Avian Influenza Disease Antibody Test Kit (AI)

BioChek
SMART VETERINARY DIAGNOSTICS

BioChek Poultry Immunoassays

Product Number CK 121

Description of Test
The AI ELISA kit will measure the amount of antibody to AI in the serum of chickens. Microtitre plates have been pre-coated with inactivated AI antigen. Chicken serum samples are diluted and added to the microtitre wells where any anti-AI antibodies present will bind and form an antigen-antibody complex. Non specific antibodies and other serum proteins are then washed away. Anti-chicken IgG labelled with the enzyme alkaline phosphatase is then added to the wells and binds to any chicken anti-AI antibodies bound to the antigen. After another wash to remove unreacted conjugate, substrate is added in the form of pNPP chromogen. A yellow colour is developed if anti-AI antibody is present and the intensity is related to the amount of anti-AI antibody present in the sample.

The validation data for this kit have been certified by the OIE, based on expert review, as fit for the following purposes:
Serological diagnosis of type A avian influenza in chickens (specific to IgG in serum) and for the following purposes:
1. *To demonstrate historical freedom from infection in a defined population (country/zone/compartment/flock);*
2. *To demonstrate re-establishment of freedom after outbreaks in a defined population (country/zone/compartment/flock);*
3. *To confirm diagnosis of suspect or clinical cases;*
4. *To estimate prevalence of infection to facilitate risk analysis in non-vaccinated populations (surveys/herd health schemes/disease control);*
5. *To determine immune status in individual animals or populations (post-vaccination)*
   (As stated in the resolution adopted by the OIE World Assembly of Delegates).

Reagents provided:
1. **AI Coated plates**. Inactivated viral antigen on microtitre plates.
2. **Conjugate reagent**. Anti-Chicken: Alkaline Phosphatase in Tris buffer with protein stabilisers, inert red dye and sodium azide preservative (0.1% w/v).
3. **Substrate tablets**. pNPP (p-Nitrophenyl Phosphate) tablets to dissolve with Substrate buffer.
4. **Substrate reagent**. Diethanolamine buffer with enzyme co-factors.
5. **Stop solution**. Sodium Hydroxide in Diethanolamine buffer.
6. **Sample diluent reagent**. Phosphate buffer with protein stabilisers and sodium azide preservative (0.2% w/v).
7. **Wash buffer sachets**. Powdered Phosphate Buffered Saline with Tween.
8. **Negative control**. Specific Pathogen Free serum in Phosphate buffer with protein stabilisers and sodium azide preservative (0.2% w/v).
9. **Positive control**. Antibodies specific to AI in Phosphate buffer with protein stabilisers and sodium azide preservative (0.2% w/v).

Materials and Equipment required (not provided with kit):
Precision Pipettes and disposable tips
8 or 12 channel pipette/repeater pipette
Plastic tubes for sample dilution
Distilled or deionised water
Microtitre Plate Reader with 405 nm filter
Microtitre Plate Washer

Warnings and Precautions:

1. Handle all reagents with care. STOP SOLUTION contains STRONG ALKALI which can be CAUSTIC. If in contact with skin or eyes, wash with copious amounts of water.
2. Treat all biological materials as potentially biohazardous, including all field samples. Decontaminate used plates and waste including washings with bleach or other strong oxidising agent before disposal.
3. NEVER pipette anything by mouth. There should be no eating, drinking or smoking in areas designated for using kit reagents and handling field samples.
4. This kit is for IN VITRO use only.
5. Strict adherence to the test protocol will lead to achieving best results.

Reagent preparation:

1. **Substrate Reagent**. To make substrate reagent, add 1 tablet to 5.5ml of substrate buffer and allow to mix until fully dissolved (approx. 10 minutes). The prepared reagent should be made on day of use but will be stable for one week if kept in dark at +4 °C. Drop tablets into clean container and add appropriate volume of substrate buffer.
   DO NOT HANDLE TABLETS WITH BARE FINGERS
2. **Wash Buffer**. Empty the contents of one wash buffer sachet into one litre of distilled or deionised water and allow to dissolve fully by mixing.
3. All other kit components are ready to use but allow them to come to room temperature (22-27°C) before use.

Sample preparation:

1. Dilute each test sample 1:500 in Sample diluent reagent.

POSITIVE AND NEGATIVE KIT CONTROLS DO NOT REQUIRE DILUTING.

Test procedure:

1. Remove AI coated plate from sealed bag and record location of samples on template.
2. Add 100 μl of negative control into wells A1 and B1.
3. Add 100 μl of positive control into wells C1 and D1.
4. Add 100 μl of diluted samples into the appropriate wells. Cover plate with lid and incubate at room temperature (22-27°C) for **30 minutes**.
5. Aspirate contents of wells and wash 4 times with wash buffer (350μl per well). Invert plate and tap firmly on absorbent paper until no moisture is visible.
6. Add 100 μl of Conjugate reagent into the appropriate wells. Cover plate with lid and incubate at room temperature (22-27°C) for **30 minutes**.
7. Repeat wash procedure as in 5.
8. Add 100 μl of Substrate reagent into the appropriate wells. Cover plate with lid and incubate at room temperature (22-27°C) for **15 minutes**.
9. Add 100 μl of Stop solution to appropriate wells to stop reaction.
10. Blank the microtitre plate reader on air and record the absorbance of controls and the samples by reading at 405 nm.

Results:

For the assay to be valid the mean negative control absorbance should read below 0.30. The difference between the mean negative control and the mean positive control should be greater than 0.20.

The AI positive control has been carefully standardised to represent significant amounts of antibody to AI in chicken serum. The relative amounts of antibodies in chicken samples can then be calculated by reference to the positive control. This relationship is expressed as S/P ratio (Sample to Positive Ratio).

Interpretation of results

Samples with an S/P of 0.5 or greater contain anti-AI antibodies and are considered POSITIVE.

1. Calculation of S/P ratio

$$\frac{\text{Mean of Test Sample - Mean of negative control}}{\text{Mean of Positive control - Mean of negative control}} = S/P$$

2. Calculation of Antibody Titre

The following equation relates the S/P of a sample at a 1: 500 dilution to a titre

$$\text{Log10 Titre} = 1.1 * \text{Log (SP)} + 3.156$$

Antilog = Titre

S/P value	Titre Range	Antibody status
0.499 or less	667 or less	No antibody detected
0.500 or greater	668 or greater	Positive

This test is highly specific for antibodies against Avian Influenza Virus. However, be aware that false positive reactors can occur in rare circumstances. Therefore confirmation with an established reference method is required for a final diagnosis.

BioChek has a software program available which can be used with the AI kit to calculate S/P values, titres and provide general flock profiling.

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KI/CK121REV04

Appendix to Avian Influenza Disease Antibody Test Kit (AI) Kit Insert
Product Number: CK121
OIE Registration Number: 20080203

Summary of Validation Data.

Analytical characteristics

Group specificity   - A panel of sera representing all avian influenza (AI) haemagglutinin serotypes (H1 – H15) were tested. This panel represents antibody standards currently used by the VLA for serotyping strains of AI virus by haemagglutination inhibition (HI). All sera in this panel are also positive by agar gel immunodiffusion (AGID). These sera were derived from chickens experimentally infected with isolates from wild and domestic birds with blood samples collected 14-21 days post exposure. All samples supplied by VLA Weybridge tested positive on the BioChek ELISA. These results confirm that the BioChek ELISA is capable of detecting group specific antibody across all the H-types of AI virus.

Analytical sensitivity   - The BioChek ELISA was compared with two other commercial ELISA kits in the titration of a standard reference serum from VLA Weybridge. The BioChek ELISA demonstrated an endpoint titre of 1:64,000 compared to titers of 1:64,000 (Idexx ELISA) and 1:1,600 (KPL ELISA) for the other two kits.

Analytical specificity   - Chicken antisera raised against other pathogens commonly present in poultry were tested in the ELISA. They included antisera to adenovirus, avian encephalomyelitis, avian reovirus, E. coli, fowlpox, infectious bronchitis virus, infectious bursal disease, infectious laryngotracheitis, mycoplasma gallisepticum, mycoplasma synoviae, paramyxovirus 1, salmonella pullorum and turkey rhinotracheitis). Panels of monospecific hyperimmune sera derived from SPF chickens that had been either experimentally infected or vaccinated with the different pathogens were obtained from different suppliers. All samples tested negative on the BioChek ELISA.

Repeatability data   - The Quality objectives of BioChek are to keep all %CVs below 20% and also to make sure that the overall mean of the control samples stays within 2SDs of the mean in every production run. Ranges are generated and must be adhered to; they are quoted in the summary table below raw data. When a new batch of control is produced it is run in Quality Control assays for 1 year alongside existing controls to generate ranges prior to introduction. Over a 2 year period, the diagnostic kits have been manufactured consistently and this has been confirmed by customer satisfaction with the reference control repeatability in many different laboratories.

Diagnostic Characteristics

Threshold determination

The BioChek (BC) AI ELISA cut off was determined by direct comparison to competitive ELISAs in the commercial market. Based on comparative endpoint titrations (see Analytical sensitivity, above), a cut off was selected that represented optimal analytical sensitivity. The cut off was set prior to specificity studies to ensure the analytical sensitivity was consistently maintained.

Diagnostic sensitivity (DSe) and specificity (DSp) estimates with 95% confidence limits (CI)

The following results document the fitness for purposes 1, 2, 3 and 4 mentioned above. The diagnostic sensitivity (DSe) was estimated at:

- Apparent diagnostic sensitivity of 100.00% (95% confidence interval: [91.19% to 100.00%], number of flocks tested: 2, number of chickens tested: 40, flock infection status determined using HI tests conducted at VLA) in clinically affected chickens (vaccinated for IBD NDV IBV REO and TRT) in Rawda/Saudi Arabia. The BC AI ELISA identified 40/40 sera as positive compared to HI VLA in which 11 of 35 were positive (5 samples had insufficient sera) which suggests that the BC AI ELISA might be more sensitive than the HI.

- A further study, (number of flocks tested: 26, number of chicken tested: 253, flock infection status determined clinically and using an HI test specific for H9) in non-vaccinated chickens of different breeds and ages in South Korea, showed a higher detection rate of the BC AI ELISA (185/253) compared to HI H9 (137/253). These results again suggest that the BC AI ELISA might be more sensitive. The sensitivity of the BC AI ELISA in detecting infected flocks was estimated at 96.15% (25/26) in the same study.

The following results document the fitness for purposes 1, 2 and 4 mentioned above. The diagnostic specificity (DSp) was estimated at:

- 100.00% (95% confidence interval: [90.26% to 100.00%], number of flocks tested: 2, number of chickens tested: 200 but only 36 samples from a pool of 100 samples from each flock were tested) in SPF chickens from Germany,

- 100.00% (95% confidence interval: [96.45% to 100.00%], number of flocks tested: 1, number of chickens tested: 102, true status determined historically/clinically) in chickens (vaccinated against NDV IB, IBD, TRT, REO and CAV) from Scotland,

- 99.23% (95% confidence interval: [98.72% to 99.58%], number of flocks tested: 76, number of chickens tested: 1825, true status determined historically/clinically) in chicken (vaccinated against IBD, IBV, REO, etc.) from broad geographical and age spread, and

- 99.61% (95% confidence interval: [97.83% to 99.99%], number of chickens tested: 254, true status determined after Deventer AHS HI screening for all 16 H-types) in broiler and layer chickens (vaccinated against IBD, NDV, IB, REO, CAV and TRT) from the Netherlands.

The following results document the fitness for purposes 5.

- One hundred and eleven (111) independent samples collected randomly from 3 flocks (group 1, 2 and 3) of H5N2-vaccinated chickens (origin: Holland) were tested 2, 3, 4, 5 weeks post vaccination and the sensitivity in detecting a vaccination response was estimated 85.71% (24/28, 95% confidence interval: [67.33% to 95.97%]) in week 2 p.v. and 100.00% (83/83, 95% confidence interval: [95.65% to 100.00%]) in all other weeks tested. One hundred and seven (107) independent samples collected randomly from 3 flocks (group 4, 5 and 6) of H5N6-vaccinated chickens (origin: Holland) were tested 2, 3, 4, 5 weeks post vaccination and the sensitivity in detecting a vaccination response was estimated 88.89% (24/27, 95% confidence interval: [70.84% to 97.65%]) in week 2 p.v. and 98.75% (79/80, 95% confidence interval: [93.23% to 99.97%]) in all other weeks tested. All samples from control birds tested negative in both the HI and the BC AI ELISA (64 samples).

- Twelve (12) birds vaccinated with AIV H5N2 MSV+5 were tested 3, 4, 5 weeks post vaccination by Intervet HI and BioChek ELISA for comparison. Of 36 samples tested; 32 were positive by the HI test

and 4 negative. The sensitivity in detecting a vaccination response was estimated at 31/32: 96.88%, 95% confidence interval: (83.78% to 99.92%) by the BioChek ELISA kit relative to the HI test.

- Birds experimentally infected or vaccinated [1) 50 wk old SPF layers 28 days p.i. with LP H7N1; 2) 4 wk old SPF layers 10 days p.i. with LP H7N1; 3) 50 wk old SPF layers; 4) 4 wk old SPF broilers 28 days p.v. with inactivated Nobilis H5N2 vaccine; 5) 4 wk old SPF broilers 28 days p.i. with H9N2; 6) 4 wk old SPF broilers 28 days p.i. with LP H5N2: 7) 4 wk old SPF layers 10 days p.i. with LP H5N2 and 8) 50 wk old SPF layers 28 days p.i. with H6N2] were tested by AHS Deventer (ring trial for avian influenza antibody detection in serum [2006]) using AGID, HI and BioChek ELISA. BioChek ELISA showed a good level of sensitivity compared to the two others tests in this trial:

	Biochek		AGID		HI (H5 specific)	
Group 1	100%	POS	91%	POS	-	
Group 2	100%	POS	94%	POS	-	
Group 3	100%	NEG	94%	NEG	100%	NEG
Group 4	89%	POS	35%	POS	100%	POS
Group 5	100%	POS	100%	POS	-	
Group 6	78%	POS	26%	POS	93%	POS
Group 7	83%	POS	53%	POS	59%	POS
Group 8	89%	POS	82%	POS	-	

Agreement between tests

For this calculation, the results of the following studies have been used:

1. Vaccination trials – Biochek + HI AIV H5 tests compared, 218 samples from vaccinated chickens tested + 64 negative control samples.
2. Comparison of BioChek with HI test on Intervet Vaccinated flocks, H5N1, 36 samples from vaccinated chickens tested + 8 negative control samples.
3. Field samples, Deventer AHS HI screening for all 16 haemagglutinins, 254 samples tested.

		HI test		
		Reactor	Non-Reactor	Totals
BioChek ELISA	Reactor	241	1	242
	Non-Reactor	9	329	334
	Totals	250	330	580

Relative Diagnostic Sensitivity (BioChek relative to HI): 241/250 or 96.40%, 95% confidence interval: (93.28% to 98.34%)
Relative Diagnostic Specificity ((BioChek relative to HI): 325/326 or 99.69%, 95% confidence interval: (98.30% to 99.99%)
Apparent prevalence HI test: 250/576 or 0.43
Apparent prevalence BioChek test: 242/576 or 0.42

Agreement can be quantified using the kappa statistic:

Observed proportion agreement	(241 + 325)/576	=	0.983
Chance proportion agreement (both +)	0.43 x 0.42	=	0.181
Chance proportion agreement (both -)	0.57 x 0.58	=	0.331
Chance proportion agreement	0.181 + 0.331	=	0.512
Observed minus chance agreement	0.984 – 0.512	=	0.471
Maximum possible agreement beyond chance	1 – 0.512	=	0.488
Kappa	0.471/0.488	=	0.965

This shows a strong kappa value close to 1.0 and therefore high degree of agreement between tests.

<u>Reproducibility</u>

A first international ring trial for avian influenza antibody detection in serum was conducted in 2006 with experimentally infected and vaccinated birds (126 samples from SPF layers and broilers, 4 or 50 weeks old and infected with AIV [either LP H7N1, H9N2, LP H5N2,or H6N2] or vaccinated with inactivated Nobilis H5N2 vaccine). The samples were prepared at the Animal Health Service of Deventer in the Netherlands and sent to 49 laboratories worldwide to be tested by ELISA (including BioChek ELISA), AGID, and HI. The results were collected and summarized by the Animal Health Service.

The BioChek ELISA was used by 9 laboratories on 8 duplicated samples (7 positives + 1 negative). Seven out of the 9 laboratories demonstrated reproducible results and identified all samples correctly. The two other laboratories experienced problems in correctly identifying some of the positive samples. Overall, reproducibility was considered to be acceptable.