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United States Department of Agriculture
Center for Veterinary Biologics

Production Protocol

Production of Chicken Anemia Virus (CAV) DNA Positive Control

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INTERNAL USE ONLY

Table of Contents

1. Introduction

This Production Protocol (PP) describes the production of the CAV DNA control used for CAV extraneous agent testing using PCR analysis.

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 Cabinets (BSCs) (NuAire Inc., Labgard)

Note: Use good laboratory practices to perform the DNA template preparation of the CAV positive control in an area not related to the testing of other poultry viruses. Also, BSCs should be designated as "Clean" and "Dirty." The clean BSC is only used to prepare master mix. DNA should only be worked with in the dirty BSC.

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 Gel imaging system (Carestream, Gel Logic 2200)

2.1.8 Micro-spectrophotometer (Thermo, Nanodrop 2000)

2.1.9 Automated 250-µL 12 channel pipette (Matrix, Catalog # 2012)

2.1.10 8-channel screw cap decapper (Thermo, Catalog # 4105MAT)

2.1.11 500µL screw top tubes and rack (Matrix, Catalog # 3744)

2.1.12 Qiacube Instrument (Qiagen, Catolog # 9001292) – optional, can be used for extraction.

2.2 Reagents/supplies

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

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

2.2.2 CAV virus, Del-Ross strain, Lot 9-2-88, harvested and diluted 1:100

2.2.3 HotStar Taq Master Mix Kit (Qiagen, Catalog # 203443)

Note: Although a different brand name of Taq polymerase can be used, it does need to be a hot start enzyme.

2.2.4 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'

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

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

2.2.7 2% E-gel with ethidium bromide (EtBr) (Invitrogen, 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.

2.2.8 Screw cap glass bottle or flask

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

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

2.2.11 96-well PCR reaction tube tray/retainer (Applied Biosystems, MicroAmp, Part # N8015530)

2.2.12 96-well PCR reaction tube base (Applied Biosystems, MicroAmp, Part # N8015531)

2.2.13 Cap Installing Tool (Applied Biosystems, Part # N8010438)

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

2.2.15 Aerosol resistant filter tips for micropipettes, various sizes

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

2.2.17 Latex, vinyl, or nitrile powder free disposable gloves

2.2.18 1.5-mL centrifuge tubes

2.2.19 Kimwipes

2.2.20 QIAquick PCR Purification Kit (Qiagen, Catalog # 28104)

2.2.21 200-µL snap-cap microcentrifuge tubes

2.2.22 Liquid reservoir (Matrix, Catalog # 8086)

3. Preparation for Production

3.1 Personnel qualifications/training

Personnel must have experience or training in this protocol. This includes knowledge of aseptic biological laboratory techniques, including the preparation, 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

4. Production of Reagent

4.1 DNA extraction

Wear disposable, powder free vinyl, nitrile, or latex gloves for all portions of the test procedure. Perform the extraction in a dirty BSC.

4.1.1 Thaw CAV virus suspension.

4.1.2 Transfer 200 µL of virus suspension to a 1.5-mL centrifuge tube.

Note: At this point, the Qiacube Instrument (Qiagen, Catolog # 9001292) can be used for extraction using the program "CVB DNA Blood and Tissue."

4.1.3 Continue extraction following 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. The Qiagen Kit will use the heating block or water bath, the microcentrifuge, and the 200 proof ethanol.

4.1.4 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 In a clean BSC or area, prepare master mix for amplification of CAV DNA as follows:

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

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

Item	µL
HotStar Taq Master Mix 2x	25.0
Nuclease Free Water	11.0
Coral Load 10x	2.0
Primer cav-1 50 pmol/µL	1
Primer cav-2 50 pmol/µL	1
Sample	10
Total Volume	50

Note: For producing CAV DNA reagent, running multiple repeats is needed. For the current lot of CAV DNA control, 20 repeated samples were amplified by CAV PCR.

4.2.2 Insert PCR reaction tube strips into two rows on the tray/retainer assembly. Snap top of assembly to secure strips. Place assembly on base.

4.2.3 Transfer 40 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) BSC.

4.2.4 In the template BSC, add 10 µL of sample template to a PCR reaction tube. The total reaction volume will be 50 µL.

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

4.2.6 Place the tube tray in the thermocycler and close the heated cover.

4.2.7 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.

4.2.8 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 **CVB-TWS-0124**, *HotStarTaq Plus – PCR Assay*, for the gel worksheet.

1. Connect power base for E-gel to electrical plug.

2. The gel used is a 2% pre-cast agarose gel with 12 wells that hold 20 µL per well. Remove gel cassette from packaging.

3. Insert gel cassette into base, making sure the gel cassette is pushed all the way in and the light at the top turns on.

4. Using a filtered micropipette tip, load 20 µL of the reaction product (from **Section 4.2**) into the 2nd well. Repeat for remaining wells. Leave the first well and last well empty. These are loaded with ladder.

Note: Samples can be pooled, so twenty PCR samples can be pooled into two microcentrifuge tubes, and the pooled samples from those two tubes the DNA can by analyzed on the E-gel.

5. Record the order that the samples are loaded on the current version of the gel lane area on **CVB-TWS-0124**.

6. Pipette the 100 bp ladder into the first lane of the gel and the last lane of gel, if needed. Record the addition of the ladder on the lane worksheet.

4.3.2 Running gel

1. Press the button for the 30 minute run. Make sure the light turns green.

2. When the run is over, the light will be red and blinking and the apparatus will beep. Unplug the E-gel base and remove gel cassette.

4.3.3 Visualizing and documenting gel

1. Place gel in UV light box.

2. 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, *PCR Report Out Worksheet*, and retain the original copy with the batch records. After photographing, dispose of gel in a container designated for solid EtBr waste.

4.4 PCR purification

4.4.1 Using the microcentrifuge instructions for the QIAquick PCR purification kit, start with adding 5 volumes of buffer "PB" to 1 volume of PCR product. Continue through to elution following the kit instructions.

Note: DNA that was pooled after the PCR run should be run in multiple tubes, using no more then 100-200 µL of DNA per reaction tube.

4.4.2 The purified DNA may be visualized using a gel to make sure DNA is still intact. Follow steps from **Section 4.3.**

4.4.3 The DNA concentration is measured using a Nanodrop 2000. From the DNA concentration, the total number of copies can be determined.

Note: Purified DNA is pooled again before Nanodrop reading.

4.4.4 After PCR purification, the DNA is stored at -20°C.

5. Preparation and Packaging of the Product

5.1 Determination of dilution of DNA to use for reagent

5.1.1 CAV DNA is tenfold serially diluted using nuclease free water.

5.1.2 Following the steps in **Section 4.2**, each dilution of CAV DNA is amplified.

5.1.3 Following the steps in **Section 4.3**, the dilutions of CAV DNA are visualized using a gel. Based on the results of the gel analysis, a dilution is picked that will work as the dilution for the final reagent. Ideally, this value should be at the dilution that is one or two folds more concentrated then the dilution with the last visible band on the gel.

5.2 Bottling and filling

The volumes given in this section are for a concentration of 1 x 10^5 copies/µL based off of the dilutions made in **Section 5.1**.

5.2.1 500-µL screw cap Matrix tubes are labeled and placed in trays.

5.2.2 300 µL of 1 x 10^{11} copies/µL dilution is transferred to 29.7 mL of nuclease free water, resulting in a final concentration of 1 x 10^5 copies/µL.

5.2.3 The final reagent solution of 1 x 10^5 copies/µL is transferred to a liquid reservoir.

5.2.4 Tubes are uncapped using a Thermo automated decapper.

5.2.5 Using a 12-channel automated pipette, 100 µL of the reagent was transferred to each labeled tube. Tubes are then capped using the automated decapper.

5.2.6 After filling and capping, the labeled tubes are stored at -20ºC.

6. Testing

6.1 Sequencing

The CAV DNA standard is tested for sequence identity to Chicken Anemia Virus.

6.2 Final reagent inhibition study

The final reagent, after diluting and bottling, is tested using steps from **Sections 4.2 and 4.3** and following **CVB-PRO-0032**, *DNA PCR Assay using Qiagen HotStar Taq Polymerase Kit,* to make sure the product is inhibited by tissue samples, including chicken embryo fibroblasts, ground chicken bursa, chicken embryo, chicken liver and kidney. (Chicken bursa has shown to partially inhibit the amplification of CAV DNA.)