Animal and Plant Health Inspection Service

Veterinary Services

Center for Veterinary Biologics

1920 Dayton Avenue
PO Box 844
Ames, IA 50010

(515) 337-6100

DNA PCR Assay Using Qiagen HotStar Taq Polymerase Kit

Document Number: CVB-PRO-0032 **Revision: 03**

Previous Number: VIRPRO0126.02

Vault: CVB-Released

Section/Area: CVB-PRO

Effective Date: 05 Jan 2021

Notes:

DNA PCR Assay Using Qiagen HotStar Taq Polymerase Kit

Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by USDA and does not imply its approval to the exclusion of other products that may be suitable.

Table of Contents

1. Introduction

2. Materials

   2.1 Equipment/instrumentation for DNA extraction
   2.2 Equipment/instrumentation for PCR
   2.3 Reagents/supplies

3. Preparation for the Test

   3.1 Personnel qualifications/training
   3.2 Preparation of equipment/instrumentation
   3.3 Preparation of reagents/control procedures

4. Testing Procedure

5. Sequencing Sample with Detected DNA

6. Interpretation of the Test Results

7. Report of Test Results

8. Recordkeeping and Report of Test Results

9. References

10. Summary of Revisions

11. Appendices

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 (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-45-11 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 (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 96-well Rack

2.2.8 Quick Spin Minifuge (6 x 1.5/2.0-mL 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 45µl 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 1.5-mL microcentrifuge tubes

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

2.3.1.4 PCR Reaction Tube Caps (Applied Biosystems, Micro Amp, Part #N8015535)

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 and 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, Product #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 HotStar Taq® Plus Master Mix Kit (Qiagen, Catalog #203203) or HotStar Taq (Qiagen, Catalog #203443)

2.3.3.3 Forward and Reverse Primers at approximately 10pmol/µl each (A list of primer pairs can be found on the CVB VIR SharePoint Site or **Section 11.1** in the **Appendices**. Not all are listed and may change/update. Be sure to verify current primer sets before starting.)

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 Blue Juice (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 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 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 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.)

3.3.2 Prepare the pDNAcIAC WS Lot #19-01 to a concentration of 10^3 copies/µl by adding 900µl of nuclease free water to the 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 to two 1.5 mL centrifuge tubes. In one of the duplicate sample tubes inoculate 10µl of pDNAcIAC diluted at 10^3 copies/µl.

4.1.3 Add 200µl of nuclease free water to two 1.5 mL centrifuge tubes. In one of the duplicate sample tubes inoculate 10µl of pDNAcIAC 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 and Tissue Mini Kit-spin protocol or most current version of **CVB-WI-0264**, *DNA Extraction Assay – Qiagen Dneasy Blood and Tissue Kit*.

4.1.5 Final elution of samples – Buffer AE (provided in kit) or Dnase/Rnase free water may be used for elution of samples.

4.1.6 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**.

Example of Extraction Label
YYYYMMDD DNA initials
#1 Sample Name
Additional information

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

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 (**CVB-TWS-0124** 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.

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. Return all unused master mix reagents back to -20°± 5°C storage. (Either HotStar

Taq kit or HotStar Taq Plus kit can be used. Only difference is HotStar Plus includes Coral Load as a dye marker for better visualization when loading gels.)

Master Mix (HotStar Taq Plus)

Ingredient Name	Concentration	25µL/Rxn	50µL/Rxn	100µL/Rxn
HotStar Taq Plus Master Mix	2X	12.50	25.00	50.00
RNase-free water	N/A	5.3	15.60	31.2
Coral Load	10x	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	[redacted]	20	45	90
DNA Template (extracted Sample)		5	5	10

Master Mix (HotStar Taq)

Ingredient Name	Concentration	25µL/Rxn	50µL/Rxn	100µL/Rxn
HotStar Taq Master Mix	2X	12.5	25.00	50.00
RNase-free water	N/A	6.3	17.60	35.2
Forward Primer	10pMol/µl	0.6	1.2	2.4
Reverse Primer	10pMol/µl	0.6	1.2	2.4
Total Master Mix	[redacted]	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 reactions. 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-0124.**

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 HotStar amplification program (see **Section 3.2**).

Note: Although all DNA HotStar Taq 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 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 4^0C 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 agrose gel into the E-Gel® electrophoresis docking system and remove the comb.

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

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-0124**.

3. After photographing, dispose of E-Gel® in a container designated for solid EtBr 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. Sample 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 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 DNA 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 appropriate sized DNA ladder/marker. (Not all are listed and may change/update. Be sure to verify current primer sets before starting.)

6.1.2 Test Criteria

1. No visible wild type only bands in sample only lanes.
2. No visible wild type only bands in negative control lanes.
3. Lanes containing pDNAcIAC have a visible band at expected base pairs when 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, testing is considered invalid. 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 analyzed DNA sequences from Section 5 are unsatisfactory if the blast results are identified as the virus being tested.

7. Report of Test Results

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 Test 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 HotStar Taq® Plus Master Mix Handbook (October 2010)

9.2 Operator manual for thermocycler used

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

9.4 Invitrogen DNA Ladder/Marker Reference

10. Summary of Revisions

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.

Version VIRPRO0126.02

- Sections 5.1.1 and 5.1.2 were removed to follow current practices.
- **7.1:** Removed reference to VIRTWS0128 as the document has been inactivated.

- Included the use of pDNAcIAC Lot# 19-01
- Updated the test criteria and interpretation of results to reflect the use of the pDNAcIAC
- Updated the primer List

11. Appendices

11.1 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	720 bp	~921bp
	PCV2R7	AGTTGAGGAGTACCATTCCA			
Chicken Anemia Virus (CAV)	CAV_190F	TCCGAGTACAGGGTAAGCGA	60°C	620 bp	~846bp
	CAV_SR1	CCGTGGGCTGCATCATCATT			

Signature Manifest

Document Number: CVB-PRO-0032 **Revision:** 03
Title: DNA PCR Assay Using Qiagen HotStar Taq Polymerase Kit
Effective Date: 05 Jan 2021

All dates and times are in Central Standard Time.

CVB-PRO-0032 02 DNA PCR Assay Using Qiagen HotStar Taq Polymerase Kit

Review for Doc Format

Name/Signature	Title	Date	Meaning/Reason
CROSLEY HERR (CRHERR)	QM Program Asst	19 Oct 2020, 04:37:44 PM	Approved

Lab Supervisor Final Approval

Name/Signature	Title	Date	Meaning/Reason
ALETHEA FRY (AFRY)	Supvy Micro	07 Dec 2020, 06:26:54 AM	Approved

Final Quality Check

Name/Signature	Title	Date	Meaning/Reason
MARK PAGALA (MPAGALA)	Assistant Director	05 Jan 2021, 11:35:15 AM	Approved