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Enzyme-Linked Immunosorbent Assay (ELISA): A Narrative Literature Review

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A B S T R A C T

Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) are both widely used as diagnostic tools in medicine and as quality control measures in many industries. This literature review was aimed to describe enzyme linked immunosorbent assay in practical. ELISA has been the system of choice when testing soluble antigens and antibodies. EIA/ELISA uses the basic immunological concept of antigen binding to specific antibodies, which allows the detection of small amounts of antigens such as proteins, peptides, hormones or antibodies in fluid samples. In all protocols, solid-phase reagents are incubated with secondary or tertiary reactants covalently coupled with the enzyme. The unbound conjugate is washed and a chromogenic or fluorogenic substrate is added. In conclusion, EIA / ELISA is a powerful method not only for general biomedial research but also as a diagnostic tool. This procedure allows the detection of all types of biological molecules at very low concentrations and quantities. Despite their limitations, EIA/ELISA remains an important tool in both clinical and basic research, and in clinical diagnosis.

1. Introduction

Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) are both widely used as diagnostic tools in medicine and as quality control measures in many industries. These two procedures are also used as analytical tools in biomedical research to detect and measure a specific antigen or antibody in a specific sample. These two procedures share similar basic principles and are derived from radioimmunoassay (RIA).[1,2] RIA was first described as endogenous plasma insulin evaluation. RIA was then developed into a new technique for detecting and measuring biological molecules present in very small numbers, paving the way for the analysis and detection of countless other biological molecules, including hormones, peptides, and proteins. Due to safety concerns regarding their use of radioactivity, the RIA test was modified by replacing radioisotopes with enzymes, creating the modern EIA and ELISA.[3,4]

ELISA has been chosen to test for soluble antigens and antibodies. Factors that contribute to its success include sensitivity, the long shelf life of the reagent (alkaline phosphatase conjugates typically lose only 5% to 10% of their activity per year), lack of radiation hazard, ease of reagent preparation, test speed and reproducibility, and variation of the ELISA format which can be produced with several well-chosen reagents. In addition, no sophisticated equipment is required for many ELISA applications, including screening of the hybridoma supernator for specific antibodies and screening of biological fluids for antigen content.[5]

General principles of ELISA

EIA/ELISA uses the basic immunological concept of antigen binding to specific antibodies, which allows the detection of small amounts of antigens such as proteins, peptides, hormones, or antibodies in fluid samples. EIA and ELISA use enzyme-labeled antigens and antibodies to detect biological molecules. The most commonly used enzymes are alkaline phosphatase and glucose oxidase. The antigen in the fluid phase is immobilized, usually in a 96-hole microtiter plate. The antigen is allowed to bind to certain antibodies, which are then detected by secondary antibodies combined with the enzyme. The chromogenic substrate for the enzyme produces a visible color change or fluorescence, which indicates the presence of an antigen. Quantitative or qualitative measurements can be assessed based on these colorimetric readings. The fluorogenic substrate has a higher sensitivity and can accurately measure the level of antigen concentration in a sample.

A key step in the ELISA test is direct or indirect antigen detection by attaching or immobilizing the specific antigen-capturing antigen or antibody, respectively, directly to the well surface. For sensitive and robust measurements, antigens can be specifically selected from mixed antigen samples by means of antibody "capture". The antigen is thus "caught" between the capturing antibody and the detecting antibody. If the antigen to be measured is small or has only one epitope for antibody binding, a competitive method is used in which the antigen is labeled and competes for the formation of an unlabeled antigen-antibody complex or an antibody is labeled and competes for the bound antigen and the antigen in the sample. Each of these modified ELISA techniques can be used for both qualitative and quantitative purposes.[4]

Although many variants of ELISA have been developed and used in different situations, they all rely on the same basic elements; coating/capture, plate blocking, probing/detection, and signal measurement. The coating is the direct or indirect immobilization of the antigen to the surface of the polystyrene microplate well. Plate blocking is the addition of irrelevant protein or other molecules to cover all unsaturated surface binding sites of micro-holes. Probing or detection is incubation with antigen-specific antibodies that bind affinity to the antigen. In contrast, signal measurement is the detection of signals generated by direct or secondary tags on specific antibodies.

In a typical test designed to detect antigens in a complex protein mixture, the antigen is immobilized either by direct adsorption or via adsorbed antibody into the microplate well. The plates are blocked, and the antigen is checked with specific detection antibodies. Detection antibodies can be directly labeled with signaling enzymes or fluorophores or can be examined secondarily with secondary antibodies labeled with enzymes or fluorine (or the avidin-biotin chemical). For enzymatic detection, a suitable enzyme substrate is added. The observed signal is proportional to the number of antigens in the sample. Inter-step washing ensures that only the specific binding event (high affinity) is maintained to cause the signal in the last step.[5]

Variations between ELISA protocols

In the first four protocols, solid-phase reactants are prepared by adsorbing antigens or antibodies onto a plastic microtiter plate; in the next two protocols, solid-phase reactants are cell-related molecules. In all protocols, solid-phase reagents are incubated with secondary or tertiary reactants covalently coupled with the enzyme. The unbound conjugate is washed, and a chromogenic or fluorogenic substrate is added.[6,7]

As the substrate is hydrolyzed by conjugate-bound enzymes, a colored or fluorescent product is produced. Finally, the product is detected visually or with the microtiter plate of the reader. The amount of product produced is proportional to the amount of the test mixture analysis. The first support protocol can be used to optimize different ELISAs. The second supporting protocol presents a method for preparing alkaline phosphatase conjugates.[6,7]

Antigen immobilization

Antigen immobilization varies between the two principle techniques. In traditional ELISA (direct coating), the antigen is directly attached to the plate by passive adsorption, usually using a carbonate/bicarbonate buffer at pH> 9. Most, but not all, proteins firmly bind to the surface of the polystyrene microplate under alkaline conditions. However, if the antigen is present at low levels or does not adhere well to the plastic, an alternative sandwich or capture ELISA can be used. In ELISA capture (indirect coating), antigen-specific antibodies are adsorbed onto the plastic, which in turn binds to and immobilizes the antigen after incubation with the antigen sample. Antibody attachment is usually achieved using the same carbonate/bicarbonate buffer at pH> 9, or in rare cases, pre-activation plates are used for a more targeted adhesion approach.[6,7]

ELISA sandwiches have become very popular when using complex protein samples because only the specific antigen becomes immobile rather than the entire protein sample. The more antigen that is immobilized, the higher the potential for the sensitivity of the assay. The ELISA sandwich requires two different antibodies that bind specifically to the antigen (each reacts with a different epitope). The first antibody (bound to the plate) is called a trapping antibody or coating antibody, while the second antibody detects an immobile antigen and is called a detection antibody. Such antibodies are known as "matched pairs"; they must be validated to work in combination because they do not have to compete for antigen binding for accurate results to be possible. A combination of monoclonal and polyclonal antibodies can be used. It is more common to use monoclonal as a coating antibody and polyclonal as the detection antibody. ELISA sandwiches sometimes require more optimization than traditional ELISAs, but typically, signal-to-noise (S: N) ratios are higher.[6,7]

Direct versus indirect detection

Direct detection involves labeling the detecting antibody with an enzyme or alternative signaling molecule such as a fluorophore. Indirect detection involves an additional screening step using antibodies or other streptavidin that are labeled with a detectable tag. These additional probes are called secondary antibodies because their primary purpose is to transmit a measurable signaling signal by binding to the detection antibody (primary). Direct detection is generally faster than indirect detection, and potential background signals from secondary antibody cross-reactivity with coating antibodies are also eliminated. Even under the best of circumstances, direct detection cannot provide the amplification of the signal obtained from the use of secondary antibodies or the avidin/biotin system. As a result, direct detection is generally less sensitive than indirect detection and is best used only when a relatively large number of targets are involved.[6,7]

Biotin signal booster

Biotin is a small vitamin molecule (MW 244) that is easily modified so that it can be chemically attached to proteins, antibodies and other biomolecular probes of interest. Avidin and streptavidin are proteins that come from different sources but have almost identical functions in their very strong and specific binding to the biotin molecule. Thermo Scientific NeutrAvidinTM Protein is a specially modified form of avidin. Later in this document, "avidin" and "streptavidin" will be used interchangeably to denote one of these biotin-binding proteins. The avidin-biotin affinity system is frequently used in the design of marking and detection systems for ELISA.[6,7]

A common adaptation of indirect detection is amplifying the signal using the avidin-biotin chemical. There are two approaches. The first method involves the use of biotinylated detection antibodies, which are examined using avidin or streptavidin proteins conjugated to the enzyme horseradish peroxidase (HRP) or alkaline phosphatase (AP). A second approach also uses biotinylated detection antibodies but is investigated with an incubated mixture of avidin and a biotinylated enzyme, a process known as signal amplification of the "avidin-biotin complex" (ABC).[6,7]

Signal amplification occurs via two mechanisms with this approach. First, biotinylation (biotin labeling) typically results in multiple biotin tags per antibody molecule, thereby allowing more than one streptavidin molecule to bind to each antibody. Its binding is aided by the fact that avidin-type protein is tetrameric and has four biotin-binding sites per molecule. Second, the process of labeling the streptavidin molecule with an enzyme or using a mixture of incubated streptavidin plus a biotinylated enzyme produces conjugates that have more than one enzyme. The combined effect of this double labeling is to increase the number of enzyme molecules in the final immune complex. This enhances catalysis of the corresponding substrate and provides a stronger signal compared to conventional enzyme-labeled secondary antibodies.[6,7]

Fluorescence

The relatively recent increase in the number of stable fluorophores in the infrared and ranges have made the detection of fluorescent signals an attractive option for ELISA applications. This kind of approach is common when performing multiplex arrays because more than one antigen can be detected simultaneously with antibodies conjugated to different fluorophores. In fluorescence testing, the detection antibody is directly labeled, or a secondary antibody (or sometimes avidin) is labeled for indirect detection. When multiplexing using labeled secondary antibodies, it is important to use detection antibodies from different species to differentiate between separate signals. The detection limit is usually around 100pg/well, which is less sensitive than colorimetric or chemiluminescent detection.[6,7]

Enzymatic detection

Two enzymes are commonly used in ELISA applications. They are alkaline phosphatase and horseradish peroxidase. Alkaline phosphatase (AP) is a large enzyme used in a small proportion of tests. Its size (140 KDa) makes it difficult to conjugate more than one or two enzyme molecules to each antibody or avidin molecule, and this limits the number of signals that can be generated. APs are also prone to stability issues unless properly stored and handled.[6,7] Horseradish peroxidase (HRP) is a more commonly used enzyme. Its small size (40KDa) allows more molecules to combine with antibodies or avidin, and this can enhance signal formation. HRP is the enzyme of choice for most researchers performing ELISAs and can be used with a variety of substrates, most of which are more sensitive than the AP equivalent.[6,7]

Substrate

Enzymatic signal generation requires substrate catalysis to produce colored or fluorescent compounds or chemiluminescence (visible light). The signal is measured using a spectrophotometric plate reader, a fluorometer with a suitable filter or a luminometer set to read the total light output.[6,7] The colorimetric substrate forms a soluble colored product that accumulates over time relative to the number of enzymes present in each well. When the desired color intensity is reached, the absorbance of the product is measured directly or in some cases, a stopping solution is added to provide a fixed end point for the test. Colorimetric substrates are available for horseradish peroxidase (TMB, OPD, ABTS) and alkaline phosphatase (PNPP).[6,7]

Type of ELISA

ELISA indirect

The sample which has to be analyzed for a specific antigen is attached to the hole of a microtiter plate, followed by an unreacted protein solution such as bovine serum albumin to block any area of the well not coated with the antigen. Primary antibodies, which bind specifically to the antigen, are then added, followed by enzyme-conjugated secondary antibodies. A substrate for the enzyme is introduced to measure the primary antibody through color change. The concentration of primary antibodies present in serum directly correlates with color intensity. One of the indirect ELISA methods was shown by Haapakoski et al., who investigated the role of receptor activation, such as tolls during cytization of skin allergens, using

ovalbumin (OVA) in the modulation of asthma allergy. In one trial, dermal exposure to receptors such as Toll ligands (lipopolysaccharide, Pam3 Cys, P (I: C)) was demonstrated to downregulate OVA-specific IgE antibody in serum, as measured by the indirect ELISA technique.[8]

ELISA sandwich

The sandwich technique is used to identify specific antigen samples. The well surface is prepared with a known amount of bound antibody to capture the desired antigen. After the nonspecific binding site was blocked using bovine serum albumin, a sample containing the antigen was smeared onto the plate. Specific primary antibodies are then added, which "envelop" the antigen. The enzyme-linked secondary antibody is applied, which binds to the primary antibody. The unbound antibody-enzyme conjugate is washed off. The substrate is added and enzymatically converted to a color which can then be measured. Canady et al. analyzed patient serum using the sandwich method to detect elevated levels of keratinocyte growth factor (KGF) in the serum of keloid and scleroderma patients compared to healthy controls to measure human KGF.[9] One of the advantages of using specific purified antibodies to capture antigens is that it eliminates the need to purify the antigen from a mixture of other antigens, thus simplifying testing and increasing their specificity and sensitivity.[4,9]

Competitive ELISA

Competitive ELISA is a competitive reaction process between antigen samples and antigen binding to a microtiter plate with primary antibodies. First, the primary antibody is incubated with the antigen sample, and the resulting antibody-antigen complex is added to a plate that has been coated with the same antigen. After the incubation period, any unbound antibody is washed off. The more antigens in the sample, the more primary antibodies will bind to the sample antigen. Therefore, there will be a small amount of primary antibody available to bind to the antigen coated on top of the well. Secondary antibodies conjugated to the enzyme are added, followed by the substrate to acquire a chromogenic or fluorescent signal. The absence of color indicates the presence of an antigen in the sample. The main competitive advantage of ELISA is its high sensitivity to composition differences in complex antigen mixtures, even when specific detection antibodies are present in relatively small amounts. This method can be used to determine the extract potency of U.S. standardized allergens and to measure total antibodies to the capsular polysaccharide of *Haemophilus influenzae* type b in the human serum of vaccinated subjects. Competitive ELISA is often used to detect HIV antibodies in patient serum. HIV antigen is coated on the surface of the microtiter plate orifice, and two specific antibodies are applied: one conjugated with the enzyme and the other from the patient serum.

Cumulative competition occurs between two antibodies for the same antigen. If antibodies are present in the serum, then an antigen-antibody reaction occurs, leaving a very low quantity of antigen available for binding with antibody-labeled enzymes. Most of the unbound enzyme-labeled antibody is washed off, resulting in little or no color change. The absence of color is an indication of an HIV-positive sample.[3,10]

Multiple and portable ELISA

Multiple and portable ELISA is a novel technique that uses a multicatcher device with 8 or 12 immunosorbent protrusion pins on a central stick that can be dipped into the collected sample. Washing and incubation with enzyme-conjugated antigen and chromogen are carried out by dipping a pin in a microwave and pre-filling with reagents. The main advantages of these ready-to-use lab kits are their relatively low cost, can be used for screening large populations, and do not require skilled laboratory equipment or equipment, making them the ideal tool for low resources. Clinical applications include the detection of infectious diseases in the setting of care, bacterial toxins, oncological markers, and drug

screening.[1,4]

Factors affecting immune complex binding

Although ELISA is a powerful application and has good characteristics, it can be difficult to develop and optimize specific tests. This method involves assembling a large immune complex with many components. Therefore, failure to pick up a signal is potentially due to one of these factors, and optimization is essential. The list of these factors and their associated variables is described in Figure 4, followed by a discussion of some of the related issues.[11-13]

Assay plate and coupling buffer

The plates used in conventional ELISA applications are usually made of polystyrene. Other materials such as polypropylene, polycarbonate, and in some instances, nylon are sometimes used; many plates are now irradiated with gamma rays to provide a positive charge, which assists the plating procedure. The absorbance of the colorimetric substrate is measured by shining the laser upward through the bottom of each well, so it is important to use a flat bottom plate with a clear base. Fluorescent detection requires the use of an opaque black or white plate, and a fluorometric plate reader measures either from the top or bottom of the plate. Chemiluminescent detection requires the use of a black or white opaque plate and can also be measured from above or below. For this reason, fluorescent and chemiluminescent testing should be carried out on a clearly grounded plate. Although black plates are preferred for fluorescence (due to lower background), and white plates are preferred for chemiluminescence (because they amplify the signal), plates can be used interchangeably.[11-13]

Antibodies

Not all antibodies are used successfully in ELISA applications, and individual antibodies must be evaluated. During the adsorption process, the three-dimensional structure of an antigen may change and can no longer bind to the target epitope. Some antibodies against peptides; if the peptide represents the internal sequence of the antigen, then the antibody may not bind if the entire antigen cannot move across the plate. In addition, for antibodies to work successfully in ELISA, antibodies must react specifically with antigens but not cross-react with blocking buffer components. For a sandwich test that requires two different antibodies, the two antibodies must react with different epitopes on the antigen or epitopes that appear multiple times on the antigen. For example, if the antigen is immobilized on the plate via the capture antibody, then the detection antibody must be able to interact with its own epitope without steric resistance from the first antibody or plate. In ELISA applications where secondary antibodies are used as part of the detection complex, it is also important to enhance the capture and detection antibodies in different animal species so that secondary antibodies do not react with coating antibodies. Antibodies that act in combination with each other are generally known as "matched pairs". Another important factor to consider when preparing for an ELISA is the antibody concentration.[11-13]

Blocking buffer

Blocking buffers usually consist of protein formulations designed to prevent the binding of non-specific proteins to plates. Optimal buffer blocking maximizes signal-to-noise ratio and does not react with antibodies or target proteins. If cross-reactivity is observed, then different inhibitors should be tested, and if repeated cross-reactivity is observed, it is advisable to switch to non-mammalian protein inhibitors such as salmon serum or protein-free blocking solutions.[11-13]

Target antigen

The target antigen must be present in a buffer or matrix that allows it to interact with capture antibodies that have been pre-coated or attached to the plate directly. Direct coupling may require the antigen to be exchanged into a suitable clutch buffer.

In rare cases, the three-dimensional structure of the antigen can be altered during the adsorption process so that it no longer binds to the target epitope. In such cases, the use of plates coated with binding proteins (such as trapping antibodies) can eliminate this problem. If the antigen is present in the form of a biological sample, then the matrix effect (i.e., serum, plasma component) must be controlled by performing spike-and-recovery and linearity-of-dilution experiments.[11-13]

When performing quantitative ELISA, it is important to have an equivalent standard protein (generally recombinant purified) for which the amount of a specific protein is known in advance. Standards are used to prepare a series of known solution concentrations by serial dilutions of the protein stock solution followed by a standard curve construction describing concentration versus absorbance. The absorbance value for samples of unknown concentration is extrapolated from this curve to determine the actual amount of specific protein in the sample.[11-13]

Conjugated enzymes

Enzyme conjugate concentration is one of the most important parameters in the optimization process. The number of enzymes that bind directly affects the amount of signal generated. Too little enzyme and the signal may be very weak with a poor signal-to-noise ratio. Too much and the background may be too high, again resulting in a poor signal-to-noise ratio and slight differences between different concentration standards. For recommended enzyme conjugate concentrations, see the Appendix at the end of this document.[11-13]

Washing plate

The two most common wash buffers used in ELISA applications are Tris-buffered saline (TBS) and phosphate-buffered saline (PBS) containing 0.05% (v /v) Tween®-20. To wash the plates, the well must be filled and emptied repeatedly by either aspiration or inversion of the plate (i.e., dumping and flicking of solution into a suitable container). Generally, a wash of at least 3 x 5 minutes should be applied after incubation of the coating antibody, sample and detection antibody; Washes of 6 x 5 minutes should be given after incubation with conjugating enzymes. There is no need to wash after the blocking step, although this is not detrimental to the test.[11-13]

Substrate and signal detection

The choice of specific media will depend on the available equipment but also on the level of sensitivity required. Chemiluminescent substrates are the most sensitive substrates, with antigen detection possible in sub-picograms per good range. Colorimetric and chemifluorescent substrates are usually capable of detecting low to medium picogram antigen levels per well. The settling substrate is not used with the plate test because the sediment settles in the well and prevents absorbance measurements.[11-13]

Colorimetric media is measured using a standard plate reader with a suitable filter. Chemifluorescence is measured using a fluorometer with suitable excitation and emission filters. Chemiluminescence is most often measured using a luminometer, although some plate readers have the option to read chemiluminescence or can be adjusted to measure the total light output. If a wavelength is to be chosen, a measurement at 425nm gives a rough indication of chemiluminescence. Some fluorescent plate readers can also be used if the excitation is off.[11-13]

2. Conclusion

EIA/ELISA is a powerful method not only for general biomedial research but also as a diagnostic tool. This procedure allows the detection of all types of biological molecules at very low concentrations and quantities. Despite their limitations, EIA/ELISA remains an important tool in both clinical and basic research and in clinical diagnosis. The basic principle of ELISA is to use enzymes to detect antigen (Ag) and antibody (Ab) binding. Enzymes change colorless substrates (chromogen) into colored products, indicating the binding of antibody antigens. ELISA can

be used to detect the presence of an antigen or antibody in a sample, depending on how the test is designed.

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