Specificity of the ELISA kit

This kit is designed to detect antigen specific (user supplied) antibodies in serum or egg yolk. It is not designed to measure the actual concentration of Ig's in serum. We have other ELISA kits to measure IgG, IgM IgA, and IgE levels in chicken serum or IgY in Egg yolk (contact ADI or see web site for details). The conjugate supplied in the kit is anti-Chicken IgG (H+L)-HRP. It will detect IgG+IgA+IgM. If specific antibody detection is desired then it is possible to use isotype specific (IgG, IgM, or IgA) conjugates (available from ADI).

Quality Control of the assay.

This kit is primarily designed to detect chicken antibodies (IgG, IgM or IgA) in serum or egg yolk. It is therefore, important to establish that the user can detect both purified control IgG and IgM antigens using the suggested protocol. Purified IgG (#20010-1) and IgM (20010-2-1) are available that can be coated on the ELISA plates and a model ELISA established before attempting to test the antigen specific antibodies. Control antigen assay will establish the concn of IgG or IgM that can detected under defined ELISA conditions.

Related Items (Chicken antigen, antibodies, and ELISA Kits)

80012 High binding ELISA Strips plates (8 wellsx12 strips)

20010-1 Chicken (non-immune) IgG, purified
20010-2-1 Chicken (non-immune) Serum IgM, purified

60320 Anti-Chicken IgG (H+L)-HRP conjugate
60322 Anti-Chicken IgG (H+L)-AP conjugate
60325 Anti-Chicken IgG (H+L) aff pure, unconjugated
60419 Anti-Chicken IgG (Fc), aff pure, unconjugated
60420 Anti-Chicken IgG (Fc)-HRP conjugate
60519 Anti-Chicken IgM (mu chain), aff pure, unconjugated
60520 Anti-Chicken IgM (mu chain)-HRP conjugate
60219 Anti-Chicken IgA (alpha chain), aff pure, unconjugated
60220 Anti-Chicken IgA (alpha chain)-HRP conjugate

6010 Chicken Egg Ovalbumin ELISA Kit, 96 tests, Quantitative
60100 YolkY® Chicken Egg Yolk IgY antibody purification kit
6020 Chicken IgG ELISA Kit, 96 tests, Quantitative
6030 Chicken IgY ELISA Kit, 96 tests, Quantitative
6040 Chicken IgM ELISA Kit, 96 tests, Quantitative
6050 Chicken Egg Ovalbumin ELISA Kit, 96 tests, Quantitative

920-100-AIV Chicken Anti-Avian Influenza A virus (AIV) IgG ELISA kit
920-110-AV Chicken Anti-Anemia Virus (AV) IgG ELISA kit
920-120-IBV Chicken Anti-Infectious Bronchitis Virus (IBV) IgG ELISA kit
920-130-MDV Chicken Anti-Marek's Disease Virus (MDV) IgG ELISA kit
920-130-MDV Avian Marek's disease virus antibody ELISA kit
920-140-NDV Chicken Anti-Newcastle Disease Virus (NDV) IgGs ELISA kit
920-300-H51 Chicken Anti-Avian Influenza virus (H5N1) IgG ELISA kit

Instruction Manual No. M-80150-80188

ELISA "Ensemble" for the Detection of Various Primary Antibodies in Chicken Serum or Whole Egg Yolk

For Chicken Serum (#80176) and Whole Egg Yolk (#80177) primary antibodies

A Colorimetric Assay Using a Sensitive and ready-to-Use Single Solution TMB

ALPHA DIAGNOSTIC INTERNATIONAL

6203 Woodlake Center Drive • San Antonio• Texas 78244 • USA.

Phone (210) 561-9515 • Fax (210) 561-9544

Toll Free (800) 786-5777

Email: service@4adi.com

Web Site: www.4adi.com

ELISA "Ensemble" for the detection of Primary Antibodies

for Cat #80176-80177

Kit Contents: (Sufficient for 1000-2000 samples or 10-20 plates)

Components	Cat. No.
Anti-*chicken IgG/IgY-HRP Conjugate, 150 ul,* (dilute 1:2K-1:10K before use)	60321
Coating Buffer (10X), 25 ml	80050
Blocking Buffer (Milk-based) 25 ml (10X)	80062
Serum Sample/Conjugate Diluent (Red color), (10 X); 25 ml (supplied in kit #80176)	80070
Egg Yolk Sample/Conjugate Diluent, Green Color, (10 X); 25 ml (supplied in kit #80177)	80071
Wash buffer concentrate (100X), 25 ml	80081
Ready-to-Use TMB Substrate Soln, 100 ml	80091
Stop Solution for TMB, 10X (10 ml)	80100
Complete Instruction Manual	M - 80185

All of the above components are available as independent items. We also supply antibody sub-isotype specific antibody conjugates for sub-isotyping or detecting specific antibody isotypes. Please call us for specific details. Additional items related to ELISA are listed below to increase the efficiency of sample handling.

INTRODUCTION

Immunoassays using enzyme labeled antibodies are highly specific for the analyses of a particular protein. Use of an enzyme labeled antibody together with a highly sensitive TMB substrate provides an excellent method for the detection and characterization of sample proteins bound to solid surface by ELISA techniques. Following attachment of protein to the solid surface (ELISA plate), a primary antibody is used to selectively bind the protein of interest. Alternatively, a known protein is bound to the surface for the screening of specific monoclonal/polyclonal antibodies in serum or other samples. An enzyme labeled second antibody directed against the primary antibody is then applied. The TMB substrate reacts with bound HRP to produce blue color. Upon addition of stop solution, the color is converted to yellow color (measure at 450 nm). Yellow color is more sensitive than the blue color. Intensity of color, up to a certain range, is directly proportional to the amount of bound primary antibody.

This kit is designed to detect the presence of a antibodies in chicken serum (Cat# 80176) or whole egg yolks (#80177) using the antigen-coated plates (prepared by the user). It is not suitable for the quantitation a chicken IgG/IgY. We have other ELISA kits to measure Chicken IgG (#6020) and Chicken IgY (#6030).

Note: At low serum dilution, there could be high background as compared with the control. It is possible to reduce the coating antigen concentration and/or reduce the 2-ab concentration. Control serum at 1:100 should be <0.500. There should be a clear distinction of A450 reading in control and samples at dilutions of 1:200 or above (as in above example at 1:1000). After an initial test as above, it is necessary to expand the antibody dilution at desired dilution.

TROUBLE SHOOTING AND GENERAL NOTES

1. Always incorporate a positive control, negative control, and reagent blanks.
2. Remove as much buffer as possible from the wells after washes, but do not allow the plates to dry out. In order to compare results between experiments., it is important to observe various experimental conditions.

Causes of Excess Signal or Background

1. Insufficient dilution of the peroxidase labeled antibody is the most common cause of high background. Try more diluted enzyme conjugate solution.
2. Excessive antibody (use more diluted antibody) or incubation times (Try short incubation at RT).
3. Inadequate washing or blocking procedures. Increase number of washings or increase blocking time.
4. High concentrations of coating antigen (>10 ug/ml) should be avoided. Make sure your antibody does not react with the blocking

Causes of No or Poor Signal

1. Procedure was not followed properly, a reagent may have been omitted or prepared improperly.
2. Antigen was not coated in sufficient amount. Increase concentration of coating antigen. Some antigen may not bind directly to an ELISA plates. please call us to discuss your situation..
3. Specificity of the peroxidase labeled antibody was not appropriate for the primary antibody (for Bovine antibodies you must use anti-Bovine-IgG HRP conjugate, etc.). Conjugate may be too dilute (use more concentrated solution).
4. Primary antibody was absent or present in non-detectable concentration. Try increasing antibody concentration or increase incubation time with primary antibody or perform incubation at 37^{o}C.
5. All solutions must be at room temperature prior to their use. Cold solution will diminish or eliminate reaction rates.
6. All plates are not suitable for a sensitive ELISA. Make sure the plates are intended for ELISA.

5. Dispense 100 µl of **TMB substrate soln/well**. Incubate plates for 15 min. at RT (incubation can be continued for up to 60 min. to increase color). Plates should be shaken gently once or twice during incubation to mix color evenly throughout the well. Blue color develops in antibody positive wells.

6. Stop reaction by adding 100 µl of **Stop soln**. Blue color turn yellow. Mix gently for few seconds and read at 450 nm within 15-30 min (Yellow color will fade over time).
4. Stop reaction by adding 100 µl of **Stop soln**. Blue color turn yellow. Mix gently for few seconds and read at 450 nm within 15-30 min (Yellow color will fade over time).

Suggested ELISA Template for the placement of samples

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

1st incubation with antisera-From_____ to _____ (time _____ min)
2nd incubation with conjugate-From_____ to _____ (time _____ min)
3rd incubation with substrate-From_____ to _____ (time _____ min)
Read plates at 450 nm at _____ (time).

Example of ELISA Results

	Sample 1 (dilution) A450		Non-immune **Control serum** (dilution) A450	
	1	**2**	**3**	**4**
A	(1:100) **3.885****	(1:100) **3.9905****	(1:100) **0.350**	(1:100) **0.320**
B	(1:1000) **1.950****	(1:1000) **1.86****	(1:1000) **0.200****	(1:1000) **0.190****
C	(1:10,000) **0.785****	(1:10,000) **0.820****	(1:10,000) **0.155****	(1:10,000) **0.145****
D	(1:100,000) **0.320**	(1:100,000) **0.350**	(1:100,000) **0.088**	(1:100,000) **0.090**

PRECAUTIONS

The ADI ELISA kit is intended for *in vitro research* use only. For proper analysis of results, be sure to include positive and negative controls, blanks, and/or protein standards as appropriate.

Applicable **MSDS**, if not already on file, for the following reagents can be obtained from ADI or the web site.

TMB (substrate), H2SO4 (stop solution), and Prolcin-300 (0.1% v/v in, sample diluent and HRP-conjugates).

STORAGE AND STABILITY

The reagents are stable at 2-8^{o}C until the expiration date printed on the label. ***Diluted*** enzyme conjugate should not be stored. Any unused mixture of substrate must be discarded and not returned to the bottle.

Items Required but not supplied with this kit

High binding ELISA plates (Cat #80011) *Strip Plates* (Cat # 80012 are suitable for coating antigen or antibody. High binding plates, non-strip, from other manufacturers can be used as well.

An ELISA plate reader (Cat # MPR-504 or from other manufacturers) will be necessary to measure color and express results in a quantitative fashion. The presence of antigen or antibody can still be detected by visual inspection of color.

ELISA plate washer (Cat # MPW-30) is recommended for washing the whole ELISA plate in a consistent manner. in the absence of an automated ELISA plate washer, plates can be washed by using semi-automatic ELISA, gravity-fed *Precision pipettes* (1-200 µl; preferably multichannel 8 or 12 channels) for processing and dispensing samples.

Disposable pipette tips for dispensing reagents. *Microtubes* for test tubes for dilution of samples and reagents.

8-channel washers or manually using a wash bottle filled with wash buffer.

Preparation of Solutions for ELISA

Antibody-HRP Conjugate preparation

ADI's ELISA "Ensemble" is provided with anti-Chicken IgG/IgY-HRP conjugate stock **(0.15 ml).** The conjugates is made against highly pure antigen (whole molecule). It will detect all Ig's (IgA, IgG, and IgM). The antibody-HRP conjugates are supplied as concentrated solution in a stabilizing buffer and must be diluted before use. A suggested starting dilution is 1:2000 with the working solution of antibody/conjugate diluent. It can be diluted up to 1:10,000-1:20,000 to suppress background in preimmune samples or in control wells. Store at 4^{o}C.

***Wash Buffer Concentrate* (100X)**. Occasionally, crystals may form at 4°C but these redissolve at room temperature or slight warming with warm water. Before use, dilute 1:100 with distilled or deionized water. Store at room temp or 4°C. Diluted solutions can be stored at room temp. for about 1-2 week.

***Coating Buffer*, 10x,** A neutral pH buffer solution. **Dilute 1:10 before use**. Prepare as necessary and keep the unused buffer at 4oC.

***Blocking buffer*, 10 X**, **Dilute 1:10 with water and prepare as necessary**. It contains bovine milk proteins and it should be used at 200 ul/well. It contains an antigen/antibody stabilizing substance and Proclin-300 (0.1%). Store at 4oC. If milk interferes with your assay, you must use other blocking buffers (please call us for alternate buffers).

***Ready-to-Use TMB Substrate Solution**, 100 ml.* (do not contaminate the bottle; withdraw as much as needed and do not return solution to the bottle to avoid contamination. Store at 4°C.

***Stop Solution**,* 10X, 10 ml (A diluted H2SO4 solution). Dilute 1:10 before use. Store at room temp. or 4oC.

Serum Sample/Conjugate Diluent, Red Color (#80170), 10 X, Dilute 1:10 with water and prepare as necessary. It contains Proclin-300 (0.1%) as preservative. This is a general purpose diluent for chicken serum, plasma or culture medium or allantoic fluid samples.

Whole Egg Yolks/IgY Sample/Conjugate Diluent, Green Color (#80171), 10 X, Dilute 1:10 with water and prepare as necessary. It contains Proclin-300 (0.1%) as preservative. This sample diluent is specially developed to dilute the viscous egg yolks. Samples diluted in this buffer can be used directly without purifying the IgY.

Preparation of whole egg yolk for antibody assay

Notes: Chicken (birds) not only produce antibodies to a given antigen and secrete it into the serum just like mammals but they also lay eggs and concentrate the antibody in egg yolks (designated IgY). Antibodies (IgG or IgY) are the same and the anti-IgG-HRP will detect both IgG and IgY. Due to the presence of high concentration of many other proteins and lipids, it is not possible to detect antibodies directly in egg yolks unless the IgY can be dissociated from other proteins and uniform solution is prepared. ADI has developed a special diluent that allows egg yolk dilution and used directly for the detection of antibodies.

We recommend collecting whole eggs and separating the egg yolk. Measure the volume of each egg yolk (e.g. 20 ml) and adding at least equal volume of egg yolk sample diluent (dilution 1:2). Mix it thoroughly using a pipette and them make further dilutions (1:100, 1:1000, 1:10,000 etc) or other dilution scheme. Egg yolk diluted 1:2 can be stored frozen in small aliquots and used to prepare further dilutions as necessary. Do not store highly diluted solution of egg yolks.

A GENERAL ELISA PROCEDURE

Prepare all working solutions of antigen coating buffer, blocking buffer, wash buffer, sample/conjugate diluents as specified on page 4. All stock solutions are diluted in distilled water. Prepare in the amounts that are required for the assay and store all unused stock in cocn form at 4oC.

COATING

1. Make 1-10 μg/ml solution of antigen in **Coating buffer** (do not store diluted antigen).
2. Coat **high binding ELISA plates** by dispensing 100 μl of solution/well. Incubate at RT for 3-6 h or overnight at 4°C.

BLOCKING

1. Aspirate coating solution and Block plates with 200 μl of **Blocking buffer/well.** Incubate plates for 2-6 h at RT or overnight at RT.
2. Aspirate Blocking buffer and tap over paper towels to remove traces of liquid. There is no need to wash plates again as it will wash out the stabilizing solution present in the blocking buffer. Air dry plates by leaving at RT for 30-60 min. Store plates in sealed bag at 4°C until used (Plates blocked in the above blocking buffer will remain stable for up to several months depending upon the nature of antigen).

ANTIBODY ASSAY

7. Dilute preimmune serum and antiserum in **Antibody/conjugate diluent** (serial dilution 1:100-100,000 K). Following dilution scheme can be used.

Serum sample designation	Serum Sample	Antibody diluent	Total Volume	Sample Dilution
A	25 ul undiluted serum	225 ul	250 ul	1:10
B	25 ul of A	225 ul	250 ul	1:100
C	25 ul of B	225 ul	250 ul	1:1K
D	25 ul of C	225 ul	250 ul	1:10K
E	25 ul of D	225 ul	250 ul	1:100K

Notes: It is recommended to perform the assay in duplicate using 100 ul per well. A total of 200 ul will be used per assay. It is possible to modify the dilution scheme if more or less sample is available or used for the assay. Other sample dilution scheme such as 1K, 1:5K, 1:25, 1:125 can also be used.

Dispense 100 μl/well. Cover plates with **Adhesive film** ELISA plates or saran wrap to prevent evaporation. Incubate at RT for 30 min. (incubation time may be increased to increase binding). After incubation, wash plates with Washing buffer (3X, 200 μl/well; tap plates over paper towels between washings to remove traces of liquid). Improper washing will lead to high back ground. It is recommended to use automated plate washer for economy and consistency.

8. Dispense 100 μl/well of diluted antibody-HRP conjugate. Cover plate and incubate at RT for 30 min.. After incubation, wash plates **4X** as in step 1. Remove all traces of liquid by tapping over a clean paper towel.