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Enzyme-Linked Immuno-Sorbent Assay (ELISA), basics and it's application : A comprehensive review

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ABSTRACT

Enzyme immunoassay, a bioanalytical method incorporating an antigen–antibody reaction to capture the analyte of interest and an enzyme reporter system to detect the captured analyte, is one of the most widely used immunoassay formats. The method is sometimes applied only qualitatively to indicate the presence of an antigen in a matrix. However, in the more common quantitative implementation, a calibration curve is incorporated, from which the concentration of the analyte in unknown samples is interpolated. Immunoassays have been widely applied in support of medical practice and drug development. However, in recent years, there has been a decline in the application of immunoassays to the quantitation of low-molecular weight xenobiotics, primarily due to the advent of liquid chromatography–mass spectrometry (LC–MS) methods, which have high sensitivity and specificity. Immunoassays remain the method of choice for the quantitation of protein macromolecules and antibodies in complex matrices. Another major application of immunoassays is in the detection and quantitation of biomarkers, which are evolving to be of pivotal importance in the evaluation of pharmacological, toxicological, and clinical activities of candidate drugs[1]. It's a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample[2].

Key words: Enzyme, Immuno assays, biomarkers, antibody, antigen.

INTRODUCTION

Immunoassays generally vary in the type of critical antibody binding reagent or the detection and reporter systems used to monitor the end-point of the binding reaction. Enzyme immunoassay formats fall broadly into two categories, namely heterogeneous and homogeneous. In a heterogeneous assay, at least one key reagent is immobilized on a solid surface and there is at least one ''washing'' step before the final detection step. In contrast, in a homogeneous assay, all reagents are in solution together and there is no ''washing'' step prior to signal generation and detection. Both categories of assay include formats described as competitive and non-competitive. In a competitive assay, there is direct competition between the labeled and the unlabeled antigen (analyte or ligand) in solution or, in some cases, between immobilized and soluble antigen for a limited number of antibody binding sites. In non-competitive assays, antibody binding sites to capture and detect the antigen are not limiting because the antigen is incubated with excess capture antibody and enzyme-labeled detection antibody. An example of a competitive homogeneous assay format is the enzyme-multiplied immunoassay (EMIT) system, in which enzyme-labeled antigen competes directly in solution with unlabeled antigen in the biological sample (or calibration standard and quality control samples) for a limited number of antibody binding sites. The reaction endpoint is detected and quantitated spectrophotometrically without any intervening wash steps. Enzyme-linked immunosorbent assay (ELISA) is an example of a heterogenous noncompetitive immunoassay[3].

ELISA- (ENZYME LINKED IMMUNOSORBENT ASSAY)

Enzyme immunoassay (EIA) and enzyme-linked immunosorbent assay (ELISA) have become household names for medical laboratories, manufacturers of in vitro diagnostic products, regulatory bodies, external quality assessment and proficiency-testing organizations (Rudolf M). It is a biochemical technique used mainly in immunology to detect the presence of an antibody or an antigen in a sample[2]. In principle, the method is similar to that of the RIA which involves competitive binding, it does not involve in use of any radio-labelled material and the sensitivity of the method is equally good 0.0001-0.001 µg/ml. The method is safe and cheaper as compared to RIA. Here the enzyme conjugated to an antibody reacts with a colorless substrate to generate a colored reaction product. The color is measured spectrophotometrically. The most commonly used enzymes are horseradish peroxidase, alkaline phosphatase, p-nitrophenyl phosphatase ß galactosidase[4]. These enzymes have been used to link to the antibody or antigen molecule as may be the case. When mixed with a suitable substrate each of these enzymes generates a colored reaction product[5]. ELISA procedure are popular primarily because they require little interpretive skill to read the results tend to be clearly positive or clearly negative[6].

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HISTORY

Before the development of the EIA/ELISA, the only option for conducting an immunoassay was radioimmunoassay, a technique using radioactively -labeled antigens or antibodies. In radioimmunoassay, the radioactivity provides the signal which indicates whether a specific antigen or antibody is present in the sample. Radioimmunoassay was first described in a paper by Rosalyn Sussman Yalow and Solomon Berson published in 1960.

Because radioactivity poses a potential health threat, a safer alternative was sought. A suitable alternative to radioimmunoassay would substitute a non-radioactive signal in place of the radioactive signal. When enzymes (such as peroxidase) react with appropriate substrates (such as ABTS or 3,3',5,5'-Tetramethylbenzidine), this causes a change in color, which is used as a signal. However, the signal has to be associated with the presence of antibody or antigen, which is why the enzyme has to be linked to an appropriate antibody. This linking process was independently developed by Stratis Avrameas and G.B. Pierce. Since it is necessary to remove any unbound antibody or antigen by washing, the antibody or antigen has to be fixed to the surface of the container, i.e. the immunosorbent has to be prepared. A technique to accomplish this was published by Wide and Jerker Porath in 1966 [2].

In 1971, Peter Perlmann and Eva Engvall at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen in The Netherlands, independently published papers which synthesized this knowledge into methods to perform EIA/ELISA.

Numerous variants of ELISA

A number of variation ELISA have been developed, allowing qualitative detection or quantitative measurement of either antigen or antibody. Each type of ELISA can be used qualitatively to detect the presence of antibody or antigen. Alternatively, a standard curve based on known concentration of antibody or antigen is prepared, from which the unknown concentration of a sample can be determined.

1.Indirect ELISA :

Antibody can be detected or quantitatively determined with an indirect ELISA. Serum or some other sample containing primary antibody (Ab_1) is added to an antigen- coated microtiter well and allowed to react with the antigen attached

to the well. After any free Ab_1 is washed away, the presence of antibody bound to the antigen is determined by adding an enzyme- conjugated secondary anti-isotype antibody (Ab_2), which binds to the primary antibody. Any free Ab_2 then is washed away, and a substrate for the enzyme is added. The amount of colored reaction product that forms is measured by specialized spectrophotometric plate readers, which can measure the absorbance of all of the wells of a 96- well plate in seconds[7].

Indirect ELISA is the method of choice to detect the presence of serum antibodies against human immunodeficiency virus (HIV), the causative agent of AIDS. For detection of anti- HIV-1 and anti- HIV-2 antibodies in the patient serum. The well of the polystyrene micrititre plate are coated with purified HIV-1 and HIV-2 antigens or synthetic peptides representing immunodominant epitopes of HIV-1 and HIV-2, which constitutes the solid phase antigens. Diluted test serum or plasma sample is added to such a well and incubated. If antibodies specific for HIV-1 and/or HIV-2 is/are present in the test sample they will form stable complexes with antigens coated on the well. Well is then washed and a conjugate of goat anti-human immunoglobin, which has been labelled with the enzyme horse-radish peroxidase, is added. If the antigen- antibody complex is present, the peroxidase conjugate will bind to the complex and remains in the well is removed by washing and the presence of enzyme immobilized on the complexes is shown by incubation in the presence of enzyme substrate (ortho-phenylene-diamine dihydrochloride solution). Incubation with enzyme substrate produces a yellow-orange colour in the test well. If the sample contains no anti-HIV-1 and/or anti-HIV-2, then the labeled antibody cannot be found and no colour develops. The absorbance value of each well is read by an ELISA plate read at wavelength of 492±2 nm[8].

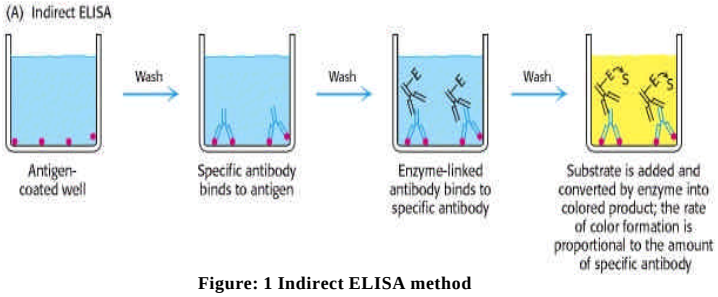

Figure: 1 Indirect ELISA method

2.Sandwich ELISA :

One of the most useful of the immunoassays is the two antibody "sandwich" ELISA. This assay is used to determine the antigen concentration in unknown samples. This ELISA is fast and accurate, and if a purified antigen standard is available, the assay can determine the absolute amount of antigen in an unknown sample. The sandwich ELISA requires two antibodies that bind to epitopes that do not overlap on the antigen. This can be accomplished with either two monoclonal antibodies that recognize discrete sites or one batch of affinity-purified polyclonal antibodies. Antigen can be detected or measured by a sandwich ELISA.

In this technique, the antibody (rather than the antigen) is immobilized on a microtiter well. A sample containing antigen is added and allowed to react with the immobilized antibody. After the well is washed, a second enzyme- linked antibody specific for a different epitopes on the antigen is added and allowed to react with the bound antigen. After any free second antibooby is removed by washing, substrate is added, and the colored reaction product is measured[7].

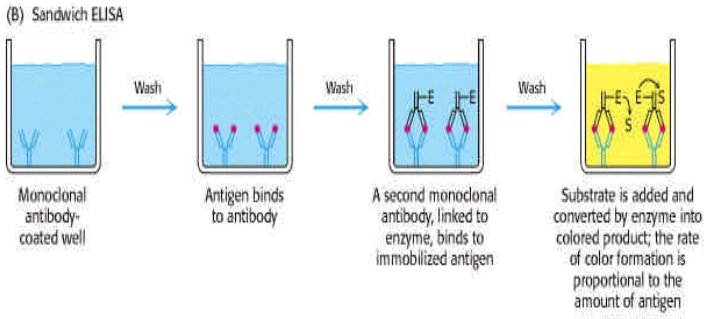

Figure : 2 Sandwich ELISA method

The Sensitivity of the Sandwich ELISA is Dependent on Four Factors:

1. The number of molecules of the first antibody that are bound to the solid phase.
2. The avidity of the first antibody for the antigen.
3. The avidity of the second antibody for the antigen.
4. The specific activity of the second antibody.

The amount of the capture antibody that is bound to the solid phase can be adjusted easily by dilution or concentration of the antibody solution. The avidity of the antibodies for the antigen can only be altered by substitution with other antibodies. The specific activity of the second antibody is determined by the number and type of labeled moieties it contains[9].

1.Competitive ELISA :-

When two "matched pair" antibodies are not available for your target, another option is the competitive ELISA. Another advantage to the competitive ELISA is that non-purified primary antibodies may be used. Another variation for measuring amounts of antigen is competitive ELISA[9].

In this technique, antibody is first incubated in solution with a sample containing antigen. The antigen- antibody mixture is then added to an antigen-coated micrititer well. The more antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well. Addition of an enzyme conjugated secondary antibody (Ab_2) specific for the isotype of the primary antibody can be used to determine the amount of primary antibody bound to the well in an indirect ELISA[7].

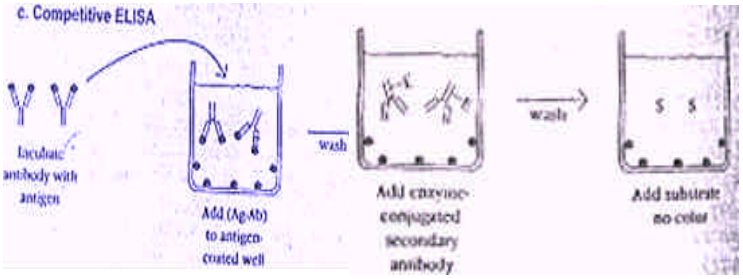

Figure: 3 Competitive ELISA method

1.Reverse ELISA :

A new technique uses a solid phase made up of an immunosorbent polystyrene rod with 4-12 protruding gives. The entire device is immersed in a test tube containing the collected sample and the following steps (washing, incubation in conjugate and incubation in chromogenous) are carried out by dipping the gives in microwells of standard microplates pre-filled with reagents.

The advantages of this technique are as follows:

1.The gives can each be sensitized to a different reagent, allowing the simultaneous detection of different antibodies and different antigens for multi-target assays;

2.The sample volume can be increased to improve the test sensitivity in clinical (saliva, urine), food (bulk milk, pooled eggs) and environmental (water) samples;

3.One give is left unsensitized to measure the non-specific reactions of the sample;

4.The use of laboratory supplies for dispensing sample aliquots, washing solution and reagents in microwells is not required, facilitating ready-to-use lab-kits and on-site kits[2].

FACTORS THAT INFLUENCE ASSAY PERFORMANCE

1.The greatest impact of the behavior of the assay is typically based on the antibody pair itself. If the pair is a "good" match for the desired analyte, other unwanted factors are greatly minimized. If the pair is a "poor" match with lower affinity for the desired analyte, it may take a lot of time and creativity to achieve acceptable parameters.

2.Non-specific binding can cause high background values and poor recovery and linearity, which can often be controlled with IgG antibodies or serum.

3.False positive values can occur on Non-specific binding of samples to one or both of the antibodies present which is often detected when performing spiked recovery and linearity testing. In the ELISA format, these samples can typically be controlled by the use of IgG antibodies and/or heterophilic blockers.

4.Hook Effect may give incorrect sample values for highly concentrated samples and can usually be detected during linearity studies.

VALIDATION OF ELISA ASSAYS

1.Spiked recovery tests are performed using the blank, low standard, medium standard and high standard points spiked into at least 8 different samples in duplicate. This test should be repeated at least two times to determine if results are reproducible and within acceptable criteria for the coefficient of variation (CV). Acceptable criteria are CVs of 80–120%.

2.Linearity tests are performed on at least 8 different samples in duplicate. At least three dilution of the neat samples should be performed and more if possible. This test should be repeated at least two times to assure that the results are reproducible and acceptable (80–120% CVs).
3.Intra-assay variation is tested by running 8 different samples (of varying concentration) in replicates of ten across the microtiter plate and determining the % CVs of the samples. Acceptable criteria are typically CVs of 80–120%.
4.Inter-assay variation is determined by evaluating at least 8 samples (of varying concentration) in duplicate on at least three different microtiter plates on different days using the same reagent lots. The % CVs are then calculated. Acceptable criteria is typically CVs of 80–120%.
5.The microtiter plates coated with the capture antibody must also be validated by running the blank, low, and high standard points in replicates of 32, on a minimum of three plates, and the % CVs calculated. Acceptable criteria is typically CVs of =10%.
6.The sensitivity of the assay is determined by evaluating the blank and the lowest standard point in replicates of at least 20. The following calculation is

$$\text{Sensitivity} = \frac{\text{Background Mean}}{\text{Low Std. Mean} - \text{Background Mean}} \times \text{Low Std. Mean}$$

Sensitivity = Above calculation+2 std. deviations

used to determine the sensitivity of the assay: 1. Further specificity of the assay is evaluated by running standards of several similar analytes of available species in the assay to determine if any similar analytes can be detected by the assay.
7If available, the sample values are compared to sample values in the most popular competitor kit or our own Millipore RIA kit to determine the correlation of sample values.
8Compare serum vs. plasma values and determine the correlation. Also evaluate if assay can be performed on cell extracts or cell culture media[10].

APPLICATIONS11 [11,14-21]

Because the ELISA can be performed to evaluate either the presence of antigen or the presence of antibody in a sample, it is a useful tool for determining serum antibody concentrations (such as with the HIV test or West Nile Virus). It has also found applications in the food industry in detecting potential food allergens such as milk, peanuts, walnuts, almonds, and eggs. ELISA can also be used in toxicology as a rapid presumptive screen for certain classes of drugs. ELISA provides a means to quantitatively measure extremely small amount of proteins in biological fluids and serves as a tool for analyzing specific protein during purification[11].

The ELISA was the first screening test widely used for HIV because of its high sensitivity. In an ELISA, a person's serum is diluted 400-fold and applied to a plate to which HIV antigens are attached. If antibodies to HIV are present in the serum, they may bind to these HIV antigens. The plate is then washed to remove all other components of the serum. A specially prepared "secondary antibody" — an antibody that binds to other antibodies — is then applied to the plate, followed by another wash. This secondary antibody is chemically linked in advance to an enzyme. Thus, the plate will contain enzyme in proportion to the amount of secondary antibody bound to the plate. A substrate for the enzyme is applied, and catalysis by the enzyme leads to a change in color or fluorescence. ELISA results are reported as a number; the most controversial aspect of this test is determining the "cut-off" point between a positive and negative result.

A cut-off point may be determined by comparing it with a known standard. If an ELISA test is used for drug screening at workplace, a cut-off concentration, 50 ng/mL, for example, is established, and a sample will be prepared which contains the standard concentration of analyte. Unknowns that generate a signal that is stronger than the known sample are "positive". Those that generate weaker signal are "negative.

ELISA can also be used to determine the level of antibodies in faecal content,specifically the direct method.

ELISA test are utilized to detect substances that have antigenic properties, primarily proteins (as opposed to small molecules and ions such as glucose and potassium). Some of these include hormones, bacterial antigens and antibodies. ELISA methods are fundamental tools in the pharmaceutical industry with applications in drug discovery, animal studies, and clinical trials[12].

There are variations of this test, but the most basic consists of an antibody attached to a solid surface. This antibody has affinity for (will latch on to) the substance of interest, for example, human chorionic gonadotropin (HGC), the commonly measured protein which indicates pregnancy. A mixture of purified HCG linked (coupled) to an enzyme and the test sample (blood, urine, etc) are added to the test system. If no HCG is present in the test sample, then only HCG with linked enzyme will bind. The more HCG which is present in the test sample, the less enzyme linked HCG will bind. The substance the enzyme acts on is then added, and the amount of product measured in some way, such as a change in color of the solution[13].

ELISA tests are generally highly sensitive and highly specific and less expensive technique used in serology to detect antigens or antibodies[14]. They have the added advantages of not needing radioisotopes or a radiation-counting apparatus ELISA assays are widely applied in clinical laboratory testing[15].

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