

# ELISA Technique

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**Abstract::** The sensitivity and convenience are important to the biological measurement. ELISA (enzyme-linked immunosorbent assay) supplies a sensitive and convenient technique in the biological research, especially in the quantity. This article is describing the basic protocol of ELISA for the general purpose.

## Introduction

ELISA is the abbreviation of enzyme-linked immunosorbent assay. It is a useful and powerful method in estimating ng/ml to pg/ml ordered materials in the solution, such as serum, urine and culture supernatant (Savige et al. 1998).

The basic principle of an ELISA is to use an enzyme to detect the binding of Ag and Ab. The enzyme converts a colorless substrate (chromogen) to a colored product, indicating the presence of Ag:Ab binding. An ELISA can be used to detect either the presence of Abs or Ags in a sample, depending on how the test is designed.

## Materials and Methods

Homogenize tissue with 5 times of protein extract buffer → centrifuge 10,000 rpm 20 minutes → 0.1 ml supernatant each well → over night at 4°C → PBS with 0.5% BSA washing 3 X 3 minutes → 0.1 ml diluted primary antibody 1-2 hour at room temperature → PBS washing 3 X 3 minutes → 0.1 ml diluted secondary antibody 1-2 hour at room temperature → PBS washing 3 X 3 minutes → dye (0.2 ml pNPP) → 0.05 ml 3 N NaOH → O.D. (405 nm) measurement.

### 1. Extract buffer:

50 mM Tris-HCl or 50 mM HEPES (pH 7.4)  
150 mM NaCl  
0.02% sodium azide  
0.1% SDS  
0.1 mg/ml phenylmethylsulfonyl fluoride (PMSF)  
0.001 mg/ml aprotinin  
1% Nonidet P-40 (NP-40) or 1% Triton X-100  
(The half-life of a 0.02 mM aqueous solution of PMSF is about 35 minutes. PMSF is usually stored as a 10 mM or 100 mM stock solution 1.74 or 17.4 mg/ml in isopropanol at -20°C).

### 2. Homogenize sample under ice.

3. **Centrifuge sample** at 10,000 rpm for 20 minutes at 4°C.

4. **Keep supernatant** at -70°C until usage.

5. **PBS:** Phosphate-buffered saline (PBS), pH 7.4, 1000 ml (NaCl 8 g, KCl 0.2 g, Na<sub>2</sub>HPO<sub>4</sub> 1.44 g, KH<sub>2</sub>PO<sub>4</sub> 0.24 g, adjust to pH 7.4 with HCl). Add 0.5% BSA of 1% milk into PBS when washing processed. It can also use Dulbecco's PBS or try

others. Instead of BSA, it can use gelatin or milk. Skim (0.5% to 1%) milk could reduce the non-specific reaction.

6. **Primary and secondary antibodies** are normally 1:1000 to 1:2000 diluted by PBS and 0.1 ml each well.
7. **Use alkaline phosphatase yellow (pNPP)** liquid substrate as the dye for the ELISA (Derango et al. 1996). This product is supplied as a ready-to-use buffered alkaline phosphatase substrate p-nitrophenylphosphate (pNPP). Prior to reaction with alkaline phosphatase, the substrate should appear as a colorless to pale yellow solution. It will develop a yellow reaction product when reacted with phosphatase in microwell applications. For the end-point assays, the reaction can be stopped with 0.05 ml/well of 3 N NaOH for every 0.2 ml of substrate reaction. Following the reaction with alkaline phosphatase, a yellow reaction product forms can be read at 405 nm.

## More Information

### 1. Using Polyclonal Antibodies:

- (1) Antibody purification: Protein G column is the best for this purpose.
- (2) Conjugate: Making conjugate is the most important part (e.g. horseradish peroxidase).
- (3) 96-well plate: Making the solid phase using the 96-well plate.

### 2. Buffers and other reagents:

- (1) Plate buffer: 0.1 M Sodium carbonate buffer, pH 9.5.
- (2) Reaction buffer: 0.01 M Sodium phosphate buffer, pH 7.2, 0.15 M NaCl (PBS), 0.5% BSA, 0.05% thimerosal; You can also use Dulbecco's PBS or try others. Instead of BSA, you can use gelatin. Skim (0.5% to 1%) milk could reduce the non-specific reaction.
- (3) Washing buffer: 0.05% Tween-20, 0.01 M Sodium phosphate buffer, pH 7.2 or 0.05% Tween-20, 0.15 M NaCl.
- (4) Developing buffer: 0.05 M Sodium acetate buffer, pH 5.5.
- (5) TMB stock solution: Tetramethylbenzidine 1 mg/ml in DMSO.

### 3. Making Conjugate

- (1) Nakane's method.
- (2) Glutaraldehyde method.
- (3) Maleimide method.

### 4. Steps.

- (1) 2 mg Horseradish peroxidase (HRP) in 1 ml water: A.
- (2) 21.4 mg NaIO<sub>4</sub> (never to be NaIO<sub>3</sub>!) in 1 ml water: B.
- (3) 100 micro-l of B into A: Color will change to the dark green!
- (4) Wait for 10 min at room temperature.
- (5) Put into the dialysis tube (such as Molecular cut off 20,000).
- (6) F. Put the tube into 5 mM NaAcetate buffer, pH 4.0 in a 2 to 3 l flask.
- (7) Dialysis overnight: Color will change to the gold.
- (8) Raise the pH of the HRP solution to pH = 9.0 by the addition of 0.2 M NaCarbonate buffer, pH 9.5 (try an aliquot of 0.05 ml).
- (9) Mix with the antibody solution (8 mg of IgG in 1 ml), which has been pre-dialyzed to 0.01 M NaCarbonate buffer, pH 9.0 overnight.
- (10) Incubate the mixture for 2 hr at room temperature.
- (11) Put freshly prepared 0.1 ml, 0.1 M NaHBr<sub>4</sub> in water to the solution.
- (12) Incubate at 4 degree for 2 hr.
- (13) Put the mixture into a dialysis tube and dialyze against PBS overnight.
- (14) Now the conjugate solution is ready for use. Add thimerosal to a final concentration of 0.02% for preservation. Add glycerol to a final concentration of 10% (optional). If you stock the conjugate solution for a long period such as years, stock it at -80 degree. But, in this case, don't repeat freeze-thaw. You can stock the solution at 4 degree at least 6 months.

### 5. Preparation of ELISA Plate

This will take 2 hr to overnight. Overnight is preferable.

- (1) Dilute antibody (IgG) by Plate buffer: 5 to 10 micro-g/ml.
- (2) Put the diluted antibody solution, 0.1 ml to the wells of 96-well ELISA plate.
- (3) Incubate for 2 hr at room temperature or overnight at 4 degree.

- (4) Discard the solution and wash the plate three times by washing buffer. Put 200 micro-l into wells using micro-pipette or just put the Washing buffer using some devices.
- (5) Discard the Washing buffer by tapping against paper towel.
- (6) Put 0.15 to 0.2 ml of reaction buffer. Now, the plate is ready for use. You can stock the plate at least for 6 months. Take care not to dry up the plate.

### 6. Using Monoclonal Antibodies

Antibody purification: Antibody purification step is the only special part comparing with materials and methods in using polyclonal antibody. For most monoclonals, except for IgM, Protein G column will be good for the practical use. If you failed by this method, confirm your procedure again before proceeding to the other methods such as DEAE column. When your monoclonal antibody is IgM, try Protamine column combined with molecular sieving column. Others are the same as above mentioned in "Using polyclonal antibodies.

Try skim milk (any kind of powdered milk such as powdered milk for babies) instead of BSA. It's really cheap! Try 1% to 3%. It will decrease the background!! Thing is stability. It will form precipitate if you keep it for a few months. If you are running many plates, it is good alternative.

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