

Introduction

Enzyme-linked Immunosorbent Assays (ELISAs) are useful tools for the identification of unknown quantities of antibodies and or antigens. ELISA's combine the specificity of antibodies with the sensitivity of simple enzyme assays, by using antibodies or antigens coupled to an easily-assayed enzyme. ELISAs can provide a useful measurement of antigen or antibody concentration typically in the pg range. There are two main variations on this method: ELISA can be used to detect the presence of antigens that are recognized by an antibody or it can be used to test for antibodies that recognize an antigen.

There are three common Types of ELISAs

1. Direct ELISA: the simplest method for detecting of antigen. Requires the primary antibody to be labeled to an enzyme. No secondary antibody
2. Indirect ELISA: Capable of detecting either antigen or antibody. Used to screen tissue culture fluids for monoclonal antibodies. The Primary antibody is unlabeled and the secondary antibody is labeled to an enzyme.
3. Sandwich ELISA: This is the preferred method when the amount of unknown antigen is too dilute to be detect\able when directly adsorbed onto the Microtiter plate surface or when the presence of unknown constituents interferes with antigen detection. In this method an unlabeled antibody is absorbed onto the plate first followed by the antigen. Then a secondary enzyme labeled antibody directed against the antigen is added.

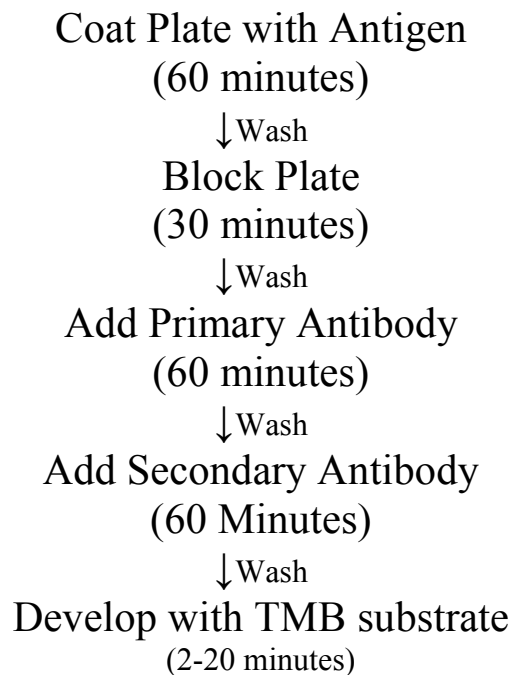
Described below is a simple guideline for an indirect ELISA.

The Indirect ELISA is a five-step procedure:

- 1) Coat the microtiter plate wells with antigen.
- 2) Block all unbound sites to prevent false positive results.
- 3) Add antibody to the wells.

- 4) Add the secondary antibody coupled to an enzyme.
- 5) Reaction of a substrate with the enzyme to produce a colored product, thus indicating a positive reaction.

Flow Diagram of the EZ-Assay ELISA Kit Procedure (indirect ELISA method)



Preparation of Reagents

1. For all 10 X concentrate solutions: Dilute concentrate solution 1/10 with reagent quality water. (i.e. mix 1 ml of the coating solution concentrate to 9 mls of reagent quality water).If crystals appear in any of the concentrate solutions then warm to room temperature or 37° C with mixing to redissolve crystals. Only dilute what will be used **within 2 weeks**. After 2 weeks discard any remaining solution.

2. HRP labeled secondary antibody: We recommend a dilution range of 1:1000 to 1:2500 in ELISA diluent/wash solution.
3. Positive Control: Dilute positive control 1:100 in ELISA coating buffer.

Instructions

The following method is recommended as a guideline for the EZ-Assay ELISA kit.

1. Dilute your antigen or antibody to a concentration of 1-10 ug/ml in the 1 X coating buffer. Add 100 ul of the diluted antigen/antibody to the appropriate wells. (Incubate at room temperature for 1 hour while shaking or overnight at 4° C. At this point you may include a positive control by preparing the control the same as the antigen and adding the diluted protein to the appropriate well.
2. Add 200ul of the blocking solution to each well. Incubate for one hour at room temperature. Empty plate and tap out residual liquid.
3. Add 100 ul of the diluted primary antibody to each well. Incubate at room temperature for one hour with gentle shaking.
4. Fill each well with 100 ul of wash solution. Invert plate to empty, tap out residual liquid
Repeat 3 times.
5. Add 100 ul of the diluted secondary antibody (diluted 1:500 to 1:2500 in the diluent buffer) to each well. Incubate at room temperature for one hour with gentle shaking.
6. Empty plate, tap out residual liquid and wash as in step four.

7. Add 100 ul of substrate to every well after sufficient color development either read plate in a plate reader set at 650nm or add stop 100ul of stop solution (1 N HCL) to every well and read plate in plate reader set at 450 nm. If measuring absorbance using a dual wavelength mode then subtract the absorbance at 650nm from 450 nm.

Additional Notes

Plate Coating

There are a number of factors that can influence plate coating such as antigen or antibody concentrations, pH, ionic strength, temperature and incubation time. In addition the amount of protein that binds has been shown to be inversely proportional to the molecular weight of the protein.

In the first step the antigen or antibodies diluted in the coating buffer is added to the plate. It is important that the antigen/antibody is the purest preparation possible. Any contaminating proteins could compete with the antigen/antigen for binding to the plate. In general, a concentration of 1-10 ug/ml of protein incubated for one hour at room temperature with gentle agitation will give good coating.

Following coating, the plate is blocked by the addition of the blocking solution. This step blocks all unreactive sites and reduces non specific binding thereby reducing background.

Plate Washing

The plate is washed after addition of the sample, primary antibody and secondary antibody. This will help reduce background by

removing unbound reactants for the wells. During the wash steps make sure the washes are uniform with no carryover from well to well. Between washes the plate should be completely emptied. This can be done by tapping the plate upside down on a clean absorbent material like a paper towel or Kim wipe. If the procedure is interrupted at any point, make sure the wells have wash solution in them. Do not let the plates dry out at any time.

Dilution of the primary antibody.

The concentration of the primary antibody is critical to the success of the assay. If too high a concentration of the primary antibody is used the result will be too much background. If too low a concentration of the primary antibodies is used then a low or no signal will result. Therefore the concentration of the primary antibody needs to be determined empirically. The signal generated by a sample containing analyte, relative to the signal of the same sample without analyte, is the signal: noise ratio. As the signal to noise ratio increases, the assay becomes better at measuring small amounts of antigen.

To establish the optimal dilutions of the antibodies, a checkerboard titration, is performed. A checkerboard titration is a single experiment in which the concentration of the two components is varied in a way that will result in a pattern. This method is used to optimize reagent concentrations. The primary antibody is serially diluted across the top of the plate and the enzyme labeled secondary antibody is serially diluted down the plate. This experimental design permits analysis of different concentrations of the two reagents in each well to obtain the best signal to noise ratio.

Troubleshooting Guide

To increase specific signal:

1. Increase concentration of the primary or secondary antibody (perform checkerboard titration to determine optimal antibody concentrations)
2. Incubate substrate for a longer period of time before stopping.
3. Increase concentration of the antigen used or increase incubation time.
4. Use fewer, more gentle plate washes to check wash procedure.
5. Make sure to include control IgG as a sample in ELISA to ensure that the components of the kit are performing.

To reduce non-specific signal:

1. Decrease the concentration of the antigen used.
2. Decrease concentration of the primary or secondary antibodies (perform checkerboard titration to determine optimal antibody concentrations).
3. Incubate substrate for shorter period of time before stopping.
4. Increase the number of washes.