ELISA PROCEDURE SUMMARY
Total Assay Time - 75 min. (30 + 30 + 15 min.)

	Bring all reagent to room temp. Prepare working dilutions of all required solutions (coating, blocking, antibody-conjugates, conjugate diluent, and stop solution).
Step 1	Coat ELISA plates with protein (1-10 ug/ml; 100 ul/well in coating buffer.
Step 2	Aspirate antigen soln and block plates with diluted blocking buffer (200 ul/well). Drain excess liquid. There is no need to wash; Air dry plates or use directly.
Step 3	Dilute primary antibody/serum samples (use optimized concentrations) in antibody/conjugate diluent. Incubate with the plates for **30 min.** at room temp (Gentle rocking may be used).
Step 4	Wash the membrane 3X with diluted wash buffer. Improper washing will cause high background.
Step 5	Add 100 ul Ab-enzyme conjugate (diluted 1:2K or more in antibody/conjugate diluent). Incubate at room temp. for **30 min.**
Step 6	Wash plates 4X as above to remove unbound enzyme labeled antibodies.
Step 7	Add 100 ul of ready-to-use TMB substrate/well. Incubate plates at room temperature for **15 min.** (positive samples will give blue color). Shake gently.
Step 8	Stop reaction by the addition of 100 ul/well stop solution (blue color turns yellow). Measure absorbance at 450nm. The color should be read within 30 min.

KIT PROFILE

Date received:_____ **Cat #** **Lot #** _____ **Exp.** _____

Date kit opened _____ **Technician:** _____

Date used: _____

Remarks: _____

_____

_____

Instruction Manual No. M-80150-80188

ELISA "Ensemble" for the Detection of Various Primary Antibodies

For mouse (#80150), rat (#80155), rabbit (#80160), goat (#80165), sheep (#80166), human (#80170), G. pig (#80171), donkey (80172), horse (80173) monkey (#80175), chicken (#80176), hamster (#80180), bovine (#80185), pig (#80186), turkey (#80187), ferret (#80188), cervid (Deer, Elk, Moose #80120) primary antibodies

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

ALPHA DIAGNOSTIC INTERNATIONAL

India Contact:

Life Technologies (India) Pvt. Ltd.
306, Aggarwal City Mall, Opposite M2K Pitampura, Delhi – 110034
Ph: +91-11-42208000, 42208111, 42208222

Mobile: +91-9810521400
Fax: +91-11-42208444
Email: customerservice@atzlabs.com
Web: www.atzlabs.com

LIFE TECH Life Technologies™
Your Molecular & Cell Technology Partner

DRAFT MANUAL: PLEASE CONSULT THE MANUAL SUPPLIED WITH THE KIT FOR ANY LOT SPECIFIC CHNAGES.

ELISA "Ensemble" for the detection of Primary Antibodies

for Cat #80120, 80150-80188

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

Components	Cat. No.
The kit contain one of the following secondary antibody-HRP conjugate stock, 150 ul (dilute 1:2K-1:10K before use) *mouse (#40321), rat (#50321), rabbit (#20321), goat (#30321), sheep (#30326), human (#10321), G. pig (#50326), monkey (#70021), chicken (#60321), hamster (#80321), bovine (#80521), pig (#90321), turkey (#80187-C), ferret (#80188-C) and Cervid (80120-C)*	
Coating Buffer (10X), 25 ml	80050
Blocking Buffer (Milk-based) 25 ml (10X)	80062
Sample/Conjugate Diluent (10 X); 25 ml	80070
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 given species (mouse, rat, monkey etc) antibodies in serum and determine antibody titer using the antigen-coated plates. It is not suitable for the quantitation a given species IgG (mouse, rat, monkey etc). We have other ELISA kits to measure mouse, rat, bovine, Human, goat and sheep IgGs.

TROUBLE SHOOTING AND GENERAL NOTES

1. Always incorporate a positive control, negative control, and reagent control (blank).
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 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.

Specificity of the ELISA kit

This kit is designed to detect antigen specific (user supplied) antibodies in animal's serum. Typically, the antibody presence is expressed in dilution. 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 bovine serum (contact ADI or see web site for details).

The conjugate supplied in the kit is anti-IgG (H+L; animal species)-HRP. It will detect IgG+IgA+IgM. If specific antibody detection is desired then it is possible to use isotype specific antibody conjugates (available from ADI). This kit has not been tested in other animals. ADI also supplied animals specific antibody detection kits.

ELISA Template for the placement of samples –(The ELISA template can be used to plan for the preparation of blank (no sample; antibody diluent only), various samples dilutions).

	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**

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.

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.

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.

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

Following Items will increase sample processing, efficiency, and accuracy and but they are not an absolute requirement (Optional)

Microtube racks (96 tubes/rack; 10 racks/case, 1.1 ml capacity, compatible with multichannel pipettes, Cat # 80020). Samples can be diluted in these tubes and dispensed using a multichannel pipette avoiding delays in dispensing from single tubes.

Reagent Reservoirs (sterile, 50 ml capacity, cat # 80030; 100/Cs), Ideal for dispensing conjugate and substrate using a multichannel pipette.

Adhesive films for ELISA plates (Cat # 80040; 50/pk), Peel-off film made specially for covering ELISA plate during incubations.

ELISA plate reader (Cat # MPR-504), capable of reading the 96-well ELISA plates in just a few seconds, processing and plotting the data.

ELISA plate Washer (Cat # MPW-30), capable of washing the ELISA plate using programmed washing (2-5 times).

Preparation of Solutions for ELISA

Antibody-HRP Conjugate preparation

ADI's ELISA "Ensemble" is provided with anti-IgG (see species on the vial)-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 10K (or more) to suppress background in preimmune samples or in control wells. Store at 4°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**.

***Blocking buffer*, 10 X**, The milk-based blocking buffer can 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). **Dilute 1:10 with distilled water before use.**

Antibody/HRP-Conjugate Diluent, 10X; A red dye has been added for better visibility and contains Proclin-300 (0.1%) as preservative. Store at 4oC. If BSA or goat/calf serum interferes with your assay, you must use other buffers (please call us for alternate buffers). **Dilute 1:10 before use**. *Do not store diluted conjugate solutions.*

***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.

A GENERAL ELISA PROCEDURE

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

1. 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 Bovine 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.

2. 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.

3. 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.

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). 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).