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United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

Reverse Transcription (RT)PCR Assay Using Qiagen OneStep to Detect Bovine Viral Diarrhea Virus (BVDV), Porcine and Reproductive Respiratory Virus (PRRSV) or Extraneous Reticuloendotheliosis Virus (REV)

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1. Introduction

This testing protocol (PRO) describes the performance of reverse transcriptase polymerase chain reaction (RT-PCR) for detection of RNA viruses for purity testing of biologic products.

2. Materials

2.1 Equipment/instrumentation for RNA extraction

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

2.1.1 Laminar Flow Class II Biological Safety Cabinet (BSC) (NuAire Inc., Labgard)

2.1.2 Freezer (-20°± 5°C, -65°C or colder)

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

2.1.4 Eppendorf Adjustable Volume Research Micropipettes (1000µl, 200µl, 100µl, 20µl, 10µl, 2.5µl)

2.1.5 Micro-tube storage racks

2.1.6 Pipette Aid

2.1.7 Centrifuge (Eppendorf Centrifuge 5415 D 13,200 RPM or Eppendorf Mini Spin Plus F-45-12-11 14,500 RPM)

2.2 Equipment/instrumentation for PCR

2.2.1 Laminar Flow Class II Biological Safety Cabinet (BSC;NuAire Inc., Labgard)

2.2.2 Freezer (-20°± 5°C, -65°C or colder)

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

2.2.4 Eppendorf Adjustable Volume Research Micropipettes (1000µl, 200µl, 100µl, 20µl, 10µl, 2.5µl)

2.2.5 Micro-tube storage racks

2.2.6 Refrigerator

2.2.7 Microcentrifuge

2.2.8 Reagent cooling block (store at 4º± 2ºC)

2.2.9 Freezer microtube racks (store at 20º± 2ºC)

2.2.10 Quick Spin Minifuge (6 x 1.5/2.0mL microtube rotor)

2.2.11 Thermocycler (Applied Biosystems Veriti 96-well thermocycler or ThermoFisher Scientific ProFlex 2x32-well PCR System)

2.2.12 E-Gel™ electrophoresis system

2.2.13 UV Gel imaging system

Note: The master mix should be prepared in a designated "clean room" area and extraction/template should NEVER be introduced into the "clean room." Once the master mix is prepared, add 20 or 45 or 90µl of master mix to each PCR reaction tube in the clean hood where the master mix was made, or before opening the RNA template (extracted samples) in the DNA/RNA template (dirty) hood.

2.3 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

2.3.1 Laboratory supplies

2.3.1.1 Sterile RNase-free aerosol-resistant pipette tips, various sizes

2.3.1.2 1.5mL microcentrifuge tubes

2.3.1.3 14mL centrifuge tube

2.3.1.4 PCR Reaction Tubes, 0.2mL (Applied Biosystems™, MicroAmp™ 8-Tube Strip, Catalog #N8010580)

2.3.1.5 PCR Reaction Tube Caps (Applied Biosystems™, MicroAmp™ 8-Cap Strip, clear, Catalog #N8010535)

2.3.1.6 Latex, vinyl, or nitrile powder free disposable gloves

2.3.2 RNA Extraction reagents/supplies

2.3.2.1 CVB-TWS-0113, *RNA Extraction Assay – QIAamp Viral RNA Mini Kit*

2.3.2.2 QIAamp Viral RNA Mini Kit (Qiagen, Catalog #52904)

Note: Carrier RNA is rehydrated with Buffer AVE; once rehydrated, Carrier RNA-AVE is stored in -20°± 5°C freezer.

2.3.2.3 Ethanol 200 proof molecular grade (Sigma-Aldrich, Catalog #E7023)

2.3.2.4 Nuclease-free water

2.3.2.5 Internal amplification control (IAC;pRNAcIAC, WS Lot# 19-02)

2.3.3 RT-PCR reagents/supplies

2.3.3.1 CVB-TWS-0123, *Qiagen OneStep RT - PCR Assay*

2.3.3.2 Qiagen OneStep RT-PCR Kit (Qiagen, Catalog #210212)

2.3.3.3 Forward and Reverse Primers at approximately 10 pmol/µl each (A list of primer pairs can be found in **Appendix A**.)

Reconstitution of the primers is determined by the synthesis data sheet that is received after the primers are made by IDT, an accredited ISO 9001 certified company or equivalent. Primers are rehydrated to a concentration of 10pmol, aliquoted in 50µl increments and stored in a -20°C freezer until further use. Vials are ready for use for the amplification process.

2.3.4 Analysis Reagents

2.3.4.1 Molecular Weight DNA Ladders based on the PCR fragment size (Invitrogen™)

2.3.4.2 Loading Buffer, 10X BlueJuice™ (Invitrogen™, Catalog #10816015)

2.3.4.3 E-Gel™ Agarose Gels with SYBR™ Safe DNA Gel Stain, 2% (A42135)

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological techniques and preparation and the proper handling of biological agents, reagents, tissue culture samples, and chemicals. Personnel must have knowledge of safe operating procedures and policies and adhere to guidelines, with training in the operation of the necessary laboratory equipment to run this test.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturers' instructions and monitor for compliance with current corresponding standard operating policies/procedures. Wear non-powdered disposable gloves. Program the thermocycler with the following program:

Amplification of RNA Viruses

Step	Time	Temperature	Cycles
Reverse Transcription	30 minutes	50°C	1
PCR Activation	15 minutes	95°C	1
Denaturation	1 minute	95°C	35x
Annealing	1 minute	Variable based on primers used (50-60°C)	
Extension	1 minute	72°C	
Final Extension	10 minutes	72°C	1
Storage	∞	4°C	1

3.3 Preparation of reagents/control procedures

3.3.1 All samples being tested for extraneous BVDV and PRRSV will be tested directly from the vial and from the 3rd pass in Bovine turbinate cells (BT cells). Each sample, including a negative extraction control (NEC; either nuclease-free water or negative cell culture passage), is ran in duplicate. Duplicate samples are spiked with an RNA internal amplification control (IAC;PRNAcIAC WS Lot# 19-02).

3.3.2 Prepare a sufficient volume of AVL Buffer and Carrier RNA mixture for the number of samples being extracted + 1 reaction according the QIAamp Viral RNA Mini Kit Extraction Protocol. Record this information **CVB-TWS-0113.**

3.3.3 Prepare appropriate DNA ladder by adding 50µl of 10x BlueJuice™ with 900µl of RNase-free water for use in a 2% E-Gel™. (This dilution is less concentrated than manufacturer's suggestion and is for use in E-Gel™s only).

3.3.4 Prepare the IAC to a concentration of 10^3 copies/µl by adding 900µl of nuclease-free water to the 100µl of pRNAcIAC (bottled at 10^4 copies/µl).

4. Testing Procedure

4.1 Viral RNA extraction

4.1.1 Thaw, rehydrate, and rebottle samples if needed. Record sample information on the current version of **CVB-TWS-0113**.

4.1.2 Add 140µl of sample into two 1.5mL centrifuge tubes. In one of the duplicate sample tubes, inoculate 10µl of IAC diluted at 10^3 copies/µL.

4.1.3 Add 140µl of nuclease-free water into two 1.5mL centrifuge tubes to serve as NEC. In one of the duplicate NEC tubes, inoculate 10µl of IAC diluted at 10^3 copies/µL.

4.1.4 From this point, follow the most current available protocol that is provided in the QIAamp Viral RNA Mini kit-spin protocol or the most current version of **CVB-WI-0265** starting with the addition of 560µl of AVL/carrier RNA added to each 1.5mL centrifuge tube.

Note: Samples can be eluted in 60µl of AE buffer provided with the kit or in nuclease-free water.

4.1.5 Once the Qiagen extraction is complete, label each extraction tube and store extracted samples at -20°C until used for amplification in **Section 4.2**.

4.2 Amplification of Viral RNA

4.2.1 See the current version of **CVB-TWS-0123** for the master mix worksheet. Prepare the master mix for DNA amplification from the RNA extractions as follows:

1. Calculate the amount of master mix needed by determining the total number of reactions needed plus one.

   [The amount of each reaction may vary with the purpose of testing. Reactions of 25 or 50µl are used for initial amplification. The 100µl reactions are only used to verify detected DNA and to purify for sequencing. To conserve supplies, most initial testing will be done with a 25µl reaction.]

2. Disinfect the “clean” (master mix) hood and “dirty” (extraction) hood. (Wipe down all surfaces with 70% alcohol and turn on ultraviolet prior to use.)

3. Thaw the master mix reagents in a cooling block. A cooling block is used to keep the kit reagents chilled while working with the master mix, and a freezer chill block is used to keep the master mix chilled once it has been dispensed into reaction tubes. (Ice can be used as an alternative.)

4.2.2 Lightly mix and spin master mix reagents. Combine reagents according to calculation on **CVB-TWS-0123**. This mixture is now referred to as master mix.

Master Mix

Ingredient Name	Concentration	25µL/Rxn	50µL/Rxn	100µL/Rxn
5X QIAGEN OneStep RT-PCR Buffer	1X	5.00	10.00	20.00
Nuclease-free water	N/A	11.80	28.60	57.20
QIAGEN OneStep RT-PCR Enzyme Mix	N/A	1.00	2.00	4.00
dNTP Mix (10mM each)	(400 µM)	1.00	2.00	4.00

Forward Primer	10pMol/µl	0.60	1.20	2.40
Reverse Primer	10pMol/µl	0.60	1.20	2.40
Total Master Mix		20.00	45.00	90.00
RNA Template		5.00	5.00	10.00

4.2.3 Insert PCR reaction tubes into a tray. Add 20 or 45 or 90µl of master mix to each PCR reaction tube in the clean hood where the master mix was made, or before opening the RNA template in the DNA/RNA template (dirty) hood.

4.2.4 Using the same nuclease-free water source as the master mix, add 5 or 10µL of water into one of the PCR reaction tubes and label as the negative template control (NTC) on CVB-TWS-0123. Only one NTC is needed per PCR reaction. After the NTC is added, return all unused master mix reagents back to -20± 5°C storage.

4.2.5 In the template hood, add 5µl of sample template to the corresponding PCR reaction tube for the 25 or 50µl reactions, or 10µl for the100µl reactions. Repeat this process for each sample. **Change pipette tips between each sample**. **Add the non-spiked samples first,** followed by the spiked samples.

4.2.6 Cap all the tubes (if necessary, use a capping instrument). Number or identify each individual tube with a corresponding ID to what's listed on **CVB-TWS-0123**.

4.2.7 Place each PCR reaction tube in the thermocycler and close the heated cover.

4.2.8 Run the Qiagen OneStep amplification program (see **Section 3.2**).

Note: Although all reverse transcription Qiagen OneStep PCRs are run with the similar program listed in Section 3.2, it is important to use the correct annealing temperature based on the primer set being used. Annealing temperatures are chosen based on a temperature gradient study done when primers sets are developed. Primer set annealing temperatures can be found in the Appendix..

4.2.9 Disinfect BSC hoods after the PCR has been started and the testing materials have been cleaned and removed (refer to **Section 4.2.1.2**).

4.2.10 Once the thermocycler program is complete, store PCR reaction tubes at 4°C until an analysis is ready to be performed.

4.3 Analysis of amplified Viral DNA

4.3.1 E-Gel™ electrophoresis system or equivalent

1. Insert a 2% E-Gel™ precast agarose gel into the E-Gel™ electrophoresis docking system and remove the comb.

2. Load 20µl of the appropriate DNA Ladder and sample in the corresponding wells as recorded on **CVB-TWS-0123**.

3. Run the E-Gel™ electrophoresis system preset program for 30 minutes.

4.3.2 Visualizing and documenting gel

1. Remove the E-gel™ from the ~~E-Gel™~~ electrophoresis docking system and place it in a UV light box.

2. Photograph the gel according to available UV imaging programs and save a copy of the image to the CVB-L drive. Attach a photograph or electronic picture to the current version of **CVB-TWS-0123**.

3. After photographing, dispose of the E-Gel™ in a designated container for autoclave waste.

5. Sequencing Samples with Detected RNA

5.1 Sanger Sequencing

5.1.1 If DNA is detected in a sample, an additional 100µl reaction is run to verify and send for sequencing. Samples for sequencing are purified using Qiagen Purification kit according to kit directions. **CVB-WI-0267**, (*QIAquick PCR DNA Purification Kit Procedure)*, and **CVB-WI-0267 (***PCR Processing for Sequencing)*, gives steps to prepare amplified DNA to send for sequencing.

5.1.2 Once the sample has been amplified, verified, purified and prepared; the sample and corresponding primers are taken to the Iowa State University's DNA facility.

5.1.3 Sequences received from the DNA facility are analyzed using Geneious software (or equivalent). A nucleotide blast is performed on NCBI to match the sequence and identify the sample.

5.2 Whole Genome Sequencing

Whole genome sequencing may also be used to sequence samples that were detected to confirm sample identity.

6. Interpretation of the Test Results

6.1 Interpretation of test results for DNA extraneous Agent Testing

6.1.1 The size of the PCR product for an RNA extraneous primer set can be found in **Section 10.1** in the **Appendices**. The size of the band from the PCR product can be interpreted by comparing it to the appropriately sized DNA Ladder.

6.1.2 Test criteria

1. No visible wild type bands in sample only lanes.
2. No visible wild type bands in NTC or NEC lanes.
3. Lanes containing the IAC have a visible band at expected base pair size and no wild type band is present.

Note: Amplification of BVDV and PRRSV for extraneous agent testing often generates amplicons differing from the expected length. Of the 268 BVDV 5'UTR sequences available in GenBank as of 23Jun17, the amplicon sizes using the BVDV Ridpath primers had a range of 278-293 bp. Of the 353 PRRSV 5'UTR sequences available in GenBank as of 23Jun17, the amplicon size range was 96-106 bp. Previous work has shown that amplicons not falling in these size ranges derive from mispriming on the host cell genome. Therefore, spurious amplicons may be ignored.

6.1.3 If all three criteria are met, testing is considered satisfactory, and samples are reported as undetected for the virus tested. If criteria #2 or #3 are not met, testing is considered invalid. ~~If.~~ If criterion #1 is not met, but criteria #2 and #3 are met, the sample is considered unsatisfactory.

6.1.4 Invalid testing will be repeated.

6.1.5 Unsatisfactory testing will be repeated using a new vial of sample. If repeated samples meet all three criteria, the sample is considered undetected for that virus and are considered satisfactory. If repeated samples do not meet the three criteria, then samples are sequenced as described in **Section 5**.

6.1.6 It is up to the supervisory discretion if samples are considered unsatisfactory after identification of viral RNA by sequencing analysis.

7. Report of Test Results

Report results for purity viral RNA testing on worksheet **CVB-TWS-0123**. Results are reviewed and entered into proper computer databases for record retention.

8. Recordkeeping and Report of Test Results

All records are kept in accordance with the current version of **CVB-SOP-0098**, *Laboratory Record Keeping*. Results of testing will be reviewed and signed by the agent contact or designee. For firm testing, results may be entered into LSRTIS or the current reporting system and released to the Reviewer for distribution to the firm.

9. References

9.1 QIAGEN OneStep RT-PCR Handbook (October 2012)

9.2 Operator manual for thermocycler used

9.3 Kit insert for Qiagen, QIAamp Viral RNA Mini Kit

9.4 Invitrogen DNA Ladder/Marker Reference

9.5 Ridpath, J. F. and S. R. Bolin (1998). "Differentiation of types 1a, 1b and 2 bovine viral diarrhea virus (BVDV) by PCR." Mol Cell Probes **12**(2): 101-106.

9.6 Vilcek, S., Herring, A.J., Herring, J.A., Nettleton, P.F., Lowings, J.P., Paton, D.J., 1994. Pestiviruses isolated from pigs, cattle and sheep can be allocated into at least three genogroups using polymerase chain reaction and restriction endonuclease analysis. Archives of virology 136, 309-23.

9.7 Nicholson, T.L., Kukielka, D., Vincent, A.L., Brockmeier, S.L., Miller, L.C., Faaberg, K.S., 2011. Utility of a panviral microarray for detection of swine respiratory viruses in clinical samples. J Clin Microbiol. 49, 1542-1548.

10. Summary of Revisions

Version CVB-PRO-0033.05

- Changed gel type to reflect laboratory usage and deleted warning regarding EtBr in gels since the new gels don't contain EtBr
- Made revisions across multiple sections to correct errors regarding sample type and IAC dilutions
- Added NTC to be used during PCR

Version CVB-PRO-0033.02

- Addition of Master mix 25 and 100ul reactions.
- Change in name to include the names of viruses to be tested.
- Documents identification number has changed from VIRPRO0128.04 to CVB-PRO-0033.02 due to the transition to MasterControl.

Version VIRPRO0128.04

- 5.1.1 and 5.1.2 were removed.

Version VIRPRO0128.03

- PRSV was changed to the correct acronym of PRRSV.
- Note on spurious band was changed to reflect current practices for BVDV
- PRRSV testing in the laboratory

Version VIRPRO0128.02

- Changed rehydration of primers Section 2.3.3.
- Added note on BVDD and PRRSV primers to Section 6.1.2.

Appendices-Extraneous Agent PCR Primer Information

RNA Virus	Primer Name	Primer Sequence 5'-3'	Annealing Temp	Wild Type Fragment Size	pRNAcIAC Fragment Size
Porcine and Reproductive Respiratory Syndrome (PRRSV)	PRRSV-F	TCAGCTGTGCCAGATGCTGG	60ºC	~100bp	436 bp
	PRRSV-R	AAATGCGGCTTCTCCGGGTTT			
Bovine Viral Diarrhea Virus (BVDV)	BVDV Ridpath-F	CATGCCCATAGTAGGAC	50ºC	~250bp	471 bp
	BVDV Ridpath-R	CCATGTGCCATGTACAG			
Bovine Viral Diarrhea Virus (BVDV)	BVDV Vilcek-F	ATGCCCTTAGTAGGACTAGCA	55ºC	~288bp	486bp
	BVDV Vilcek-R	ACAACTCCATGTGCCATGTAC			
Reticuloendotheliosis (REV)	REV-F	TAAGAAGACGCCTCCGGGTA	58ºC	~349bp	549bp
	REV-R	CACAAGACGCCCTTCAGACT			

* T was replaced with an A on the 5' end of BVDV Vilcek Reverse primer. Roughly 2/3 of BVDV2 isolates contain an adenine residue at this locus.