Animal and Plant Health Inspection Service

Veterinary Services

Center for Veterinary Biologics

1920 Dayton Avenue
PO Box 844
Ames, IA 50010

(515) 337-6100

Polymerase Chain Reaction Assay for Detection and Identity of Extraneous Chicken Anemia Virus (CAV) DNA

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Notes:

Polymerase Chain Reaction Assay for Detection and Identity of Extraneous Chicken Anemia Virus (CAV) DNA

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1. Introduction

This Testing Protocol (PRO) describes a polymerase chain reaction (PCR) assay for detection and identity of extraneous chicken anemia virus (CAV) DNA in biologic products as specified in the guidelines of Veterinary Services Memorandum No. 800.89.

2. Materials

2.1 Equipment/instrumentation

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

2.1.1 Equipment for DNA Extraction/Loading of Template

1. Laminar Flow Biological Safety Cabinet (BSC) (NuAire Inc., Labgard)

2. Microcentrifuge (Eppendorf 5415 C)

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

4. Quick Spin Minifuge (6 x 1.5/2.0 ml microtube rotor)

5. Eppendorf Adjustable Volume Research micro-pipettes (20 µl, 100 µl, 200 µl, 1000 µl)

Note: Separate Micro-pipettes are assigned to the individual BSCs and should not be interchanged between designated hoods to avoid contamination

2.1.2 Equipment for Master Mix

1. Laminar Flow Biological Safety Cabinet (BSC) (NuAire Inc., Labgard)

2. Quick Spin Minifuge (6 x 1.5/2.0-mL microtube rotor)

3. Eppendorf Adjustable Volume Research micro-pipettes (1000 µl, 200 µl, 100 µl, 20 µl, 10 µl, 2.5 µl)

4. 96-well Eppendorf PCR cooler (stored at -20°C)

2.1.3 Equipment for PCR and PCR analysis

1. Thermocycler (Applied Biosystems Veriti 96-well thermocycler)
2. E-Gel® electrophoresis system
3. UV imaging system and computer software

2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below.

2.2.1 Laboratory Supplies

1. Aerosol Resistant Filter tips for micropipettes, various sizes
2. Powder-free disposable gloves
3. 1.5 ml centrifuge tubes
4. Nuclease-free water
5. PCR reaction tubes (Applied Biosystems, MicroAmp, Part #: N8010580)
6. PCR reaction tube caps (Applied Biosystems, MicroAmp, Part #: N8010535)
7. 96-well PCR reaction tube tray/retainer (Applied Biosystems, MicroAmp, Part #: N8015530)
8. 10% hypochlorite solution (bleach)
9. 70% Ethanol solution

2.2.2 DNA Extraction Reagents and Supplies

1. DNeasy Blood and Tissue Kit (Qiagen, Catalog #: 6504)
2. Ethanol 200 proof molecular grade (Sigma-Aldrich, Product #: E7023)
3. pCAV_IAC 10^4working stock (WS) diluted at 10^3 from master sequence lot# 17-02 prepared according to **CVB-PP-0065**, *Chicken Anemia Virus Plasmid Internal Amplification Control*
4. Current versions of Virology Testing Worksheet: **CVB-TWS-0118**, *DNA Extraction Essay – Qiagen Dneasy Blood and Tissue Kit*

2.2.3 PCR Reagents and Supplies

1. HotstarTaq® Plus Master Mix Kit (Qiagen, Catalog#: 203645)

2. CAV primers, CAV-190F and CAV-S1R at approximately 10 pmol/µl each

   Reconstitution of the primers is determined by the synthesis data sheet that is received after the primers are made by IDT or equivalent. Primers are rehydrated to a concentration of 10 pMol/µl, aliquoted in 50µl increments into 0.2mL screw cap tubes, and stored in a -20°C freezer until further use.

   Primer Sequences:

   CAV-190F: **5'**-TCCGAGTACAGGGTAAGCGA **-3'**
   CAV-S1R: **5'**- CCGTGGGCTGCATCATCATT **-3'**

3. Current versions of Virology Testing Worksheet: **CVB-TWS-0124**, *HotStarTaq Plus – PCR Assay*

2.2.4 Reagents for Analysis of PCR Amplicons

1. DNA Ladder 100 base pair (bp) ladder (Invitrogen REF #: 15628-017)

2. Loading Buffer, 10X BlueJuice (Invitrogen, Catalog #: 10816-015)

3. E-Gel® pre-cast Agarose 2% gel (Catolog#: G5018-02)

Caution: Ethidium bromide (EtBr) is a suspected carcinogen. Use gloves when handling any reagents containing EtBr. 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 laboratory techniques and preparation and the proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals.

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 CAV DNA

Time	Temperature	Cycles
15 minutes	95°C	1
1 minute	95°C	35x
1 minute	60°C	
1 minutes	72°C	
10 minutes	72°C	1
∞	4°C	1

3.3 Preparation of reagents/control procedures

3.3.1 Add 900µl of nuclease-free water to 100µl of the pCAV_IAC positive control Lot# 17-02 WS for a final concentration of 10^3 copies/µl of plasmid. Information on how the pCAV_IAC was made can be found in **CVB-PP-0065**.

3.3.2 Prepare 100bp Ladder by adding 50µl of 10x Blue Juice with 900µl of nuclease-free water for use in an 2% E-gel®

3.4 Storage of the sample

Obtain and store samples for testing as described in the current version of **CVB-TWS-0272**, *Sample Tracking in the CVB Virology Section*. When working with known or suspected live biological agents, a biological safety cabinet must be used and aseptic conditions must be maintained.

4. Performance of the Test

4.1 DNA extraction

4.1.1 Thaw, rehydrate, and rebottle samples if needed. Record sample information on the current version of **CVB-TWS-0018**.

4.1.2 Transfer 200 µL of the sample to a 1.5-mL centrifuge tube. If the sample is difficult to pipette due to viscosity, transfer 100 µL sample and add 100 µL nuclease-free water to a 1.5mL centrifuge tube.
Note: If the sample being tested is whole tissue, take approximately 1 gram of tissue, push through 5-mL syringe without a needle, and mix with 400 µL of nuclease-free water. Pull solution through a canula attached to a syringe until it is broken down enough to be brought up in a pipette tip. Use 100 µL of this solution with 100 µL of nuclease-free water. This method is recommended for chick embryo tissue.

4.1.3 In the same 1.5 mL centrifuge tube, add 10 µl of pCAV_IAC at a concentration of 10^3 copies/µl to the 200 µl of sample for a final volume of 210 µl.

Example:

Extraction #	Sample Name
1	Sample 1 (200 µl) + 10 µl of pCAV_IAC
2	Sample 2 (200 µl) + 10 µl of pCAV_IAC
3	External Negative Control (Water) + 10 µl of pCAV_IAC

4.1.4 From this point, follow the most recent available protocol that is provided in the Qiagen DNeasy Blood and Tissue Kit for tissue culture samples, starting with the addition of 20 µL of proteinase K and 200 µl of lysis buffer to each sample followed by a 10 minute incubation in a 56°C heat block.

4.1.5 Once the DNA template extraction is complete, label each extraction tube and store the extracted DNA samples at -20°C until use for amplification in **Step 4.2**.

Example of DNA Template Extraction Label
Qiagen DNA Ext #1
Sample Name
Date/Initial

4.2 Amplification of CAV DNA

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

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

2. Thaw the master mix reagents in a 96-well Eppendorf PCR cooler or an ice bucket. Add the master mix ingredients in the order listed in the clean master mix area. Do not include DNA template at this time.

Master Mix

Ingredient Name	Concentration	µL/Rxn
*HotstarTaq® Plus Master	2X	25.00
*Nuclease Free Water	N/A	15.60
*Coral Load	10X	2
CAV-190F	10pMol/µl	1.2

CAV-S1R	10pMol/µl	1.2
Total Master Mix		45

*Components of the HotstarTaq® Plus Master Mix Kit

4.2.2 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.

4.2.3 In the template BSC, add 5 µL of DNA template (extracted sample) to the corresponding PCR reaction tube. Repeat this process for each sample. Change pipette tips between each sample. Label the caps of each tube with the template number corresponding to **CVB-TWS-0124**.

4.2.4 Cap all the tubes ensuring a tight fit

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

4.2.6 Run the CAV amplification program (see **Section 3.2**).

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

4.3 Analysis of amplified CAV 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

2. Remove the comb from the E-Gel® precast agarose gel and load 20µl of prepared 100bp ladder.

3. Load 20µl of amplified DNA in the corresponding wells as recorded on **CVB-TWS-0124**, *HotStar Taq Plus – PCR Assay*.

4. 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. Interpretation of the Test Results

5.1 The wild type CAV amplicon size produced by primers CAV-190F and CAV-S1R is 620bp. The 620bp band of the PCR product can be interpreted by comparing it to the bands of the 100bp Ladder.

5.2 The pCAV_IAC amplicon size produced by primers CAV-190F and CAV S1R is 823 bp. The 823bp band of the PCR product can be interpreted by comparing it to the bands of the 100bp Ladder.

5.2 Test Criteria to determine whether a sample is negative for CAV DNA

1. Sample lanes have no visible bands at 620 bp.
2. Sample lanes have a visible band at 826 bp.
3. External negative control has no visible band at 620 bp.
4. External negative control has a visible band at 826 bp.

5.3 If all four criteria are met, testing is considered satisfactory and samples are reported as undetected for extraneous CAV DNA.

5.4 If criteria #1, 3, and 4 are met, but criterion #2 is not met, testing is considered invalid. Invalid testing will be repeated and samples will be spiked with 10^4 copies/µl of pCAV_IAC prior to extraction. If PCR analysis shows a band at 826 bp, it is likely there was some inhibition of the PCR, but samples can be reported as undetected for CAV DNA. If no 826 bp band is observed, the template is severely inhibiting the PCR reaction and the molecular team will be consulted to determine an alternate method for CAV DNA detection.

5.5 If criterion #1 is not met while criteria #2, 3, and 4 are met, testing is considered unsatisfactory. At supervisory discretion, unsatisfactory testing will be repeated until either two consecutive tests are satisfactory and negative for extraneous CAV or until two consecutive tests are unsatisfactory and positive for extraneous CAV.

6. 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.

7. References

7.1 DNeasy Blood & Tissue Mini Kit. Qiagen, Inc., Valencia, California

7.2 HotStarTaq® Plus Master Mix Kit. Qiagen, Inc., Valencia, California

7.3 Hill, R. (2011) Center for Veterinary Biologics Notice no. 11-01. United State Department of Agriculture, A.P.H.I.S. https://www.aphis.usda.gov/animal_health/vet_biologics/publications/notice_11_01.pdf

8. Summary of Revisions

Version CVB-PRO-0027.02

- Updated to the new MasterControl numbering and format.

Version VIRPRO0118.07

- Change of plasmid CAV DNA to plasmid internal amplification control CAV DNA.
- **3.4:** Replaced VIRSOP2041 with VIRWI2040 (SOP inactivated).
- **6:** CVBQMSOP0023 replaced by LABSOP0023.
- Added references.

Version VIRPRO0118.06

- Update of the PCR kit reagents used and thermocycling conditions.
- Primer sequences updated.
- Change of positive control from CAV DNA to plasmid CAV DNA.

Version VIRPRO0118.05

- The Contact information has been updated; however, the Virology Section has elected to keep the same next review date for the document.

Version VIRPRO0118.04

- The Contact information has been updated.
- **2.2.1:** Test worksheets have been updated.
- **2.2.3:** A new CAV DNA positive control has been added.

- **3.3.1:** The preparation, use, and storage of the new CAV DNA positive control have been added.

- **4.1/4.2:** These sections have been updated to reflect procedures in the laboratory that involve using an internal CAV DNA positive control before extraction of samples.

- **4.3:** This section has been updated to reflect current equipment and procedures used in the laboratory.

Version VIRPRO0118.03

- Contact information has been updated.

- **2.2.4:** "Six Paq" has been removed and the part number changed.

- **2.2.10:** This section has been updated to reflect current equipment and procedures used in the laboratory.

- **4.1.2/4.3.1:** The processes have been further clarified.

- **4.4.3:** The use of Kimwipes has been added.

- References to documents have been updated.

Version VIRPRO0118.02

- The Contacts have been changed from Scott Taylor and Karen Wineland to Sheridan Booher and Danielle Koski.

- Any mention of Reticuloendotheliosis Virus (REV) has been removed from this document. (See VIRPRO0119 for REV extraneous testing).

- **3.2:** The PCR program has been changed from "45 seconds at 47°C" to "45 seconds at 51.5°C".

- **4.2:** Further clarification of procedures has been added.
- **4.2:** The amounts of master mix components added for one reaction have been changed.

- **5:** Testing criteria have been added.