

CHAPTER 47

Enzyme-Linked Immunosorbent Assay (ELISA)

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1. Introduction

The ELISA technique (1) is a simple, sensitive, rapid, reliable, and versatile assay system for the quantitation of antigens and antibodies. Because of the extreme discriminating power of antibodies to recognize an almost infinite array of antigenic structures, the application of ELISA to analyte measurement is almost unlimited. ELISAs have been developed in many configurations depending on the particular application of the assay. In practice, ELISA as a solid-phase technique may be classified into two main types: (1) competitive assays using either an antigen-enzyme conjugate or an antibody-enzyme conjugate, and (2) noncompetitive assays using a double antibody "sandwich" technique where the second antibody has an indicator enzyme conjugated to it. In solid-phase ELISA, one of the immunoreactants (antibody or antigen) is immobilized onto a solid support (microtiter plate) by adsorption, through noncovalent interactions. The immobilized antibody is then incubated with test solution containing the analyte of interest. Following a period of incubation and washing, the bound antigen is detected, by the addition of an enzyme-conjugated antibody that binds to the remaining antigenic sites on the antigen. Following further incubation and washing, the amount of antigen present is visualized by the addition of a chromogenic or fluorogenic substrate, depending on the sensitivity

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required. The sensitivity of any immunochemical procedure depends on the amplification system used to detect the specific interaction between antigen and antibody. ELISA relies on the production of color produced by an enzyme-labeled antibody or antigen, following hydrolysis of a chromogenic substrate. Since one molecule of an enzyme can catalyze the hydrolysis of many substrate molecules, a direct and simple, high-amplification system is achieved. The concentration of the substrate product is directly proportional to the concentration of standards used in the assay, as well as the analyte antigen. The choice of ELISA system is determined primarily by the specificity and purity of available antibodies or antigens. When two purified monoclonal antibodies with specificities for different epitopes on the antigen are available, the method of choice is the "sandwich"-type ELISA. This is also the case with having one or two purified polyclonal antibodies. In the case of having only a single antigen-specific polyclonal antibody, the antibody can be directly conjugated to an enzyme to form the top layer of the "sandwich," while using the unlabeled antibody as the coating reagent. In other conditions, a competitive assay employing either a labeled antigen or antibody is the method of choice. The ELISA method described here is the noncompetitive double antibody "sandwich" method (*see* Fig. 1) using an alkaline phosphatase detection system. Depending on the affinity of the antibodies available, the limit of detection for protein antigens can be as low as 10 pg/mL.

2. Materials

(for Noncompetitive Assays)

1. 96-well flat-bottomed polystyrene microtiter plates.
2. Adjustable micropipets, 0–20, 20–200, 200–1000 μ L.
3. Multichannel pipet 0–250 μ L (eight channel).
4. Plate washing device: Automated plate washer; manual hand-held pipet connected to a reservoir; wash bottle or multichannel pipet.
5. Manual or automated microplate ELISA reader.
6. 0.1M Carbonate/bicarbonate buffer, pH 9.6: Make up 0.1M solutions of Na_2CO_3 (10.6 g/L) and NaHCO_3 (8.4 g/L), then mix 29.3 mL 0.1M Na_2CO_3 with 70.7 mL 0.1M NaHCO_3 . Note: The bicarbonate solution is unstable and should not be kept for more than 1 wk.

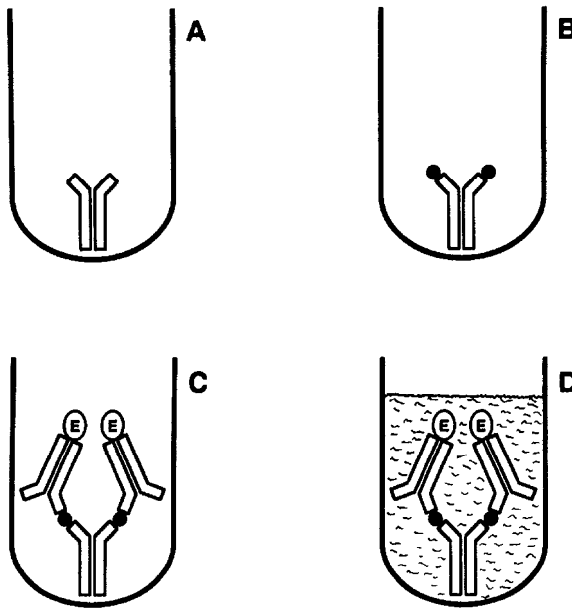


Fig. 1. Schematic representation of ELISA technique (noncompetitive assay). (A) Solid-phase adsorbed antibody on microwell plate. (B) Formation of antigen-antibody complex. (C) Detecting enzyme-labeled second antibody combining with "solid-phase" antigen "sandwich." (D) Addition of substrate results in color development.

7. PBS-Tween: Make up 0.5M phosphate-buffered saline (PBS), pH 7.2. Weight out 8.0 g NaCl, 0.2 g KCl, 1.15 g Na₂ HPO₄, and 0.2 g KH₂ PO₄. Dissolve in 1 L distilled H₂O. Then add 0.5 mL Tween 20.
8. 10% Diethanolamine buffer, pH 9.8: Dilute 9.7 mL diethanolamine with 80 mL distilled H₂O and adjust to pH 9.8 with 1M HCl. Make up to 100 mL with distilled H₂O (stable at 4°C).
9. Coating antibody: IgG fraction or affinity-purified polyclonal or monoclonal antibody, specific for the analyte (commercial sources or laboratory prepared). (*See Chapter 43.*)
10. Enzyme-conjugated antibody: Alkaline phosphatase conjugated IgG specific for the analyte (commercial sources or laboratory prepared; *see Chapter 44*).
11. Reference reagent: A pure preparation of the analyte, for setting up a reference curve.

12. Substrate: *p*-nitrophenyl phosphate (1 mg/mL) in 10% diethanolamine buffer, pH 9.8.

3. Method

1. Dilute the coating antibody to 10 $\mu\text{g/mL}$ protein in 0.1M carbonate/bicarbonate buffer, pH 9.6 (*see* Note 1).
2. Add 100 mL of diluted antibody to each well of the microtiter plate. Cover the plate with plate sealer, and incubate overnight at 4°C.
3. Aspirate each well of the plate, and wash with three to five quick changes of PBS-Tween (fill each well). After the last wash, blot the plate against clean paper toweling to remove any remaining wash buffer (*see* Note 2).
4. Add 100 mL of each dilution of standard antigen reagent, in triplicate, to the plate. Doubling dilutions of standard reagent are made up freshly, usually in the range 10–1000 pg/mL (*see* Note 3). Add 100 μL of appropriate dilutions of test antigen in triplicate, diluted in PBS-Tween, to the plate. PBS-Tween without added antigen can be used as a negative control. Cover the plate, and incubate at room temperature (approx 20°C) for 2 h to allow antigen binding to the antibody-coated plate to occur (*see* Note 8).
5. Empty and wash the plate as in step 3 above. Add 100 μL of optimally diluted enzyme-conjugated antibody (approx 1/1000 dilution) to each well (*see* Note 1). Cover the plate and incubate at room temperature for 2 h (*see* Note 6).
6. Empty and wash plate, as described in step 3. Add 100 μL *p*-nitrophenyl phosphate solution. Cover the plate and incubate at room temperature for 30 min.
7. If required, the reaction can be stopped by adding 100 μL 3M NaOH to each well.
8. Read the absorbance at 405 nm (*see* Note 10.)
9. A standard curve of absorbance vs $\log_{10}$ antigen concentration is constructed, and the concentration of the antigen in the test sample is read off the standard curve (*see* Note 4).

4. Notes

1. The optimum concentration of coating antibody and enzyme-conjugated antibody should be determined in advance, by a series of reagent combination experiments. This is best carried out by a checkerboard-type titration experiment. Doubling dilutions of both coating antibody and detecting antibodies are set up according to the experimental conditions of the test system. An “ideal” antigen concentration (e.g., 100

ng/mL) which would be expected to be within the sensitivity range of the assay, is included in the test. The optimal concentration for each antibody is the combination for each antibody giving maximum signal. Where a double antibody system is used to detect the bound antigen to the antibody-coated plate, a titration experiment should be carried out to determine the optimum concentration of each of the two antibodies in the detection system.

2. Antibody-coated plates can be stored, after thorough washing and drying, for 7–14 d at 4°C, provided the plate is well sealed.
3. Tween 20 is added to PBS in the washing solution and the reagent diluent to minimize nonspecific protein binding to the solid surface. It is sometimes necessary to add 1% protein (bovine serum albumin [BSA]) to the PBS-Tween buffer to reduce nonspecific binding of the enzyme–antibody conjugate or sample further to acceptable levels. The coating antibody can be either a monoclonal or polyclonal antibody.
4. Where the “background signal” remains unacceptably high after the addition of BSA to the diluent and washing buffers, a “blocking” step may be introduced after Section 3., step 3 as follows: Add 100 μ L PBS-Tween–1%BSA to each well. Cover the plate and incubate at room temperature for 1 h. Empty and wash the plate as described in Section 3. Continue on to Section 3., step 4.
5. If a suitable quality antibody is available, it is advisable to use a F(ab)₂ fragment of both the coating and the enzyme-labeled antibody, in order to minimize crossreactivity of rheumatoid factors in biological fluids, with the Fc portion of IgG.
6. Where only a nonenzyme-conjugated detecting antibody is available, an extra step can be introduced to detect antigen. Following incubation with the nonconjugated antibody, the plate is emptied and washed as in Section 3., step 3. One hundred microliters of optimally diluted (*see* Note 1) alkaline phosphatase conjugated antisppecies antibody (specific for the second antibody, which must be different from the species of the coating antibody) are added to each well and incubated at room temperature for 2 h.
7. When monoclonal antibodies are used, the epitope specificity of the coating antibody must be different from that of the antigen conjugated antibody detection system. Although this is also preferable when using polyclonal antibodies, it is possible to obtain perfectly acceptable data using the same polyclonal antibody on both sides of the “sandwich.”
8. Duration of incubation times with test solutions, antibody–enzyme conjugate, and substrate may be reduced or extended to achieve maximum sensitivity, depending of the quality of both antibodies.

9. Because of the extreme sensitivity of the technique, particular care should be taken with pipeting techniques, dilution steps, preparation of standard curve reagents, and so forth. Make sure all pipets are precisely calibrated and that the quality of tips is sufficiently high.
10. If a dual-wavelength ELISA plate reader with wavelength correction is available, set it to 540 nm. This will correct for optical interference owing to imperfections in the microtiter plate. Readings made directly at 405 nm can be used, but will be higher than those using the wavelength correction factor.

References

1. Engvall, E. and Perlmann, P. (1974) Enzyme linked immunosorbant assay (ELISA). Quantitative assay of immunoglobulin G. *Immunochemistry* **8**, 871–874.