$ of a Working BVDV type II MAb or Antiserum into at least 4 wells inoculated with the same Test Serial. Repeat this for every Test Serial. Pipette $75 \pm 25 \mu\text{l}$ of each Working BVDV MAb or Antiserum into at least 4 wells of Cell Control, and at least 4 wells of Media Control. Incubate in an aerobic incubator at $36^\circ \pm 2^\circ\text{C}$ for 30 ± 5 min.

4.20.2.1 Wash per **Section 4.18**. Discard the PBS.

4.20.2.2 Pipette $75 \pm 25 \mu\text{l}$ of the Working Anti-Species FITC Conjugate into each well of the Lab-Tek® Slides; incubate in an aerobic incubator at $36^\circ \pm 2^\circ\text{C}$ for 30 ± 5 min.

4.21 Wash per **Section 4.18**. Discard the PBS. Briefly dip each Lab-Tek® Slide in DW.

4.22 Allow the Lab-Tek® Slides to air dry.

4.23 Read within one hour at 100-200X with a UV-light microscope. Alternately, the Lab-Tek® Slides may be stored in the dark at $4^\circ \pm 2^\circ\text{C}$, for no longer than 48 hr, before reading. Examine the cell monolayer for typical apple-green cytoplasmic fluorescence.

4.24 Wells containing 1 or more cells displaying specific fluorescence for BVDV are positive.

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5. Interpretation of the test results

5.1 Validity requirements

5.1.1 For a valid test, the Media Control and the Cell Control wells on Lab-Tek® Slides shall not exhibit signs of contamination, CPE, or positive DFAT or IFAT.

5.1.2 The BVDV Positive Controls must show specific fluorescence for BVDV.

5.1.3 If either validity requirement in **Section 5.1.1** or **5.1.2** is not met the test is considered a **NO TEST** and may be repeated without prejudice.

5.2 Results

5.2.1 If the initial test is valid and all wells of the Test Serial on the Lab-Tek® Slides are free of BVDV contamination the serial is **SATISFACTORY**.

5.2.2 Retests

5.2.2.1 If the initial test is valid and 1 or more wells of the Test Serial on the Lab-Tek® Slides exhibits signs of BVDV contamination, the test is repeated (1st retest), using a new vial of the Test Serial. Retests are conducted using an additional BT Flask for each passage and at least 4 wells of a Lab-Tek® Slide to serve as Serum Control flask and wells, respectively. These will have contents identical to the Media Control flask and wells, plus addition of equivalent amounts of antiserum as used in the Test Flask and wells to neutralize viral fractions of the Test Serial.

5.2.2.2 If the second valid test (1st retest) confirms the initial result, and the Serum Control is negative for BVDV, the Test Serial is **UNSATISFACTORY**.

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5.2.2.3 If the second valid test (1st retest) fails to confirm the initial result, the Test Serial is tested a third time (2nd retest). The test is repeated using a new vial of Test Serial and a Serum Control as described in **Section 5.2.2.1**.

1. If the second and third valid tests (1st and 2nd retests) result in no signs of BVDV contamination, the Test Serial is **SATISFACTORY**.

2. If the third valid test (2nd retest) confirms the initial result and the Serum Control is negative for BVDV, the Test Serial is **UNSATISFACTORY**.

5.2.2.4 If at any time in a valid retest the Serum Control in the flasks or on the Lab-Tek[®] Slides is positive for BVDV, the blocking antiserum used in the testing is suspect and that test and all preceding testing is a **NO TEST**. A Test Serial may be retested without prejudice after adequate evaluation and assurance of BVDV free reagents.

6. Report of test results

Record all test results on the test record.

7. References

7.1 Code of Federal Regulations, Title 9, Parts 113.300, U.S. Government Printing Office, Washington, DC, 2000.

7.2 G. E. Cottral ed. *Manual of Standardized Methods for Veterinary Microbiology*. Comstock Publishing Assoc. Ithaca, NY.

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8. Summary of revisions

This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the Center for Veterinary Biologics-Laboratory, to provide additional detail and to reflect these changes from the superseded protocol:

- 1) replacement of simultaneous inoculation of bovine embryonic kidney (BEK) cells by inoculation of BT cell monolayers,
- 2) inclusion of BVDV type II Reference Virus for a Positive Control,
- 3) inclusion of newly added vaccine viruses to the serum neutralization step,
- 4) inclusion of two passages in 25-cm² flasks prior to inoculation of Lab-Tek[®] Slides,
- 5) inclusion of DFAT for detection of BVDV type I and II.
- 6) expanding the scope to allow testing of all modified live vaccines that are grown on cell lines that are permissive for BVDV.

9. Appendices

9.1 Cell lines that support BVDV replication

Based on testing conducted at the CVB-L and other scientific institutions, the cell lines that are permissive for BVDV include:

- 1) all bovine, ovine, and swine cell lines,
- 2) cat cell lines, especially Crandell feline kidney (CRFK),
- 3) rabbit cell lines,
- 4) MA104 (African green monkey) cell lines,

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- 5) chick embryo fibroblast cells and cell lines,
- 6) equine dermal cell lines (ED), though the BVDV titer is much reduced.

Products grown on cells that are not readily available to CVB-L, such as ferret embryo cells, are also considered candidates for testing until such time as BVDV replication on these cells is determined.

9.2 Agents that require neutralization during testing

Live vaccine viruses or live agents that replicate when grown in BT and that may potentially interfere with the detection of extraneous BVDV, are neutralized with homologous antisera.

Agents requiring neutralization	Agents not requiring neutralization
bovine coronavirus bovine parvovirus bovine rotavirus bovine respiratory syncytial virus bluetongue virus <i>Chlamydia psittaci</i> * canary pox virus canine distemper virus canine parainfluenza virus equine herpesvirus 1 and 4 equine influenza virus infectious bovine rhinotracheitis virus infectious canine hepatitis virus measles virus parainfluenza virus 3 pseudorabies virus	canine coronavirus canine parvovirus equine viral arteritis virus feline calici virus feline infectious peritonitis virus feline panleukopenia virus feline rhinotracheitis virus porcine parvovirus West Nile virus

* When present in viral vaccines, *Chlamydia psittaci* may cause unwanted effects on BT. These effects can be neutralized by the use of tetracycline.

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