ELISA TEST PROCEDURE

PREPARING THE TEST PLATE

a) Remove an AIV antigen coated test plate from the protective bag and label according to serum dilution plate identification.
b) Add 50 µl Dilution to all wells on the test plate.
c) Add 50 µl diluted AIV Positive Control Serum to wells A1, A3, and H11. Discard pipette tip.
d) 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.
e) Incubate plate for 30 minutes at room temperature.

WASH PROCEDURE

f) Tap out liquid from each well into an appropriate vessel containing bleach or other decontamination agent.
g) 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 CONJUGATE, SUBSTRATE AND STOP SOLUTION

h) 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.
i) Incubate for 30 minutes at room temperature (21° to 24° C; 70° to 75° F).
j) **WASH** as in steps f and g above.
k) Using an 8 or 12 channel pipette (or transplating device) dispense 100 µl Substrate Solution into each test well. Discard pipette tips.
l) Incubate 15 minutes at room temperature (21° to 24° C; 70° to 75° F).
m) Using an 8 or 12 channel pipette (or transplating device) add 100 µl diluted Stop Solution (prepared as described above) to each test well.
n) Allow bubbles to dissipate before reading plate.

MANUAL PROCESSING OF DATA

a) Read the plate using and ELISA plate reader set at 405-410nm. Be sure to blank the reader as directed.
b) 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.
c) Subtract the average normal control absorbance from the average positive absorbance. The difference is the Corrected Positive Control.
d) 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:

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

e) An AIV ELISA titer can be calculated by the following suggested equation:

LOG_{10} TITER = (1.464 X LOG_{10} SP) + 3.197

TITER = ANTILOG OF LOG_{10} TITER

Example:

1. Example Positive Control Absorbance:
   0.585, 0.610, 0.590
   Average = (0.585 + 0.610 + 0.590) / 3 = 0.595
2. Example Normal Controls:
   0.078, 0.067, 0.057
   Average = (0.078 + 0.067 + 0.057) / 3 = 0.067
3. Corrected Positive Control:
   (0.595) - (0.067) = 0.528
4. Example Sp value calculation:
   Absorbance of sample = 0.560
   (0.560) - (0.067) / 0.528 = 0.934
5. Example of Calculation of titer using the Sp from above:
   Log_{10} Titer = 1.464 X (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 1.200. 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 0.350 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 results. Please note that tests with O.D. values which do not fall within the suggested O.D. ranges above do not constitute an invalid test.

*Optimal conditions are at room temperature (21° to 24° C; 70° to 75° F). Higher room temperatures may result in slightly higher O.D. 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 0.350	0	Non-Reactive[a]
Greater or equal to 0.350	338 or greater	Positive[b]

a. **Non-reactive.** Serum samples with an AIV Sp ratio of less than 0.350 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[1], 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[1,2] and has yielded AIV non-reactive serologic and virus isolation results each time.

b. **Positive.** Additional conventional serologic testing (AGP, HI and NI) and virus isolation of samples collected from presumed AIV ELISA antibody **positive** flocks, using standard techniques[1,2], are needed to obtain a confirmed positive dianosis of AIV infection within a 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.

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

*U.S. Customers Only

Please contact Synbiotics Technical Service at
(800) 228-4305 or (858) 451-3771 with questions and comments.

03-3076-0906

AVIAN INFLUENZA VIRUS ANTIBODY TEST KIT

ITEM NO. 96-6552

SYNBIOTICS® CORPORATION

ProFLOK® PLUS

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 16 known serologically distinct subtypes based on surface hemagglutinins and 9 based on neuraminidases[3]. 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[3].

The ProFLOK®plus AIV ELISA Kit is a rapid and specific presumptive screening test for the detection of antibody to AIV in chicken or turkey serum samples. It was designed for screening large numbers of sera from numerous flocks; however, additional conventional AIV serologic testing [i.e. agar gel precipitin (AGP), hemagglutination inhibition (HI) test and neuraminidase-inhibition (NI)][1-4] and virus isolation techniques are needed to confirm AIV negative and AIV-infected 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 or turkeys 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, a peroxidase labeled 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 perxidase enzyme. The relative intensity of color developed in 15 minutes (compared to controls) is directly proportional to the level of AIV antbody 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

a) 1 AIV antigen coated plate
b) 10 µl AIV Positive Control Serum
c) 10 µl Normal Control Serum
d) 100 µl prepared Peroxidase Conjugate Solution
e) 40 ml Dilution Buffer
f) 10 ml ABTS-Hydrogen Peroxide Substrate Solution
g) 2.5 ml 5X Stop Solution, 5% SDS (dilute [1:5] with laboratory grade water)
h) 20 ml 20X Wash Solution (dilute [1:20] with laboratory grade water)

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

EQUIPMENT AND MATERIALS REQUIRED BUT NOT PROVIDED

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

WARNINGS TO THE USERS OF REAGENTS AND AIV ANTIGEN COATED PLATES

a) Handle all reagents and samples as biohazardous material.
b) Keep all reagents away from skin and eyes. If exposure should occur, immediately flush affected areas with cold water.
c) 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.
d) Take special care not to contaminate any of the test reagents with serum or bacterial agents.
e) 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.
f) The best results are achieved by following the protocols as they are described below, using good, safe laboratory techniques.
g) Do not use this kit after the expiration date.
h) **NEVER PIPETTE BY MOUTH**

ALLOW ALL REAGENTS TO COME TO ROOM TEMPERATURE (21° to 24° C; 70° to 75° F) 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

a) 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.
b) Add 6 µl unknown serum per well as per Figure 1 (producing 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.
c) Add 6 µl of Normal Control Serum (producing a 1:50 dilution) to wells A2, H10, and H12.
d) Aspirate and remove any liquid in dilution plate wells A1, A3, and H11.
e) Allow all diluted serums to equilibrate in Dilution Buffer for 5 minutes before transferring to an AIV antigen coated ELISA plate.
f) Diluted serum should be tested within 24 hours. This dilution format provides adequate quantities of diluted serum samples to conduct four additional ELISA tests using the same serum dilution plate.

Figure 1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	+	-	+	1	2	3	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 peroxidase labeled conjugate is supplied as a stabilized concentrate. 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.