

Technical Notes

ELISA Immunoassay

Introduction

Enzyme Linked Immunosorbent Assays (ELISA) are primarily used to determine antigen concentrations in unknown samples. Two-antibody based assays are quick and accurate, and when a source of pure antigen is available, this type assay can be transformed to define the absolute amounts of antigen.

Sandwich ELISA requires the use of two antibodies that recognize and bind non overlapping epitopes on the antigen. To this end, either two monoclonal antibodies that recognize discrete sites or one batch of affinity purified polyclonal antibodies can be used.

Usually, one purified antibody is bound to a solid phase, i.e. wells of ELISA-grade plastic microplates or strips (step 1), antigen is allowed to bind in a test solution and unbound proteins are then removed by serial washing (step 2). A second labelled antibody is subsequently added to the assay and allowed to bind to the antigen (step 3). Again, unbound proteins are removed by extensive washing. The amount of antigen is finally quantitated by measuring the amount of labeled antibody that is bound to the matrix (step 4).

Either biotinylated secondary antibodies or enzyme conjugated antibodies can be employed, depending on the sensibility to be achieved. A proper detection method should be then used to quantitate the amount of bound secondary antibody. Biotin-labeled antibodies are detected by using Streptavidin. This protein binds tightly to biotin, forming an irreversible complex. Streptavidin is conventionally labeled with enzymes such as Alkaline Phosphatase (AP) or Peroxidase (HRP) which can be used to convert chromogenic substrates. Conventionally, in those assays that are carried out in microtiter wells, soluble chromogenic substrates which are converted to a soluble colored product are used. The enzyme levels are measured by monitoring the color development and reading the absorbance of the single well by using a spectrophotometer. The most sensitive chromogenic substrate for the detection of horseradish peroxidase (HRP)-labeled reagents is tetramethylbenzidine (TMB). Metabolized TMB turns to a pale blue. Usually, to enhance the final color reaction, the enzyme activity is blocked by adding excess H_2SO_4 , this turns positive wells to a bright yellow color. Results are obtained by reading wells absorbance at 450 nm. The best substrate for AP is paranitrophenylphosphate (pNPP). An EDTA based stopping solution is used to turn metabolized pNPP to a bright yellow as well as for TMB.

Many ELISA washers and readers are also commercially available (Euroclone codes EP-HPW08-240; EP-HPW12-240; EP-RD08-240; EP-RD12-240) which allow to automate most of the procedure by using 96 wells plate. Data storage and reanalysis are greatly simplified when a computer and an ELISA analyzing software are introduced in this system.

Application Notes: Using Sandwich ELISA Immunoassay for Cytokines and Chemokines quantitation.

Cytokine sandwich ELISA are highly specific because antibodies directed against two or more distinct cytokines epitopes are required. Therefore, sandwich ELISA can discriminate between cytokines that might have overlapping biological functions which cannot be solved in a biological assay. Although cytokine sandwich ELISA are very useful for cytokine detection and measurement, they do not provide direct information on the identities and frequencies of individual cytokine producing cells.

In addition, when designing experiments that involve cytokine and chemokine protein measurements using sandwich ELISA, several key factors must be carefully considered:

Cytokine production by stimulated cell populations is transient and the kinetics of expression of different cytokine genes can vary. For these reasons, it is required to collect test samples at different time points to better characterize the kinetic of cytokine-production by a cultured cell population.

Cytokine protein concentrations, measured at any one time point, may reflect simultaneous processes of secretion, uptake by cells and degradation. For these reasons, the level of cytokine protein measured by ELISA may significantly underestimate the cytokine-producing potential of cells. In such cases, the use of complementary techniques such as multi-probe ribonuclease protection assay or intracellular cytokine staining might be required.

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The levels of cytokine detected by ELISA may or may not correlate directly with the levels of biologically active protein. Sandwich ELISA may, in fact, utilize anti-cytokine antibodies that cannot discriminate between the precursor (inactive) and mature (bioactive) forms of a cytokine. In addition, an ELISA may detect partially-degraded cytokine proteins which have retained their immunoreactive properties (i.e., at least two recognizable epitopes) but may have lost their bioactivity. In conclusion, cytokine sandwich ELISA are useful indicators of the presence and levels of cytokine and chemokine proteins but they do not actually provide information concerning their biological activity.

In addition to provide a rich source of information for clinical and research studies, sandwich ELISA for measuring cytokines and their receptors have become increasingly important as diagnostic tools and for monitoring therapeutic regimens, such as biological response modification regimens utilizing recombinant cytokine proteins. In the latter cases, highly optimized sandwich ELISA kits designed to minimize interference or nonspecific reactivities presented by patient samples are highly desirable.

ELISA Protocol: General Procedure

Capture antibody:

1. Dilute the purified capture antibody to 1-10 µg/ml in Coating Buffer (PBS pH 7.2). Add 100 µl of diluted antibody to the wells of an enhanced protein-binding ELISA plate (e.g., Nunc Maxisorp # 468667)
2. Seal plate to prevent evaporation. Incubate overnight at 4°C.

Saturation:

3. Bring the plate to RT, remove the capture antibody solution, and block non-specific binding by adding 250 µl of Saturating Buffer per well.
4. Seal plate and incubate at RT for 2 hrs.
5. Wash 3 times with approximately 400 µl/well Wash Buffer.

Standards and Samples:

6. Add standards and samples (diluted in Standard Diluent Buffer). Add 100 µl of diluted standards and controls, always in duplicate

Detection antibody:

7. Dilute the biotinylated detection antibody to 0.5-2 µg/ml in Biotinylated Antibody Diluent Buffer. Add 50 µl of diluted antibody to each well.
8. Seal the plate and co-incubate the antigen and the detection antibody at RT for 2 hrs.
9. Wash 3 times with approximately 400 µl/well Wash Buffer.

Streptavidin-Horseradish Peroxidase (HRP-Streptavidin):

10. Dilute HRP-Streptavidin conjugate to its pre-titered optimal concentration in Standard Dilution Buffer. Add 100 µl per well.
11. Seal the plate and incubate it at RT for 20-30 min.
12. Wash 3 times with approximately 400 µl/well Wash Buffer.

Substrate:

13. Distribute 100 µl/well TMB "Ready-to-Use" Substrate Solution and incubate at RT (10-15 min) in the dark (wrap plate in aluminium foil) for color development. The color reaction can be stopped by adding 100 µl of Stop Solution (1M Sulfuric Acid).
14. Read absorbance at 450nm with a reference filter set to 630nm or 650 nm.

Coating Buffer

PBS pH 7.2-7.4

Washing Buffer

PBS pH 7.2-7.4, 0.05% tween-20

Saturating Buffer

PBS pH 7.2-7.4, 5% BSA w/v

Standard Dilution Buffer

PBS pH 7.2-7.4, 1% BSA w/v

Biotinylated Ab Dilution Buffer

PBS pH 7.2-7.4, 1% BSA w/v

HRP-Streptavidin Dilution Buffer

PBS pH 7.2-7.4, 0.1% tween-20 and 1% BSA w/v

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ELISA Specimen Collection, processing and Storage

Cell Culture Supernatants: Remove particulates and aggregates by spinning samples at approximately 1000xg for 10 min.

Serum: avoid any intentional stimulation of the cells by the procedure. Use pyrogen/endotoxin free collection tubes. Serum should be removed rapidly and carefully after clotting by spinning at 1000xg for 10 min.

Storage: If not analyzed shortly after collection, samples should be aliquoted (0.2-0.5 ml) and stored frozen at -70°C. Avoid multiple freezing-thawing cycles of frozen specimens.

When possible, avoid the use of badly hemolyzed or lipemic sera. Always remove particles by centrifugation or filtration before to assay. Do not thaw reagents by heating at 37 or 56°C. Let samples and reagents to warm at room temperature. Lyophilized Standards should be discarded after use.

ELISA Helpful Hints

To determine the optimal signal and lowest background for the ELISA, the capture antibody (1-10 µg/ml) and detection antibody (0.25-5 µg/ml) should be titrated against each other in a preliminary experiment. An appropriate range of serial dilutions for the cytokine standard should be included. A suggested range is provided on the Data Sheet for either ELISA kits and matched reagents.

Handling of Standard Reagents: Check carefully the Data Sheet for each recombinant cytokine provided as standard within ELISA kit. Handling instructions are lot-specific. For maximum recovery of cytokine, add the indicated volume of Standard Dilution Buffer and allow to stand 5 minutes with gentle swirling before making dilutions. Serial dilutions of the standard must be always made before each assay and cannot be stored.

High backgrounds in blank wells (i.e., OD > 0.20) or poor consistency of replicates can be overcome by increasing the stringency of washes and optimizing the concentration of capture and detection antibodies. For example, during washes, the wells can be soaked for ~ 1 minute intervals. Moreover, lower concentrations of detecting antibody or more washes after the detecting antibody stage can reduce background.

Using peroxidase as the enzyme for color development, absolutely avoid sodium azide in wash buffers and diluents, as this is a strong inhibitor of peroxidase activity.

If no signal is observed check the activity of the enzyme/substrate system by mixing Streptavidin-HRP and TMB substrate.

References

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