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**United States Department of Agriculture
Center for Veterinary Biologics**

Testing Protocol

**Testing Protocol for Enzyme Linked Immunsorbent Assay (ELISA)
for Extraneous Avian Leukosis Virus (ALV)**

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1. Introduction

This Testing Protocol (PRO) describes a procedure for using a commercial antigen capture enzyme-linked immunosorbent assay (ELISA) kit to test for the presence of extraneous avian leukosis virus (ALV) p27 antigen in biologic products. ALV is also known as avian lymphoid leukosis. This is an acceptable Animal Plant Health Inspection Service (APHIS) approved alternative to the complement-fixation (CF) test described in title 9, *Code of Federal Regulations* (9 CFR), section 113.31.

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)

2.1.2 Micropipette, 12-channel 850 µL volume (Impact 2, Matrix)

2.1.3 Centrifuge (Beckman-Coulter J6-M1 with JS-4.2 rotor)

2.1.4 Microplate reader (Versamax)

2.1.5 Hand-held miniwasher (Skatron)

2.1.6 Freezer -70°C ± 10°C (Revco)

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:
CVB-TWS-0087, *Chicken Embryo Fibroblast (CEF) Primary Cell Culture Record*;
CVB-TWS-0084, *Record of Testing for Extraneous Avian Leukosis Virus (ALV)*;
CVB-TWS-0096, *Avian Leukosis Virus ELISA Worksheet IDEXX Avian Leukosis Test Kit*; and CVB-TWS-0101, *Detection of Extraneous Avian Leukosis Virus (ALV): p-27 ELISA Results*.

2.2.2 FlockChek Avian Leukosis Virus Antigen Test Kit, ALV-Ag (IDEXX Laboratories, Inc.)

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2.2.3 Super Q Water (National Centers for Animal Health (NCAH) Media #10206)

2.2.4 Powder-free latex or nitrile gloves

3. Preparation for the Test

3.1 Personnel qualifications/training

Personnel must have experience or training in this procedure. This includes knowledge of aseptic biological laboratory techniques and preparation, proper handling and disposal of biological agents, reagents, tissue culture samples, and chemicals. Personnel must also have knowledge of safe operation procedures, policies, and guidelines of the Center for Veterinary Biologics (CVB) and training in the operation of the necessary laboratory equipment listed in **Section 2.1**.

3.2 Preparation of equipment/instrumentation

Operate all equipment/instrumentation according to manufacturer's instructions and monitor in compliance with current corresponding standard operating policies/procedures (SOPs). Maintain aseptic conditions while working in a biological safety cabinet.

3.3 Preparation of reagents

The 20X wash solution should be at room temperature and mixed thoroughly to eliminate any precipitate before the 1X solution is made. 1X wash solution is prepared by mixing 20 mL of the concentrated 20X wash solution from the IDEXX kit with 380 mL of Super Q water.

4. Positive Control, Negative Control, and Sample Preparation

4.1 Positive (inoculated with ALV) and negative (uninoculated) chicken embryo fibroblast (CEF) cell culture controls and test samples are harvested in a biological safety cabinet and then frozen at $-70^{\circ}\text{C} \pm 10^{\circ}\text{C}$ according to current versions of CVB-SOP-0116, *Instructions for First and Second Harvests and Transfers of Chick Embryo Fibroblasts used in the COFAL and ALV Antigen Capture p27 ELISA Tests*; CVB-SOP-0117, *Instructions for Final Harvest for the COFAL and ALV p27 Antigen Capture ELISA Tests*; and CVB-PRO-0036, *Detecting Extraneous Avian Leukosis Virus in Biologic Products by p27 ELISA*. CEF preparation and harvest records are recorded on the current version of CVB-TWS-0087.

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4.2 All test samples and controls, are removed from $-70^{\circ}\text{C} \pm 10^{\circ}\text{C}$ freezer and thawed. The samples are then refrozen and thawed two more times in a $-70^{\circ}\text{C} \pm 10^{\circ}\text{C}$ freezer. After the test samples and controls are thawed for the final time, they are centrifuged at approximately 1200 X g (2400 rpm in JS 4.2 rotor) for 20 minutes and the cell culture lysate is collected.

5. IDEXX ELISA Test Kit Procedures

Note: Test instructions can be found in the insert that is enclosed in the IDEXX FlockChek Avian Leukosis Virus Antigen Test Kit. The procedure followed in this protocol includes deviations from the insert based on the albumin wash protocol and accommodates testing of cell culture lysate.

5.1 All kit components should be brought to room temperature prior to use. A p27 antibody coated microtiter plate is obtained from the test kit. Sample positions are recorded on the current version of CVB-TWS-0096. In wells A1 and A2, 100 μL of negative control **from the kit** is added. In wells A3 and A4, 100 μL of positive control **from the kit** is added.

5.2 One hundred μL of sample is added to the appropriate well in relation to CVB-TWS-0096. Each sample is run in duplicate.

5.3 After all samples have been added to the appropriate wells (including ALV positive and negative cell culture controls), incubate the plate for 60 ± 5 minutes at room temperature ($20^{\circ}\text{C} - 27^{\circ}\text{C}$) while keeping out of direct sunlight.

5.4 Aspirate samples from wells, using the hand-held miniwasher. Add approximately 350 μL 1X wash solution, using the miniwasher. Let the wash soak in the wells for 2 minutes. After the 2-minute soak, aspirate the wash from the wells using the miniwasher. Repeat addition and aspiration of the wash five more times without the 2-minute soak.

5.5 Add 100 μL of (Rabbit) Anti-p27: Horseradish Peroxidase (HRPO) conjugate (provided in the kit) to each well. Incubate the plate for 60 ± 5 minutes at room temperature ($20^{\circ}\text{C} - 27^{\circ}\text{C}$) while keeping out of direct sunlight. Repeat aspiration and wash steps from **Section 5.4**.

5.6 Add 100 μL of 3, 3'-5, 5' Tetramethylbenzidine (TMB) substrate (provided in the kit) to each well. Incubate for 15 minutes at room temperature ($20^{\circ}\text{C} - 27^{\circ}\text{C}$) while keeping out of direct sunlight. After the incubation, dispense 100 μL of Stop Solution (provided in the kit) to each well. **DO NOT ASPIRATE BEFORE ADDING THE STOP SOLUTION.**

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5.7 Using the microplate reader, measure and record the absorbance of the wells at 650 nm.

6. Results and Validity

6.1 The absorbance measurements should be recorded on the current version of CVB-TWS-0101. The readings from the kit positive control wells and the readings from the kit negative control wells are averaged. The average absorbance of the kit negative control should be less than or equal to 0.150. The readings from the cell negative control wells **for each passage** are also averaged.

6.2 For the **ELISA** to be valid, the difference between the average absorbance of the **kit positive control** and the average absorbance of the **kit negative control** should be greater than 0.200. For the **Test** to be valid, the difference between the **kit positive control** and the **cell culture negative control** for each passage should be greater than 0.200. Both the ELISA and the Test must be valid to evaluate test sample results.

6.3 To determine the relative antigen level in the samples, the sample to positive (S/P) ratio needs to be calculated. This formula is part of CVB-TWS-0101. Cell culture lysate is not listed as a sample type on the test insert. The following formula compensates for possible “background” in the cell culture lysate test samples and controls. The calculation is as follows:

$$S/P = \frac{(\text{Sample Mean} - \text{Cell Culture Negative Control Mean})}{(\text{Kit Positive Control Mean} - \text{Cell Culture Negative Control Mean})}$$

Note: For each cell culture passage, the negative control mean subtracted must be from the same passage level or harvest as the sample mean used for calculating the S/P ratio. For example, subtract 1st passage cell culture negative mean from 1st passage sample mean, subtract 2nd passage cell culture negative mean from 2nd passage sample mean, etc.

6.4 ALV cell culture positive controls must have an S/P ratio greater than 0.200 for the third and final cell culture passage. If the S/P value is less than 0.200, the Test is invalid, and the original samples will be retested in cell culture following current version of CVB-PRO-0036.

6.5 The sample is negative for ALV p27 antigen if the S/P ratio is less than 0.200. The sample is considered positive for p27 antigen if the S/P ratio is greater than 0.200. A positive sample may be retested at the discretion of the supervisor.

6.6 Record ELISA results on current version of CVB-TWS-0084. Results are reviewed by agent contact or designee and reported according to current version of

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CVB-SOP-0121, *Testing Roles, Responsibilities, and Procedures for Reporting Test Results in the Virology Section.*

7. References

Avian Leukosis Virus Antigen Test Kit, ALV-Ag, Test Kit Insert, IDEXX Laboratories, Inc.