

ELISA Protocols

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Information Enzyme-linked immunosorbant assay (ELISA) refers to a group of assay methods that have in common either an antigen or a specific antibody linked to a solid support, a “sorbant”. The cognate ligand can then bind indirectly to the solid support through binding the “immunosorbant”, enabling either detection of the “sorbant”-bound ligand.

History

In the early 1970's, methods were explored to replace the radioimmunoassay and immunoprecipitation reactions. Radioimmunoassays are sensitive but pose storage and disposal problems. By contrast, assays based on antibody precipitation or agglutination of antigens, are not sufficiently sensitive for many applications.

Engvall and Perlmann (1971) reasoned that a nonradioactive tracer could be attached to an antigen. They chose rabbit IgG as the antigen and alkaline phosphatase as the tracer. They attached anti-rabbit IgG antibodies to an insoluble support material (cellulose) to produce the “immunosorbant”. To measure the amount of rabbit IgG in an unknown sample, known amounts of enzyme-linked antigen were reacted with the immunosorbant, either with or without some quantity of the unknown. Antigen in the unknown was unlabeled, but would compete for binding with the known and labeled antigen, so that enzyme bound to the immunosorbant was reduced according to how much antigen was added with the unknown. This method is now known as a competitive ELISA, a variant that is seldom used today. A similar assay was described by Van Weeman and Schuurs (1971) at about the same time.

Because the solid support forming the immunosorbant was cellulose, centrifugation was required after every step of the process. Today, the solid support is usually plastic microtiter plates, obviating centrifugation.

Since that time, many variants of ELISA have been developed. All incorporate two central ideas: using enzymes as tracers and immobilizing a ligand. Therefore all of them can be described as ELISA, even though some of these assays measure antigen, while others measure antibody.

Purpose

While many ELISA variants have been developed, the premise of each

variant is the same – to assess the reaction between an antigen and its cognate antibody, either quantitatively or qualitatively. Using an antibody preparation of known specificity and quantity, the antigen concentration can be determined; using an antigen preparation of known specificity and quantity, the antigen concentration can be determined.

Theory

Quantification of antigen.

By allowing an excess amount of labeled (directly or through further reaction with a second, labeled antibody specific for the first antibody) antibody preparation to react with a limiting amount of immobilized antigen, the amount of enzyme bound to the antigen would be directly proportional to the amount of antigen present. The amount of bound enzyme, determined by its production of a measurable substrate, reflects the amount of antigen present.

Quantification of antibody.

By allowing a limiting amount of an antibody preparation of unknown concentration to react with immobilized antigen in excess, followed by an excess amount of a labeled antibody specific for the first antibody, the amount of enzyme bound to the antigen would be directly proportional to the amount of antigen present. The amount of bound enzyme, determined by its production of a measurable substrate, reflects the amount of antigen present.

RECIPES AND PROTOCOL

Because many variants of ELISA are in common use, two of the most common will be described here. Both are designed to detect antigen. In this variant of the first, Direct (also known as Sandwich) ELISA, a cell-bound antigen is detected. In this variant of the second, Capture ELISA, a soluble antigen is detected.

Direct (Sandwich) ELISA (Braun *et al.*, 1998.)

This assay detects class II major histocompatibility complex (MHC II) expressed by adherent murine cells.

Step 1. Adhere murine macrophages to the bottom of flat-bottom microwells and either treat them with muIFN γ (5 U/mL) or not. Incubate to allow expression of MHC II for 36 – 48 hours. (ELISA Images Figure 1)

Step 2. Flick-off (invert microplates over a sink and flick with the wrist with confidence) media and add 50 mL 0.1% formaldehyde per well to fix the cells. Incubate at room temperature for at least 10 minutes. At this point, the cells may be kept in the refrigerator for up to a week before continuing the assay.

Step 3. Wash the wells thrice with blocking buffer (Dulbecco's phosphate buffered saline, DPBS, containing 2% nonfat dry milk) by flicking-off the contents and replacing with 200 mL blocking buffer per well. (ELISA Images Figure 2)

Step 4. Flick-off the blocking buffer (ELISA Images Figure 3) and add 50 mL diluted monoclonal antibody from hybridoma 28-16-8S (Ozato and Sachs), IgM anti-Ia^{b,d}, hybridoma culture fluids diluted 1/5 in blocking buffer) to the wells and incubated for 2 hours at room temperature. (ELISA Images Figure 4)

Step 5. Wash the wells thrice with blocking buffer. (ELISA Images Figure 5)

Step 6. Flick-off the blocking buffer and add 50 mL Peroxidase-conjugated goat anti-mouse immunoglobulin antibodies (Pierce, diluted 1/2000 in blocking buffer) and incubated for 2 hours. (ELISA Images Figure 6)

Step 7. Wash the plate thrice with DPBS (without dry milk). (ELISA Images Figure 7)

Step 8. Flick-off the DPBS very well and add 100 mL substrate solution (1 mg/ml 2,2'-Azinobis[3-ethylbenzthiazolinesulfonic acid], ABTS), 0.012% H₂O₂ in 0.025M citrate/0,05% Na₂HPO₄ pH 9.0) to each well. (ELISA Images Figure 8)

Step 9. Incubate the plate for a sufficient period of time for the green colored product to develop. A green color indicates expression of MHC II, and can be quantified by spectrophotometry at 405 nm. (ELISA Images Figure 9), (ELISA Images Figure 10)

Capture ELISA (Assay Designs, Inc., Ann Arbor, MI)

This is adapted from the manufacturer's directions for use of their TiterZyme TNF-alpha assay kit.

Step 1. Obtain a microtiter plate that has been coated with immobilized anti-TNF antibodies. (ELISA Images Figure 11)

Step 2. Pipet 50 mL of the samples into the wells with immobilized anti-TNF antibodies. Tap the plate gently to mix the contents. (ELISA Images Figure 12)

Step 3. Seal the plate and incubate at room temperature on a plate shaker for 2 hours at ~500 rpm.

Step 4. Empty the contents of the wells and wash by adding 400 mL of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer. (ELISA Images Figure 13)

Step 5. Pipet 50 mL of the peroxidase-conjugated rabbit anti-TNF antibodies into each well. (ELISA Images Figure 14)

Step 6. Seal the plate and incubate at room temperature on a plate

shaker for 2 hours at ~500 rpm.

Step 7. Empty the contents of the wells and wash by adding 400 mL of wash solution to every well. Repeat the wash 3 more times for a total of 4 washes. After the final wash, empty or aspirate the wells and firmly tap the plate on a lint free paper towel to remove any remaining wash buffer. (ELISA Images Figure 15)

Step 8. Pipet 50 mL of TMB substrate solution into each well. (ELISA Images Figure 16)

Step 9. Incubate for 30 minutes at room temperature. (ELISA Images Figure 17)

Step 10. Pipet 50 mL 1 N HCl stop solution to each well. (ELISA Images Figure 18)

Step 11. Read the optical density at 450 nm.

SAFETY

All substrates for peroxidase are mutagenic and carcinogenic to some degree, and should be handled with care.

The ASM advocates that students must successfully demonstrate the ability to explain and practice safe laboratory techniques. For more information, read the laboratory safety section of the [ASM Curriculum Recommendations: Introductory Course in Microbiology](#) and the [Guidelines for Biosafety in Teaching Laboratories](#).

TIPS AND COMMENTS

Coating of Wells.

Binding of antigen or antibody to microtiter wells can be conveniently carried out overnight at 4 C. (Ross S. Johnson, Virginia State University)

Microtiter Plates.

Microtiter plates vary in properties, including protein binding and interaction with substrates and products. They can influence the outcome of assays. (Ross S. Johnson, Virginia State University)

Blocking agents.

Any material that can nonspecifically saturate protein-binding sites on microtiter plates can be used as blocking agents. Because blocking buffers are used in fairly large volumes and in fairly high blocking agent concentrations (2 – 5%, w/v), the blocking agents commonly used are bovine serum albumin and casein (in the form of nonfat dry milk), two inexpensive proteins. (Samuel Fan, Bradley University)

Enzymes.

The two most common enzymes used in ELISA are peroxidase and alkaline phosphatase. Many substrates that form colored products that are either precipitable or not are available for these enzymes. (Samuel

Fan, Bradley University)

Substitutes for enzyme-linked secondary antibodies.

Even more convenient than enzyme-linked secondary are enzyme-linked protein A or protein G. These streptococcal proteins are able to bind to the Fc of immunoglobulins of many (but not all) isotypes, so that one reagent can work with many antibodies. Likewise, enzyme-linked biotin can bind to any avidin-linked antibody at extremely high affinity. Because one antibody molecule can be labeled with several avidin molecules, this method can amplify the signal of the assay. (Samuel Fan, Bradley University)

Rates of Reactions can be increased by increasing incubation temperature, and decreased by decreasing incubation temperature. (Ross S. Johnson, Virginia State University)

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