

## Enzyme Linked Immunosorbent Assay (ELISA) Laboratory

ELISA is a test that can be used to detect either antibody (Ab) or antigen such as viral proteins. There are numerous methods for developing ELISA tests.

Commercial ELISA test kits are available to detect:

- Avian influenza virus antibody in chicken serum
- Newcastle disease virus antibody in chicken serum
- Newcastle disease virus antibody in turkey serum

These commercial ELISAs are available from two companies, IDEXX Inc., and Synbiotics. In this Lab, students will be able to run one ELISA test from either IDEXX or Synbiotics.

Although the test formats and procedures are similar, there are differences. Therefore, be sure that you have the correct protocol and reagents for the correct kit.

### **Advantages and limitations of ELISA tests for AIV and NDV antibody**

- Fast—90 samples can be tested in 2-3 hours
- Good sensitivity
- Many samples can be processed at once—about 90 samples per plate and several plates may be processed at once.
- Very little sample volume is needed, <10 µl in most cases
- Quantitative—can measure the amount of the Ab present, good for vaccine response monitoring, but not highly precise
- Easy to learn, simple procedures
- May need specialized equipment: plate reader and washer
- Specificity varies by target; over all very good, but not great specificity
- Measures exposure and vaccine response, but not acute infection. For example, once a bird is ELISA Ab positive, the infection is probably over.
- Regulatory considerations

### **Materials needed**

- ELISA plate and kit reagents IDEXX or Synbiotics
  1. ELISA plate
  2. Positive control
  3. Negative control
  4. Dilution buffer
  5. Conjugate (secondary antibody)
  6. Substrate
  7. Stop Solution
  8. Wash Solution (Synbiotics only)
- Pipet and pipet tips
- Record Sheet
- Multi-channel pipet
- dilution tubes
- Wash bottle or mechanical washer
- Reader and computer
- Unknown test samples

The manufacturers protocol for each kit is in your lab notebook. A brief protocol for each kit follows:

**IDEXX brief protocol**

1. Fill out the record sheet to record where each sample will be. Run samples in duplicate
2. Label dilution tubes
3. Add 1 ml of diluent to dilution tubes
4. Add 2  $\mu$ l of test serum to a dilution tube
5. Do NOT dilute controls
6. Add negative control to plate: 0.1 ml to wells A1 and A2
7. Add positive control to plate: 0.1 ml to wells A3 and A4
8. Add 100  $\mu$ l of diluted test serum to the plate according to your record sheet
9. Incubate for 30 minutes
10. Wash with distilled water
11. Add 100  $\mu$ l of conjugate to all wells of your test plate
12. Incubate for 30 minutes
13. Wash with distilled water
14. Add 100  $\mu$ l of TMB substrate to each well
15. Incubate for 15 minutes
16. Add 100  $\mu$ l of stop solution to each well
17. Read results

**SYNBIOTICS brief protocol**

1. Fill out the record sheet to record where each sample will be. Run samples in duplicate
2. Prepare wash solution
3. Label dilution tubes
4. Add 0.3 ml diluent to each dilution tube
5. Add 6  $\mu$ l of test serum to a dilution tube
6. Dilute controls the same way as the test samples
7. Add 50  $\mu$ l of dilution buffer to each well of the ELISA plate
8. Add positive control: 50  $\mu$ l to wells A1, A3 and H11
9. Add negative control: 50  $\mu$ l to wells A2, H10, and H12
10. Add 50  $\mu$ l of diluted test serum to the plate according to your record sheet
11. Incubate for 30 minutes
12. Wash with wash solution
13. Dilute conjugate: Add 100  $\mu$ l of conjugate to 10 ml of dilution buffer and mix
14. Add 100  $\mu$ l of diluted conjugate to all wells of your test plate
15. Incubate for 30 minutes
16. Wash with wash solution
17. Add 100  $\mu$ l substrate to each well
18. Incubate for 15 minutes
19. Dilute stop solution: Add 2.5 ml stop solution to 10 ml of distilled water
20. Add 100  $\mu$ l of diluted stop solution to each well
21. Read results

### Results Interpretation

- Results should be recorded by reading the optical densities of the plates in a plate reader at the correct absorbance:

IDEXX: 650nm

Synbiotics: 405-410nm

Each manufacturer supplies computer software specific for their test which calculates which samples are negative and the titers of positive samples.

The status of a sample is evaluated by the sample to positive ratio (S/P ratio):

$$\text{S/P ratio} = \frac{\text{Sample mean (mean of optical absorbance)} - \text{negative control mean}}{\text{positive control mean} - \text{negative control mean}}$$

-IDEXX kit S/P ratios of greater than 0.5 are considered positive

-Synbiotics kit S/P ratios of greater than 0.299 are considered positive

Example:

Sample mean = 0.820

Negative control mean = 0.053

Positive control mean = 0.563

$$0.820 - 0.053 = 0.767 = 1.5 = \text{Positive}$$

$$\cancel{0.563 - 0.053 = 0.510}$$

Values are relatively quantitative: a higher value indicates more antibody.

### Valid ranges for the positive and negative controls for each kit

IDEXX

~~Negative control:~~ 0.150 or less

The difference between the positive and negative control means must be greater than 0.075

Example: if negative control = 0.100, the positive control must be 0.176 or greater

SYNBIOTICS

~~Negative control:~~ Less than 0.200

Positive control: 0.250 – 0.900

**USDA Avian Influenza Virus-Newcastle Disease Virus Diagnostic Workshop**  
**Iowa State University, Ames, Iowa**

**IDEXX ELISA Plate**

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>A</b>	NC-	NC-	PC+	PC+								
<b>B</b>												
<b>C</b>												
<b>D</b>												
<b>E</b>												
<b>F</b>												
<b>G</b>												
<b>H</b>												

**Synbiotics ELISA Plate**

	<b>1</b>	<b>2</b>	<b>3</b>	<b>4</b>	<b>5</b>	<b>6</b>	<b>7</b>	<b>8</b>	<b>9</b>	<b>10</b>	<b>11</b>	<b>12</b>
<b>A</b>	PC+	NC-	PC+									
<b>B</b>												
<b>C</b>												
<b>D</b>												
<b>E</b>												
<b>F</b>												
<b>G</b>												
<b>H</b>										NC-	PC+	NC-

# AVIAN INFLUENZA VIRUS ANTIBODY TEST KIT

ITEM NO. 96-6539



**ProFLOK®**

SYNBIOTICS CORPORATION

11011 VIA FRONTERA SAN DIEGO, CA 92127  
800 228 4305 (OR outside U.S./Canada 858 451 3771)

U.S. VET LIC NO. 312

## AVIAN INFLUENZA VIRUS ANTIBODY TEST KIT

### GENERAL INFORMATION AND INTENDED USES

Avian Influenza Virus (AIV), also known as Fowl Plague, is a viral disease of domestic and wild birds that is characterized by a full range of responses from almost no signs of the disease to very high mortality. The causal orthomyxoviruses are type A influenza viruses. There are 14 known serologically distinct subtypes based on surface hemagglutinins and 9 based on neuraminidases<sup>1</sup>. Subtypes H5 and H7 are associated with significant to catastrophic losses. Disease signs range from only a slight decrease in egg production to a highly fatal fulminating infection. Signs of infection may include respiratory problems, edema of the head and face and diarrhea. The most severe lesions are generally characterized as congestive and hemorrhagic<sup>2</sup>.

The ProFLOK® AIV ELISA Kit is a rapid and specific presumptive screening test for the detection of antibody to AIV in chicken serum samples. It was designed for screening large numbers of chicken sera from numerous flocks; however, additional conventional AIV serologic testing [i.e. agar gel precipitin (AGP), hemagglutination-inhibition (HI) test and neuraminidase-inhibition (NI)]<sup>1,4</sup> and virus isolation techniques are needed to confirm AIV negative and AIV-infected chicken flocks.

The assay is designed to measure AIV antibody bound to AIV antigen coated plates. The principle of the test is as follows: Serum obtained from chickens exposed to AIV antigens contains specific anti-AIV antibodies. Serum, diluted in Dilution Buffer, is added to an AIV antigen coated plate. Specific AIV antibody in the serum forms an antibody-antigen complex with the AIV antigen bound to the plate. After washing the plate, an affinity purified goat anti-chicken IgG (H+L) peroxidase conjugate is added to each well. The antibody-antigen complex remaining from the previous step binds with the conjugate. After a brief incubation period, the unbound conjugate is removed by a second wash step. Substrate, which contains a chromagen (ABTS), is added to each well. Chromagen color change (from clear to green-blue) occurs in the presence of the peroxidase enzyme. The relative intensity of color developed in 15 minutes (compared to controls) is directly proportional to the level of AIV antibody in the serum. After the substrate has incubated, Stop Solution is added to each well to terminate the reaction and the plate is read using an ELISA plate reader at 405-410 nm.

### REAGENTS REQUIRED TO PERFORM 90 TESTS

- 1 AIV antigen coated plate
- 10 µl AIV Positive Control Serum
- 10 µl Normal Control Serum
- 100 µl Goat anti-Chicken IgG (H+L) Peroxidase Conjugate Solution
- 40 ml Dilution Buffer
- 10 ml ABTS-Hydrogen Peroxide Substrate Solution
- 2.5 ml 5X Stop Solution, 5% SDS (dilute [1:5] with laboratory grade water)
- 20 ml 20X Wash Solution (dilute [1:20] with laboratory grade water)

**NOTE: Store all reagents provided in the kit at 2-7°C. Reagents should not be frozen.**

### EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

- High precision pipette (i.e. 1-20 microliter pipette)
- 0.2 ml, 1.0 ml and 5.0 ml pipettes
- 8 or 12 channel pipette (or transplating device) and pipette tips
- 2 graduated cylinders (50 ml)
- 1 ml or 5 ml borosilicate glass test tubes
- Uncoated low binding 96 well plates (i.e. Nunc catalog #269620)
- Laboratory grade (Distilled or R.O.) water
- 96 well plate reading spectrophotometer with 405-410 nm filter
- Plate washing apparatus
- Waste container with bleach or other oxidizing agent

### WARNINGS TO THE USERS OF REAGENTS AND AIV ANTIGEN COATED PLATES

- Handle all reagents and samples as biohazardous material.
- Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
- Wash solution, control sera, test plates, field samples and all other test kit reagents should be properly decontaminated with bleach or other strong oxidizing agent before disposal.
- Take special care not to contaminate any of the test reagents with serum or bacterial agents.
- Humidity indicators are supplied with each plate. If any of the indicators exhibit a pink color, the plate may be compromised in some way; decontaminate (i.e. wash the plate with bleach solution) and dispose of the plate.
- The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
- Do not use this kit after the expiration date.
- NEVER PIPETTE BY MOUTH.**

**ALLOW ALL REAGENTS TO COME TO  
ROOM TEMPERATURE BEFORE  
STARTING!**

## SAMPLE COLLECTION

For routine serologic flock monitoring, it is suggested that at least **30 or more sera per flock** be randomly collected at standard time intervals (i.e. every four weeks). Proper sample collection procedures, serum harvest and serum sample storage (4 °C for up to four days or -20 °C for longer periods) are needed to provide reliable test results. **To achieve better specificity and to minimize possible false positive reactions, serum samples that are contaminated with bacteria or are very fatty should be excluded from testing.**

## SAMPLE DILUTION PROCEDURE

Dilute serum samples using Dilution Buffer in a clean, uncoated 96 well microtiter plate. Frozen serum samples should be completely thawed and thoroughly mixed before diluting. Set up samples and controls as shown in Figure 1.

## PREPARATION OF THE SERUM DILUTION PLATE

- Add 300 µl Dilution Buffer to each well of an uncoated 96 well microtiter plate. This plate is referred to as the serum dilution plate.
- Add 6 µl unknown serum per well as per Figure 1 (producing a 1:50 dilution). Start with well A4 and end with well H9 (moving left to right, row by row of wells). For example, wells 1 through 30 contain the diluted sera of flock 1, wells 31-60 contain the diluted sera of flock 2, etc.
- Add 6 µl of Normal Control Serum (producing a 1:50 dilution) to wells A2, H10 and H12.
- Aspirate and remove any liquid in dilution plate wells A1, A3 and H11.
- Allow all diluted serums to equilibrate in Dilution Buffer for 5 minutes before transferring to an AIV antigen coated ELISA plate.
- Diluted serum should be tested within 24 hours.

This dilution format provides adequate quantities of diluted serum samples to conduct four additional ProFLOK® ELISA tests (i.e. IBD, IBV, ILT and REO) using the same serum dilution plate.

Figure 1.

A	+	-	+	1	2	2	4	5	6	7	8	9
B	10	11	12	13	14	15	16	17	18	19	20	21
C	22	23	24	25	26	27	28	29	30	31	32	33
D	34	35	36	37	38	39	40	41	42	43	44	45
E	46	47	48	49	50	51	52	53	54	55	56	57
F	58	59	60	61	62	63	64	65	66	67	68	69
G	70	71	72	73	74	75	76	77	78	79	80	81
H	82	83	84	85	86	87	88	89	90	-	+	-

## Preparation of AIV Positive Control

An AIV Positive Control Serum has been provided with this kit. Dilute the appropriate volume of AIV Positive Control Serum with Dilution Buffer (1:50) in a clean, glass test tube. For example, dilute 6 µl of positive control serum in 300 µl Dilution Buffer. **Mix well.** 150 µl of diluted AIV Positive Control is needed per ELISA plate.

## Preparation of Conjugate Solution

The horseradish peroxidase conjugated anti-chicken IgG (H+L) is supplied in HRP Stabilizer. Dilute 100 µl stock conjugate in 10 ml Dilution Buffer (1:100 dilution). **Mix well.** This 10 ml preparation will supply sufficient conjugate for one 96 well ELISA plate.

## Preparation of 1X Wash Solution

Dilute 20 ml concentrated Wash Solution in 380 ml laboratory grade (distilled or R.O.) water (1:20). **Mix well.** Approximately 400 ml Wash Solution is needed for each 96 well ELISA plate.

## Preparation of the Substrate Solution

The Substrate Solution is ready to use. Each plate will require approximately 10 ml substrate solution. **For best results, the substrate solution must be equilibrated to room temperature before use.**

## Preparation of 1X Stop Solution

Dilute 2.5 ml concentrated Stop Solution in 10 ml laboratory grade (distilled or R.O.) water (1:5). **Mix well.** Approximately 12.5 ml Stop Solution is needed for each 96 well ELISA plate.

**NOTE: Storage of 5X Stop Solution at refrigerated temperatures may cause the formation of a white solid. This does not affect product performance. Warm at room temperature or 37 °C to dissolve before use.**

## ELISA TEST PROCEDURE

### PREPARING THE TEST PLATE

- Remove an AIV antigen coated test plate from the protective bag and label according to serum dilution plate identification.
- Add 50 µl Dilution Buffer to all wells on the test plate.
- Add 50 µl diluted AIV Positive Control Serum to wells A1, A3 and H11. Discard pipette tip.
- Using an 8 or 12 channel pipette transfer 50 µl/well of each of the diluted serum samples and Normal Control Serum samples from the serum dilution plate to the corresponding wells of the AIV coated test plate (yields a 1:100 dilution). Discard pipette tips after each row of sample is transferred. Transfer of samples to the ELISA plate should be done as quickly as possible.
- Incubate plate for 30 minutes at room temperature.

### WASH PROCEDURE

- Tap out liquid from each well into an appropriate vessel containing bleach or other decontamination agent.
- Using an 8 or 12 channel pipette (or comparable automatic washing device), fill each well with approximately 300 µl Wash Solution. **Allow to soak in wells for 3 minutes;** then discard contents into an appropriate waste container (waste container should contain bleach solution). Tap inverted plate to ensure that all residual liquid is removed. **Repeat wash procedure 2 more times.**

**NOTE: The wash procedure is a very critical step in any ELISA procedure. Please follow the above steps as directed.**

### ADDITION OF ANTI-CHICKEN IgG PEROXIDASE CONJUGATE, SUBSTRATE AND STOP SOLUTION

- Using an 8 or 12 channel pipette (or transplating device) dispense 100 µl diluted conjugate (prepared as described above) into each assay well. Discard pipette tips.
- Incubate for 30 minutes at room temperature.
- WASH** as in steps f and g above.
- Using an 8 or 12 channel pipette (or transplating device) dispense 100 µl Substrate Solution into each test well. Discard pipette tips.
- Incubate 15 minutes at room temperature.
- Using an 8 or 12 channel pipette (or transplating device) add 100 µl diluted Stop Solution (prepared as described above) to each test well.
- Allow bubbles to dissipate before reading plate.

### MANUAL PROCESSING OF DATA

- Read the plate using an ELISA plate reader set at 405-410 nm. Be sure to blank the reader as directed.

- Calculate the average Positive Control Serum absorbance (Optical Density [O.D.]) using the absorbance values of wells A1, A3 and H11. Calculate the average Normal Control Serum absorbance using values obtained from wells A2, H10 and H12. Record both averages.
- Subtract the average normal control absorbance from the average positive absorbance. The difference is the Corrected Positive Control.
- Calculate a sample to positive (Sp) ratio by subtracting the average normal control absorbance from each sample absorbance. The difference is divided by the corrected positive control. Use the following equation format:

$$Sp = \frac{(\text{SAMPLE ABSORBANCE}) - (\text{AVERAGE NORMAL CONTROL ABSORBANCE})}{\text{CORRECTED POSITIVE CONTROL ABSORBANCE}}$$

- An AIV ELISA titer can be calculated by the following suggested equation:  
 $\text{LOG}_{10} \text{ TITER} = (1.464 \times \text{LOG}_{10} Sp) + 3.197$   
 $\text{TITER} = \text{ANTILOG OF LOG}_{10} \text{ TITER}$

Example:

- Example Positive Control Absorbance:  
0.585, 0.610, 0.590  
Average =  $(0.585 + 0.610 + 0.590) / 3 = 0.595$
- Example Normal Controls:  
0.078, 0.067, 0.057  
Average =  $(0.078 + 0.067 + 0.057) / 3 = 0.067$
- Corrected Positive Control:  
 $(0.595) - (0.067) = 0.528$
- Example Sp value calculation:  
Absorbance of sample = 0.560  
 $(0.560) - (0.067) / 0.528 = 0.934$
- Example of Calculation of titer using the Sp from above:  
 $\text{Log}_{10} \text{ Titer} = 1.464 \times (\text{Log}_{10} 0.934) + 3.197$   
Titer = ANTILOG 3.15  
Titer = 1413

## RESULTS

### Assay Control Values:

Valid AIV ELISA results are obtained when the average optical density (O.D.) value of the Normal Control Serum is less than 0.200 and the Corrected Positive Control value range is between 0.250 and 0.900. If either of these values are out of range, the AIV test results should be considered invalid and the samples should be retested. Samples testing with an Sp value of less than or equal to 0.299 will receive a 0 titer value and are considered non-reactive for AIV antibody.

Under optimal conditions\* the suggested O.D. value ranges of **0.050 to 0.095 for AIV Normal Control Serum** and **0.400 to 1.00 for AIV Positive Control Serum** should be strived for to ensure the most consistent laboratory test results. Please note that tests with O.D. values which do not fall within the suggested O.D. ranges above does not constitute an invalid test.

\*Optimal conditions are at room temperature [70 to 75 °F (21 to 24 °C)]. Higher room temperatures may result in slightly higher OD values.

### Interpretation of Results

The AIV Sp ratio values and/or ELISA titer values obtained for sera should be interpreted using the following value ranges:

Sample to Positive (Sp) Value	AIV ELISA Titer Range	AIV Presumed Antibody Status
Less than 0.300	0	Non-Reactive*
0.300 to 0.499	270 to 569	Suspect <sup>b</sup>
Greater or equal to 0.5	570 or greater	Positive <sup>c</sup>

a. **Non-reactive.** Serum samples with an AIV Sp ratio value of less than 0.300 receive a "0" titer value and are presumed non-reactive for AIV antibody. However, a variety of factors, such as possible AIV strain variations that may exhibit atypical biological and/or antigenic properties<sup>1</sup>, prevalence of an AIV strain within a flock and timing and randomness of serum sample collection procedures could result in an AIV-infected chicken flock yielding AIV non-reactive ELISA results. It is therefore recommended that each chicken flock only be considered to be AIV non-reactive after (a) each flock has been adequately sampled and repeatedly tested several times and has yielded negative AIV ELISA results each time and (b) each flock has been adequately sampled and repeatedly tested by standard conventional serologic tests (AGP, HI and NI) and AIV virus isolation techniques<sup>1,2</sup> and has yielded AIV non-reactive serologic and virus isolation results each time.

b. **Suspect.** Presumed AIV antibody suspect denotes the ELISA Sp value range within which AIV ELISA and conventional (AGP, HI and NI) test data may suggest but **may not conclusively** detect AIV antibody within a sample. The suspect range represents a "suspect" or "gray" area in which AIV ELISA results may or may not be supported by conventional serologic (AGP, HI and NI) test results. It is highly recommended that additional conventional serologic tests and AIV virus isolation techniques<sup>1,2</sup> be conducted on serum and collected from AIV ELISA suspect chicken flocks, as recommended in parts a and c, to confirm whether each flock is an AIV non-reactive or AIV positive-infected flock.

c. **Positive.** Additional conventional serologic testing (AGP, HI and NI) and virus isolation of samples collected from presumed AIV ELISA antibody suspect and positive chicken flocks, using standard techniques<sup>1,2</sup>, are needed to obtain a confirmed positive diagnosis of AIV infection within a chicken flock. Samples may yield false positive results if the serum tested is fatty or highly contaminated with bacteria or debris. Please exclude poor quality serum samples from the ELISA analysis.

## BIBLIOGRAPHY

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- Easterday, B.C. and V.S. Hinshaw, Influenza, In: *Diseases of Poultry* (Editorial Committee of the American Association of Avian Pathologists), 9th ed., Iowa State University Press, Ames, Iowa, pp. 532-551. 1991.
- Meulemans G., M.C. Carlier, M. Gonze and P. Petit. Comparison of Hemagglutination-Inhibition, Agar Gel Precipitin and Enzyme-linked Immunosorbent Assay for Measuring Antibodies Against Influenza Viruses in Chickens. *Avian Diseases*. 31: 560-563. 1987.

All positive samples and/or results should be submitted to the National Veterinary Services Lab for H and N titration

\*U.S. Customers Only

Please contact Synbiotics Technical Service at  
800-247-1725 or (816) 454-7246 with questions and comments.



## Avian Influenza Virus Antibody Test Kit

### Name and Intended Use

FlockChek™: AI is IDEXX's enzyme immunoassay for the detection of antibody to Avian Influenza Virus (AI) in chicken serum.

### General Information

Domestic and wild avian species are affected by avian influenza viruses. The disease is characterized by a wide range of responses which include virtually no clinical signs to high mortality. Respiratory signs are common, along with drop in egg production, greenish diarrhea, bloodstained nasal and oral discharges, and cyanosis and edema of the head, comb and wattle. Due to the variation and severity of clinical symptoms, serological testing produces significant advantages to detection of infected birds. Monitoring for exposure of a flock to influenza is facilitated by the measurement of antibody to avian influenza in serum.

### Descriptions and Principles

This assay is designed to measure the relative level of antibody to AI in chicken serum. Viral antigen is coated on 96-well plates. Upon incubation of the test sample in the coated well, antibody specific to AI forms a complex with the coated viral antigens. After washing away unbound material from the wells, a conjugate is added which binds to any attached antibody in the wells. Unbound conjugate is washed away and enzyme substrate is added. Subsequent color development is directly related to the amount of antibody to AI present in the test sample.

### Reagent

	Volume
1. AI Coated Plates	5
2. AI Positive Control - Diluted chicken Anti-AI, preserved with sodium azide.	1.9 ml
3. Negative Control - Diluted chicken sera non-reactive for Anti-AI, preserved with sodium azide.	1.9 ml
4. (Goat) Anti-Chicken/(Goat) Anti-Turkey: Horseradish Peroxidase Conjugate, preserved with gentamicin.	50 ml
5. Sample Diluent buffer preserved with sodium azide.	235 ml
6. TMB Substrate	60 ml
7. Stop Solution	60 ml

### Materials Required but Not Provided

Precision pipets and multiple delivery pipetting device with disposable pipet tips, 96-well plate reader, tubes for diluting samples, distilled or deionized water and device for the delivery and aspiration of wash solution.

### Precautions and Warnings for Users

Handle all AI biological materials as though capable of transmitting AI. The antigen coated plates may be a source of AI. Prior to coating on the solid phase, the antigen has been inactivated by chemical treatment. Nevertheless, do not assume complete inactivation. Some kit components contain sodium azide as a preservative. Disposal requires flushing plumbing with large volumes of water to prevent formation of copper or lead azide complexes which may explode upon percussion. Do not expose TMB solutions to strong light or any oxidation agents. Store all reagents at 2°-7°C (36°-45°F). All wastes should be properly decontaminated prior to disposal. Do not use components past expiration date and do not intermix components from kits with different lot numbers. Careful pipetting and washing throughout this procedure are necessary to maintain precision and accuracy. Optimal results will be obtained by strict adherence to this protocol. For veterinary use only.

### Preparation of Samples

Dilute test samples five hundred fold (1:500) with sample diluent prior to being assayed (e.g., by diluting 1 µl of sample with 500 µl of Sample Diluent). **NOTE: DO NOT DILUTE CONTROLS.** Be sure to change tips for each sample. Samples must be thoroughly mixed prior to dispensing into the coated plate.

### Test Procedure

Reagents should be allowed to come to room temperature, then mixed by inverting and swirling.

1. Obtain antigen-coated plate(s) and record the sample position on a FlockChek worksheet.
2. Dispense 100 µl of UNDILUTED Negative Control into wells A1 and A2.
3. Dispense 100 µl of UNDILUTED Positive Control into wells A3 and A4.
4. Dispense 100 µl of diluted sample into appropriate wells. All samples should be run in duplicate.
5. Incubate for 30 minutes at room temperature.
6. Wash each well with approximately 350 µl of distilled or deionized water 3-5 times.
7. Dispense 100 µl of (Goat) Anti-Chicken/(Goat) Anti-Turkey: Horseradish Peroxidase Conjugate into each well.
8. Incubate for 30 minutes at room temperature.
9. Repeat step 6.
10. Dispense 100 µl of TMB substrate solution into each well.
11. Incubate for 15 minutes at room temperature.
12. Dispense 100 µl of Stop Solution into each well to stop the reaction.
13. Blank reader, with air.
14. Measure and record absorbance values at 650nm, A(650).

### Results

For the assay to be valid, the difference between the Positive Control mean and the Negative Control mean (PCx - NCx) should be greater than 0.075. The Negative Control mean absorbance should be less than or equal to 0.150. The presence or absence of antibody to AI is determined by relating the A(650) value of the unknown to the Positive Control mean. The Positive Control is standardized and represents significant antibody levels to AI in chicken serum. The relative level of antibody in the unknown is determined by calculating the sample to positive (S/P) ratio.

### Interpretation of Results

Serum samples with S/P ratios of less than or equal to 0.5 should be considered negative. S/P ratios greater than 0.5 should be considered positive and indicate exposure to AI. ELISA positive samples should be confirmed by additional serological testing such as Agar Gel Precipitation Test (AGP).

\*Note: U.S. Customers must submit all positive samples and/or results to the National Veterinary Services Laboratory for H and N titration.

### Calculations

1. Negative Control mean (NCx)  
$$\frac{\text{Well A1 A(650)} + \text{Well A2 A(650)}}{2} = \text{NCx}$$
2. Positive Control mean (PCx)  
$$\frac{\text{Well A3 A(650)} + \text{Well A4 A(650)}}{2} = \text{PCx}$$
3. S/P Ratio  
$$\frac{\text{Sample Mean} - \text{NCx}}{\text{PCx} - \text{NCx}} = \text{S/P}$$

Fluente Aviaire à

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- détecter les  
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u virus de l'influenza  
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illée ou déionisée.

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IS DILUER LES  
eur distribution

For assistance call IDEXX Customer Service

IDEXX USA

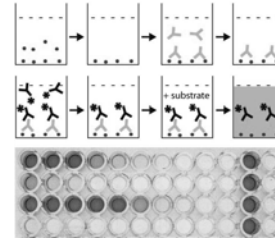
Tel: 800-943-3999 or 207-856-0890  
Fax: 800-328-5461 or 207-856-0826



## Avian Influenza Enzyme-Linked Immunosorbent Assay (ELISA)

Mary Lea Killian  
USDA APHIS VS  
National Veterinary Services Laboratories  
Ames, Iowa

## Enzyme-linked Immunosorbent Assay



<http://microvet.arizona.edu/Courses/MIC419/ToolBox/elisa.html>

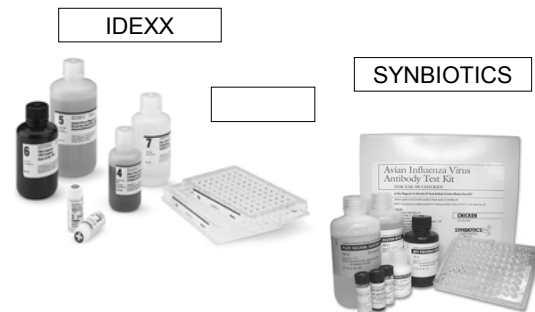
## Sample Collection for ELISA testing

- For routine monitoring, it is suggested that at least 30 or more sera per flock be randomly collected at standard time intervals (i.e. every four weeks). Proper sample collection procedures, serum harvest, and serum sample storage are needed to provide reliable results.
- **To achieve better specificity and to minimize possible false positive reactions, serum samples that are contaminated with bacteria or are very fatty should be excluded from testing.**

## ELISA Kits for Antibody Detection

- Commercial ELISA test kits are available to detect
  - Avian influenza virus antibody in chicken serum
  - Newcastle disease virus antibody in chicken serum
  - Newcastle disease virus antibody in turkey serum

## ELISA Kits for AIV Ab Detection



## ELISA for AIV and NDV

- Advantages
  - Fast—90 samples tested in 2-3 hours
  - Good sensitivity
  - High throughput—  
90 samples per plate, multiple plates at once
  - Very little sample volume required, <10 µl
  - Easy to learn

## ELISA for AIV and NDV

- Limitations
  - Need specialized equipment—plate reader and washer
  - Specificity varies by target
  - Measures exposure and vaccine response, but not acute infection
    - AGID, HI, NI, and VI are needed to confirm AIV negative and AIV-infected chicken flocks
  - Regulatory considerations

## ELISA Laboratory

- Materials Needed
  - Each person will have their own AIV ELISA plate, but everyone in each group of three will use the same kit
    - IDEXX
    - OR
    - SYNBIOTICS
  - Record Sheet
  - Test samples and dilution tubes
  - Pipets and tips

## Materials needed

- The materials for your kit (manufacturer specific).
  1. ELISA plate
  2. Positive control
  3. Negative control
  4. Dilution Buffer
  5. Conjugate (Secondary antibody)
  6. Substrate
  7. Stop solution
  8. Wash solution- Synbiotics only

## ELISA Laboratory 1

- IDEXX
  - Fill out the record sheet to record where each sample will be
  - Run samples in duplicate
- SYNBIOTICS
  - Fill out the record sheet to record where each sample will be
  - Run samples in duplicate
  - Prepare wash solution

## ELISA Laboratory 2

- IDEXX
  - Label dilution tubes
  - Add 1ml of diluent to dilution tubes
  - Add 2 $\mu$ l of test serum to a dilution tube
  - Do NOT dilute controls
- SYNBIOTICS
  - Label dilution tubes
  - Add 0.3ml diluent to each dilution tube
  - Add 6 $\mu$ l of test serum to a dilution tube
  - Dilute controls the same way as the test samples
  - Add 50 $\mu$ l of dilution buffer to each well of the ELISA plate

## ELISA Laboratory 3

- IDEXX
  - Add positive control to plate
    - 0.1ml to wells A1 and A2
  - Add negative control
    - 0.1ml to wells A3 and A4
- SYNBIOTICS
  - Add positive control
    - 50 $\mu$ l to wells A1, A3 and H11
  - Add negative control
    - 50 $\mu$ l to wells A2, H10 and H12

	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	-	-	1	1	2	2	3	3	4	4
B	5	5	6	6	7	7	8	8	9	9	10	10
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A	+	-	+		1	1	2	2	3	3	4	4
B	5	5	6	6	7	7	8	8	9	9	10	10
C												
D												
E												
F												
G												
H									-	+	-	

## ELISA Laboratory 4

### • IDEXX

- Add 100µl of diluted test serum to the plate according to your record sheet
- Incubate for 30 minutes
- Wash with distilled water

### • SYNBIOTICS

- Add 50µl of diluted test serum to the plate according to your record sheet
- Incubate for 30 minutes
- Wash with wash solution

## ELISA Laboratory 5

### • IDEXX

- Add 100µl of conjugate to all wells of your test plate
- Incubate for 30 minutes
- Wash with distilled waster

### • SYNBIOTICS

- Dilute Conjugate
  - Add 100µl of conjugate to 10mls of dilution buffer and mix
- Add 100µl of diluted conjugate to all wells of your test plate
- Incubate for 30 minutes
- Wash with wash solution

## ELISA Laboratory 6

### • IDEXX

- Add 100µl of TMB substrate to each well
- Incubate for 15 minutes
- Add 100µl of stop solution to each well
- Read results

### • SYNBIOTICS

- Add 100µl substrate to each well
- Incubate for 15 minutes
- Dilute stop solution
  - Add 2.5ml stop solution to 10ml of distilled water.
- Add 100µl of diluted stop solution to each well
- Read results

## ELISA Results

- Results should be recorded by reading the optical densities of the plates in a plate reader at the correct absorbance:

IDEXX: 650nm

Synbiotics: 405-410nm

Each manufacturer supplies computer software specific for their test which calculates which samples are negative and the titers of positive samples.

## ELISA Results

- The status of a sample are evaluated by the sample to positive ratio (S/P ratio):

$$\frac{\text{Sample mean} - \text{negative control mean}}{\text{positive control mean} - \text{negative control mean}}$$

(mean of optical absorbance)

With the IDEXX kit S/P ratios of greater than 0.5 are considered positive

With the Synbiotics kit S/P ratios of greater than 0.299 are considered positive

## ELISA Results

- Example:

Sample mean= 0.820

Negative control mean=0.053

Positive control mean=0.563

$$\frac{0.820 - 0.053}{0.563 - 0.053} = 1.5 \text{ or Positive}$$

Values are relatively quantitative: a higher value indicates more antibody.

## Valid ranges for the positive and negative controls for each kit

- IDEXX

Negative control  
0.150 or less

The difference between the positive and negative control means must be greater than 0.075

Example: if negative control = 0.100, the positive control must be 0.176 or greater.

- SYNBIOTICS

Negative control  
Less than 0.200

Positive control  
0.250-0.900