Animal and Plant Health Inspection Service

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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)

Document Number: **CVB-PRO-0033** **Revision:** **04**

Previous Number: VIRPRO0128.04

Vault: CVB-Released

Section/Area: CVB-PRO

Effective Date: 21 Jul 2020

Notes:

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 (1000-µl, 200-µl, 100-µl, 20-µl, 10-µl, 2.5-µl)

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 (1000-µl, 200-µl, 100-µl, 20-µl, 10-µl, 2.5-µl)

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 1.5/2.0-mL 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 20/45/90µl of master mix to each PCR reaction tube in the clean hood where the master mix was made, or before opening the DNA 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 1.5-mL microcentrifuge tubes

2.3.1.3 14-mL centrifuge tube

2.3.1.4 PCR Reaction Tubes (Applied Biosystems, Micro Amp, Part #N8010580)

2.3.1.5 PCR Reaction tube caps (Applied Biosystems, Micro Amp, Part #N8015535)

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, Product #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 on the CVB VIR SharePoint site or **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 10 pmol, aliquoted in 50µl increments and stored in a -20 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 Ladder based on the PCR fragment size (Invitrogen)

2.3.4.2 Loading Buffer, 10X BlueJuice (Invitrogen, Catalog #10816-015)

2.3.4.3 E-Gel® pre-cast Agarose 2% gel (Catalog #G5018-02)

Caution: Ethidium bromide is a suspected carcinogen. Use gloves when handling any reagents containing Ethidium Bromide. Refer to Material Safety Data Sheet (MSDS) and the NCAH Environmental Health and Safety Unit guidelines for safe handling, usage, and disposal.

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 (SOPs). Wear non-powdered disposable gloves. Program the thermocycler with the following program:

Amplification of RNA Viruses

Step	Time	Temperature	Cycles
Reverse Transcription	30 minutes	50ºC	1
PCR Activation	15 minutes	95ºC	1
Denaturation	1 minute	95ºC	35x
Annealing	1 minute	Variable based on Primers used 50-68ºC	
Extension	1 minute	72ºC	
Final Extension	10 minutes	72ºC	1
Storage	∞	4ºC	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 the 3rd pass in Bovine turbinate cells (BT cells). Each sample, including a negative control (Either nuclease free water or negative cell culture passage), is ran in duplicate. Duplicate sample is spiked with 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 base pair size ladder by adding 50µl of 10x Blue Juice 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-Gels only.)

3.3.4 Prepare the pRNAcIAC WS Lot# 19-02 to a concentration of 10^3 copies/µl by adding 900 µl of nuclease free water to the 100 µl 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 140 µl of sample to two 1.5 mL centrifuge tubes. In one of the duplicate sample tubes inoculate 10µl of pRNAcIAC diluted at 10^2 copies/µl for a final concentration of 10^3 copies/sample.

4.1.3 Add 140 µl of Nuclease free water to two 1.5 mL centrifuge tubes to serve as a negative control. In one of the duplicate negative control tubes inoculate 10µl of pRNAcIAC diluted at 10^2 copies/µl for a final concentration of 10^3 copies/sample.

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 1.5-mL 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^{0}C until use for amplification in **Section 4.2**.

Store samples at -20°± 5°C until ready to run PCR.

4.2 Amplification of Viral RNA

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

1. Calculate the amount of master mix needed by determining the total number of reactions needed plus one. (**CVB-TWS-0123** contains an Excel block that will calculate this once the number of samples is entered.)

   [The amount of each reaction may vary with purpose of testing. Reactions of 25 or 50µl for initial amplification. Additional 100µl reactions are used to verify detected DNA and purification 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. Return all unused master mix reagents back to -20°± 5°C storage.

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	11.8	28.60	57.2
QIAGEN OneStep RT-PCR Enzyme mix	N/A	1.00	2.00	4.00
dNTP Mix	(400 µM)	1.00	2.00	4.00
Forward Primer	10pMol/µl	0.6	1.2	2.4
Reverse Primer	10pMol/µl	0.6	1.2	2.4
Total Master Mix		20	45	90
DNA Template (extracted Sample)		5	5	10

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

4.2.4 In the template BSC, add 5µl of sample template (extracted sample) to the corresponding PCR reaction tube for 25 or 50µl reactions or 10µl for 100µl. Repeat this process for each sample. Change pipette tips between each sample. Label the caps of each tube with a corresponding number to **CVB-TWS-0123**.

4.2.5 Cap all the tubes. If necessary, use a capping instrument. Number or identify individual tubes.

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

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

Note: Although all reverse transcription Qiagen OneStep PCRs are ran 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 or on the CVB VIR SharePoint site.

4.2.8 Disinfect BSC hoods (refer to **Section 4.2.1.2**)

4.2.9 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 of 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 the UV light box.

2. Photograph E-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 E-Gel® in a container designated for solid EtBr 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 preparing 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 (ISU) DNA facility

5.1.3 Sequences received from ISU DNA facility are analyzed using Geneious software (or equivalent), blast performed to match sequence and identify sample.

5.2 Whole Genome Sequencing

Whole genome sequencing may also be used to sequence samples that were detected for RNA 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 a 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 appropriate sized DNA Ladder/marker.

6.1.2 Test criteria

1. No visible wild type bands in sample only lanes.
2. No visible wild type bands in negative control lanes.

3. Lanes containing pRNAcIAC have a visible band at expected base pairs when 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 RNA 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 testing 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 report out worksheet **CVB-TWS-0123**. Results are reviewed and entered into proper computer databases according to the current version of **CVB-SOP-0121**, *Testing Roles, Responsibilities, and Procedures for Reporting Tests Results in the Virology Section.*

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 diarrhoea 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.

9.6 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-0033.02

- 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.

11. 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	60□C	~100bp	436 bp
	PRRSV-R	AAATGCGGCTTCTCCGGGTTT			
Bovine Viral Diarrhea Virus (BVDV)	BVDV Ridpath-F	CATGCCCATAGTAGGAC	50□C	~250bp	471 bp
	BVDV Ridpath-R	CCATGTGCCATGTACAG			
Bovine Viral Diarrhea Virus (BVDV)	BVDV Vilcek-F	ATGCCCTTAGTAGGACTAGCA	55□C	~288 bp	486 bp
	BVDV Vilcek-R	ACAACTCCATGTGCCATGTAC			
Reticuloendotheliosis (REV)	REV-F	TAAGAAGACGCCTCCGGGTA	58□C	~349 bp	549 bp
	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.