United States Department of Agriculture
Center for Veterinary Biologics
Testing Protocol

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

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Table of Contents

1. Introduction

This Testing Protocol (PRO) describes a polymerase chain reaction (PCR) assay for detection and identity of extraneous chicken anemia virus (CAV) in biologic products as specified in the guidelines of Veterinary Services Memorandum Number 800.89.

2. Materials

2.1 Equipment/instrumentation

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

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

Note: Use good laboratory practices to perform the DNA template preparation of the positive controls in an area not related to the testing of other poultry viruses.

2.1.2 Thermocycler (Applied Biosystems, GeneAmp PCR System 9700 or Veriti 96-well thermocycler)

2.1.3 Thermo EC Electrophoretic gel system

2.1.4 Eppendorf Adjustable Volume Research micro-pipettes, 2.5-µL, 10-µL, 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.

2.1.5 Pipette-aid automatic pipettor (Drummond Scientific)

2.1.6 Microcentrifuge (Eppendorf 5415 C)

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

2.2 Reagents/supplies

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

2.2.1 Current versions of the following Virology Testing Worksheets: **VIRTWS1061**, **VIRTWS0118** and **VIRTWS0218**.

2.2.2 DNeasy Blood and Tissue Kit (Qiagen, Catalog #: 6504)

2.2.3 CAV DNA positive control (prepared according to **VIRNPP0001**)

2.2.4 AmpliTaq Gold with GeneAmp, containing AmpliTaq Gold, 10x PCR Gold Buffer, and $MgCl_2$ (Applied Biosystems, Part #: 4311806)

2.2.5 GeneAmp dNTP Mix with dTTPs 10mM (2.5mM each) (Applied Biosystems, Product #: N8080260)

2.2.6 CAV primers, CAV-1 and CAV-2 at approximately 50 pmol/µL each

Reconstitution of the primers is determined by the synthesis data sheet. The primer batch yield is recorded on the sheet. Convert the nmol amount to pmol by multiplying the amount in nmols by 1000 and dividing the result by 50 to determine amount in microliters of water (PCR grade) to add for a final concentration of 50 pmol per 1 µL.

Primer Sequences:

CAV-1, 5'-CTAAGATCTGCAACTGCGGA-3'

CAV-2, 5'-CCTTGGAAGCGGATAGTCAT-3'

2.2.7 PBS for HI (National Centers for Animal Health (NCAH), Media #30102)

2.2.8 Ethanol 200 proof molecular grade (Sigma-Aldrich, Product #: E7023)

2.2.9 Agarose 3:1 High Resolution Blend (Amresco, Catalog #: E776) which is optimized for molecular weights 100-1000 bp

2.2.10 PCRSizer 100 base pair (bp) ladder (Norgen, Catalog #: 11400)

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

2.2.12 Tris Borate EDTA (TBE), 10X (NCAH, Media #30387)

2.2.13 Ethidium Bromide (EtBr), 10mg/mL (Invitrogen, Catalog #: 15585-011)

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.

2.2.14 PCR reaction tubes (Applied Biosystems, MicroAmp, Part #: N8010580)

2.2.15 PCR reaction tube caps (Applied Biosystems, MicroAmp, Part #: N8010535)

2.2.16 96-well PCR reaction tube tray/retainer (Applied Biosystems, MicroAmp, Part #: N8015530)

2.2.17 96-well PCR reaction tube base (Applied Biosystems, MicroAmp, Part #: N8015531)

2.2.18 Cap Installing Tool (Applied Biosystems, Part #: N8010438)

2.2.19 Plastic 96-well Microtiter Plate (Dynatech Laboratories, Catalog #: 001-010-2801)

2.2.20 Aerosol Resistant Filter tips for micropipettes, various sizes

2.2.21 Screw Cap Micro Tubes (Sarstedt, Catalog #: 72.694.006)

2.2.22 DNase/RNase Free water appropriate for use in PCR master mix

2.2.23 Latex, vinyl, or nitrile powder free disposable gloves

2.2.24 1.5-mL centrifuge tubes

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 (see thermocycler manual) with the following steps:

Amplification of CAV DNA

Pre-PCR Heat Start Hold: 5 minutes at 95°C

Cycles 1 - 35: 30 seconds at 95°C
45 seconds at 51.5°C
1 minute at 72°C, with a 1 second increase following each cycle
Post-PCR Hold: 10 minutes at 72°C
Soak/Storage: 4°C

3.3 Preparation of reagents/control procedures

3.3.1 The CAV positive control is Qiagen spin column purified on a separate occasion to avoid contamination. See **Section 4.1** for DNA extraction process. CAV DNA is then amplified and diluted according to **VIRNPP0001**.

3.3.2 Prepare 1X TBE from 10X TBE using distilled or deionized water. If using as gel buffer, add 0.2 µL Ethidium Bromide to 1 mL 1X TBE.

3.4 Storage of the sample

Obtain and store samples for testing as described in the current version of **VIRSOP2041.**

4. Performance of the Test

4.1 DNA extraction

It is recommended that the sample preparation with the positive CAV control be prepared in a separate area than the amplification step to avoid cross contamination. Wear disposable, powder free vinyl, nitrile, or latex gloves for all portions of the test procedure. If necessary, an alternative extraction method may be used.

4.1.1 Thaw test sample(s) if needed. Rebottle if necessary or rehydrate sample if needed with an appropriate amount of Super Q water. Record sample information on the current version of **VIRTWS1061**.

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 or has a large amount of cells in it, transfer 100 µL sample and add 100 µL PBS.

Note: If the sample being tested is whole tissue, take approximately 1 gram of tissue, push through 5-mL syringe without a needle, and then mix with 400 µL of PBS. 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 PBS. This method is recommended for chick embryo tissue.

4.1.3 For an internal positive control, place 100 µL of sample in a different 1.5-mL centrifuge tube. To that tube, add 100 µL of diluted CAV DNA control for a total volume should equal 200 µL.

Note: If the sample being tested is whole tissue, take approximately 1 gram of tissue, push through 5-mL syringe without a needle, and then mix with 400 µL of PBS. 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 uL of CAV DNA control. This method is recommended for bursa and chick embryo tissue.

4.1.4 From this point, follow the protocol that is provided in the Qiagen DNeasy Blood and Tissue Kit, starting with the addition of 20 µL of proteinase K to the sample, and continue from there.

4.1.5 Once the Qiagen purification and extraction is completed, store the tubes at -20°C until ready to continue to **Section 4.2.**

4.2 Amplification of CAV DNA

4.2.1 See the current version of **VIRTWS0118** for the master mix worksheet**.** Prepare master mix for DNA amplification of CAV as follows:

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

Example:

See **Section 4.2.2** for sample tray layout. In the sample tray layout, each sample would have one reaction with just sample and one reaction of sample with CAV DNA control extracted with it as an internal positive control. For three samples, that would give a total number of six reactions. There is also one tube for an external positive control and one tube for a negative control giving another two reactions. The total number of reactions then is eight, plus one extra reaction. The amount of each ingredient listed below would then be multiplied by nine, which will give the amount of each component required in the master mix.

2. Add the master mix ingredients in the order listed in the following section (amounts are for 1 reaction) in the clean master mix area.

Note: Add Taq polymerase last to the master mix

Master Mix

Item	µL	µL
DNase/RNase free water	57.5	23.5
10X buffer	10	5
dNTPs	10	5
$MgCl_2$	8	4
primer cav-1 50 pmol/µL	2	1
primer cav-2 50 pmol/µL	2	1
Taq Polymerase	0.5	0.5
Total Master Mix	90	40
Sample	10	10
Total Volume	100	50*

*The Veriti thermocycler has a 50 µL reaction volume.

4.2.2 Insert PCR reaction tube strips into two rows on the tray/retainer assembly. Make sure to include tubes for the negative control and external positive control. Snap top of assembly to secure strips. Place assembly on base.

4.2.3 Transfer 40 or 90 µL of the master mix to each reaction tube either in the clean hood where the master mix was made, or, before any DNA template is opened, in the DNA/RNA template (dirty) hood.

4.2.4 In the template BSC, add 10 µL of sample template to a PCR reaction tube. Repeat this process for each sample, including those samples that were spiked before extraction with CAV DNA. Change pipette tips between each sample. In the second to last tube, add 10 µL of water. In the last tube, add 10 µL of CAV DNA control.

4.2.5 Cap all the tubes. If necessary, use a capping instrument.

4.2.6 Record the tray set up in the PCR tray layout area on the second page of **VIRTWS0118**. The total reaction volume will be 100 µL or 50 µL.

4.2.7 Place the tube holder in the thermocycler and close the heated cover.

4.2.8 Run the CAV amplification program (see **Section 3.2**). Make sure the reaction volume is set at 100 µL for the 9700 thermocycler and 50 µL for the Veriti thermocycler.

4.2.9 Once the program is completed, store the tubes at 4°C until ready to analyze PCR product(s).

4.3 Analysis of amplified CAV DNA

Wear disposable, powder-free nitrile, vinyl, or latex gloves for all portions of analysis.

4.3.1 Loading gel

See the current version of **VIRTWS0118** for the gel worksheet.

1. Make sure to set up the electrophoresis apparatus before heating the gel, including setting up the tray and adding the comb. Make sure the apparatus is level and the comb is not hitting the bottom of the tray. Comb sizes range from 8-12 wells for the small gel and 22-40 wells for the large gel. The volume of the wells ranges from 10 µL to 20 µL.

2. The gel used is a 2% 3:1 agarose gel. Gel size is determined by the number of lanes needed for the batch. Weigh agarose for appropriate gel size (example: 0.7 g per 35 mL - small gel, 2 g per 100 mL – large gel). The 1X TBE buffer **without EtBr** is used as a diluent for the 2% gel. EtBr is not added until after heating to avoid dissipation of the EtBr fumes. Combine buffer and powdered agarose in either a screw top glass flask or bottle. Make sure to leave the lid loose. Heat the solution in the microwave until the liquid is clear (approx. 2 to 2 1/2 minutes, depending on the microwave). The agarose is cooled to the point that the glassware can be handled, but the agarose is still fluid. Add the EtBr at this point in the quantity of 0.2 µL EtBr per 1 mL agarose. The agarose is poured onto the gel tray and allowed to solidify.

3. To the solidified gel, add the 1X TBE with EtBr as tank buffer, filling the gel box until the gel is covered.

Note: An E-gel (Invitrogen, Catalog #G5018-02), which is a sealed gel that does not use buffer and runs on a dry electrophoresis platform, can be substituted for the submarine gel. The E-gel has the ethidium bromide already added to it, and then is sealed, so there is less EtBr to handle. After use, the E-gel is simply disposed of in the EtBr solid waste container. To operate, follow instructions that are stored with the E-gels.

4. Using a filtered micropipette tip, mix the reaction product (from **Section 4.2**) with 10X BlueJuice or equivalent loading buffer at a ratio of 10:1 (reaction product to BlueJuice) in a sterile tube or 96-well microtiter plate. For example, a well that would contain 20 µL would have 18 µL of reaction product mixed with 2 µL of loading buffer. Mix by pipetting up and down. Repeat step for all samples. Make sure to change tips for each sample.

5. Pipette all of the loading buffer/product mixture into the respective gel well for that sample, reserving the first (and last lane if more than 6 samples) for the 100 bp ladder. Load all samples, making sure to change tips for each sample. Record the order that the samples are loaded on the current version of the gel lane area on **VIRTWS0118**.

6. Pipette the 100 bp ladder to the first lane of the gel and to the last lane of gel if needed. Record the addition of the ladder on the lane worksheet. The ladder is added last because some ladders have a tendency to float out of the well. Normally the volume of ladder added is the established volume of the well, but if the ladder does float easily, a volume of ladder less than that of the volume of the well may be used. For instance, for a gel with a 20 µL well volume, 10 or 15 µL of ladder may be added if floating is a concern.

4.3.2 Running gel

1. Replace the electrophoresis apparatus cover, matching black and red connectors. Attach electrodes with the appropriate positive (red) and negative (black) leads to the power supply. Verify that the entire apparatus is level. The current runs negative to positive from the top of gel.

2. Set the power supply to run at an appropriate voltage. This varies with the type of gel box used and the concentration of the gel. What is usually used is 100 volts for 60 to 80 minutes for a 2% TBE gel.

3. Power up the unit and apply current to the gel. Observe that the apparatus is operating correctly by the formation of bubbles along the black electrode end of the apparatus. If bubbles do not form, the electrophoresis apparatus is not working right. Make sure the black electrode is at the top, that all connections are connected properly, and that the gel box is on and is working properly. Allow the current to run until dye has migrated close to the bottom edge of the gel. **Do not let the dye run off the gel.**

4. Turn the power supply off.

4.3.3 Visualizing and documenting gel

1. Carefully disassemble the gel apparatus. Refer to **2.2.13** for safe handling of gels containing EtBr. Lift the tray holding the gel out of the apparatus.

2. Place the tray in a shallow container or on several sheets of Kimwipes if not moving the tray very far, and transport gel to UV light box.

3. View and photograph gel according to available programs. The current program for recording pictures is the Kodak Gel Logic 2200 imaging system and the analysis program is Kodak Molecular Imaging Software version 4.0.4 or current version image analysis software. Attach a photograph or electronic picture to the current version of **VIRTWS0218** and retain the original copy with the batch records. After photographing, dispose of gel in a container designated for solid EtBr waste. Empty the gel buffer containing EtBr into a designated container. **DO NOT pour EtBr buffer down the sink.** After disposing of the buffer, rinse the gel box and associated parts (gel tray, comb, lid, etc.) with tap water and allow to air dry.

5. Interpretation of the Test Results

Compare any visible bands to the standard ladders. CAV has a product size of 419 bp when compared to the bands on the 100 bp ladder.

Test criteria are:

1. Internal CAV positive controls have visible bands at 419 base pairs.
2. Sample only lanes having no visible bands at 419 bp.
3. External negative control has no visible band at 419 bp.
4. External positive control has a visible band at 419 bp.

If all four criteria are met, testing is considered satisfactory and samples are reported as negative for extraneous CAV. If criteria #1 or #3 are not met, testing is considered invalid. If criterion #4 is not met, testing may still be considered valid and satisfactory and negative for extraneous CAV. If criterion #2 is not met, testing is considered unsatisfactory.

Invalid testing will be repeated.

At supervisory discretion, unsatisfactory testing will be repeated until either 2 consecutive tests are satisfactory and negative for extraneous CAV or until 2 consecutive tests are unsatisfactory and positive for extraneous CAV.

6. Report of Test Results

Report results on report out worksheet **VIRTWS0218.** Results are reviewed and entered into proper computer databases according to the current version of **VIRSOP0027**.

7. Summary of Revisions

Version .05

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

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

- References to documents have been updated.

Version .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.