CONTROLLED//PROPIN//BASIC

United States Department of Agriculture
Center for Veterinary Biologics

Testing Protocol

DNA PCR Assay Using Qiagen HotStar Taq Polymerase Kit

Date: November 6, 2024

Reference Number: CVB-PRO-0032.05

Contact: Center for Veterinary Biologics, 515-337-6100

United States Department of Agriculture
Animal and Plant Health Inspection Service
P. O. Box 844
Ames, IA 50010

INTERNAL USE ONLY

Table of Contents

1. Introduction

This testing protocol (PRO) describes the performance of polymerase chain reaction (PCR) for detection of DNA viruses for purity testing of biologic products.

2. Materials

2.1 Equipment/instrumentation for DNA 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 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-45-11 14,500 RPM)

2.1.8 Heating Block or Water Bath set at 56± 2°C

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, 100-µl, 20µl, 10µl, 2.5µl)

2.2.5 Micro-tube storage racks

2.2.6 Refrigerator

2.2.7 96-well Rack

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

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

2.2.10 E-gel™ electrophoresis system

2.2.11 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 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 DNase/RNase-free aerosol-resistant pipette tips, various sizes

2.3.1.2 1.5mL microcentrifuge tubes

2.3.1.3 PCR Reaction Tubes, 0.2mL (Applied Biosystems™, Micro-Amp™ 8-Tube Strip, Catalog #N8010580)

2.3.1.4 PCR Reaction Tube Caps (Applied Biosystems™, Micro-Amp™ 8-Cap Strip, clear, Catalog #N8010535)

2.3.1.5 Latex, vinyl, or nitrile powder free disposable gloves

2.3.2 DNA extraction reagents/supplies

2.3.2.1 CVB-WI-0264, *DNA Extraction Assay – Qiagen DNeasy Blood and Tissue Kit*

2.3.2.2 Qiagen DNeasy Blood & Tissue Kit. (Qiagen, Catalog #69504)
Note: Buffer AL is used as the Lysis buffer during this DNA extraction.

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

2.3.2.4 DNase/RNase-free water

2.3.2.5 Internal amplification control (IAC;pDNAcIAC, WS Lot #19-01)

2.3.3 DNA PCR Reagents/Supplies

2.3.3.1 CVB-TWS-0124, *HotStar Taq Plus-PCR Assay*

2.3.3.2 HotStarTaq DNA Polymerase (Qiagen, Catalog #203203) or HotStarTaq Master Mix Kit (Qiagen, Catalog #203443)

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

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 DNA Viruses

Step	Time	Temperature	Cycles
PCR Activation	5 minutes	95ºC	1
Denaturation	1 minute	94ºC	35x
Annealing	1 minute	Variable based on primer used (56-60ºC)	
Extension	1 minute	72ºC	
Final Extension	10 minutes	72ºC	1
Storage	∞	4ºC	1

3.3 Preparation of reagents/control procedures

3.3.1 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).

3.3.2 Prepare the internal amplification control (IAC, pDNAcIAC WS Lot #19-01) to a concentration of 10^3 copies/µl by adding 900µl of nuclease-free water to 100µl of pDNAcIAC (bottled at 10^4 copies/µl).

4. Testing Procedure

4.1 DNA extraction

4.1.1 Thaw, rehydrate, and rebottle samples if need. Record Sample information on the current version of **CVB-TWS-0118**.

4.1.2 Add 200µl 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 200µl of nuclease-free water into two 1.5mL centrifuge tubes to serve as the negative extraction control (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 Qiagen DNeasy Blood & Tissue Mini Kit-spin protocol or most current version of **CVB-WI-0264** (*DNA Extraction Assay – Qiagen DNeasy Blood & Tissue Kit)*.

4.1.5 Final elution of samples – Buffer AE (provided in kit) or DNase/RNase-free water may be used.

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

Example of Extraction Label
YYYYMMDD DNA initials
Sample Name
Additional information

4.2 Amplification of Viral DNA

4.2.1 See the current version of **CVB-TWS-0124** for the master mix worksheet. Prepare master mix for DNA amplification 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.

4.2.2 Lightly mix and spin master mix reagents. Combine reagents according to calculation on **CVB-TWS-0124**. This mixture is now referred to as master mix. (Either the HotStarTaq kit or HotStarTaq Plus kit may be used. The only difference is HotStarTaq Plus includes CoralLoad as a dye marker for better visualization when loading gels.)

Master Mix (HotStarTaq Plus)

Ingredient Name	Concentration	25µL/Rxn	50µL/Rxn	100µL/Rxn
HotStarTaq Plus Master Mix	2X	12.50	25.00	50.00
RNase-free Water	N/A	5.30	15.60	31.20
CoralLoad	10X	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
DNA Template		5.00	5.00	10.00

Master Mix (HotStarTaq)

Ingredient Name	Concentration	25µL/Rxn	50µL/Rxn	100µL/Rxn
HotStarTaq Master Mix	2X	12.50	25.00	50.00
RNase-free Water	N/A	6.30	17.60	35.20
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
DNA 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 DNA 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-0124. 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-0124**.

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

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

Note: Although all DNA HotStarTaq Plus 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.

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 an E-Gel™ precast agarose gel into the E-Gel™ electrophoresis docking system and remove the comb.

2. Load 20µl of appropriate DNA ladder and sample in the corresponding wells as recorded on **CVB-TWS-0124**.

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 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-0124**.

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

5. Sequencing Sample with Detected DNA

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-0262** (*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 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 a DNA extraneous primer set can be found in **Section 11.1** 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 only bands in sample only lanes.
2. No visible wild type only bands in NEC and NTC lanes.
3. Lanes containing the IAC have a visible band at expected base pair size and no wild type band is present.

6.1.3 If all three criteria are met, testing is considered satisfactory, and samples are reported as undetected for the extraneous agent tested. If criteria #2 or #3 are not met, the sample is considered invalid. 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 supervisory discretion if samples are considered unsatisfactory after identification of DNA by sequencing analysis.

7. Report of Test Results

Report results for the testing on CVB-TWS-0124. 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 HotStarTaq® Plus Master Mix Handbook (October 2010)

9.2 Operator manual for thermocycler used

9.3 Kit insert for Qiagen, DNeasy Blood & Tissue Mini Spin Kit

9.4 Invitrogen™ DNA Ladder/Marker Reference

10. Summary of Revisions

Version CVB-PRO-0032.05

- Changed gel type to reflect laboratory usage and deleted warning regarding EtBr in gels since the new gels don't contain EtBr
- Added NTC to be used during PCR

Version CVB-PRO-0032.04

- Format changes.

Version CVB-PRO-0032.03

- Section 4.4.2 updated to include 25, 50 and 100ul reactions.

Version CVB-PRO-0032.02

- Alphanumeric number has changed from VIRPRO0126 to CVB-PRO-0032.

Appendices

Extraneous Agent PCR Primer Information

PCR	Primer Name	Primer Sequence	Annealing Temp	Wild Type Fragment Size	pDNAcIAC Fragment Size
Porcine Circovirus 1 (PCV 1)	PCV1B-F	GAGAAAAACAAAATACGGGAGC	56°C	~537bp	~684bp
	PCV1B-R	CCATCCCACCACTTATTTCTAC			
Porcine Circovirus 2 (PCV 2)	PCV2F1	GACGAGCGCAAGAAAATACG	56°C	720bp	~921bp
	PCV2R7	AGTTGAGGAGTACCATTCCA			
Chicken Anemia Virus (CAV)	CAV_190F	TCCGAGTACAGGGTAAGCGA	60ºC	620bp	~846bp
	CAV_SR1	CCGTGGGCTGCATCATCATT			