S. S. College, Jehanabad

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ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Enzyme-**l**inked **i**mmunosorbent **a**ssay (ELISA) is a plate-based biochemical assay technique intended to detect and measure peptides, proteins, antibodies, and hormone in a biological sample. Some examples include diagnosis of HIV infection, pregnancy tests, and measurement of cytokines or soluble receptors in cell supernatant or serum. In ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activityvia incubagtion with a substrate to produce a measurable product or colour. It is generally carried out in 96 well plates, allowing multiple samples to be measured in a single experiment. These plates need to be special absorbant plates (e.g. NUNC Immuno plates) to ensure the antibody or antigen sticks to the surface.

Before the advent of ELISA, radioimmunoassay employing radioactively-labelled antigens and antibodies were used. Radioactivity served as the reporter signal indicating specific antigen or antibody. As radioimmunoassay posed significant health risks to researchers, alternatives were sought. Therefore, enzyme linking process was developed by two different teams spearheaded by Stratis Avrameas and G. B. Pierce in 1960s. In the same period, immunosorbent preparation technique was published by Wide and Jerker Porath. Later, with the publication of independent research papers by Peter Perlmann and Eva Engvall at Stockholm University in Sweden, and Anton Schuurs and Bauke van Weemen in the Netherlands in 1971 geared the designing and development of ELISA. After that a massive development of ELISA technique has been witnessed by us. The conventional ELISA involves usage of chromogenic reporters and substrates to produce color changes to indicate the presence of specific antigen or an analyte. Newer Assay techniques make use of fluorogenic[1], electrochemiluminescent[2], and quantitative PCR reporters to create quantifiable signals. Development of newer detection techniques makes possible the subtle and accurate measurement of analyte as well as measurement of multiple analyte in a single assay.

Principle of ELISA

It is a type of sensitive immunoassay that uses an enzyme linked to an antibody or antigen as a marker for the detection of a specific protein, especially an antigen or antibody. It works by coupling antibody or antigen to assay enzyme. The assay combines the specificity of antibody and sensitivity of assay enzymes to primarily detect antigens through assay antibody or antibodies through assay antigens. It involves detection of "analyte" in a liquid sample using liquid reagent (wet lab) or dry strips (dry lab). In dry analysis, strip can be read in reflectometry[3]. The quantitative reading usually based on detection of intensity of transmitted light by spectrophotometry at specific wavelength. The sensitivity of detection depends on amplification

[1] **Fluorogenic compound** is a type of chemical compound that gives rise to **fluorescence** which is basically an emission of electromagnetic radiation, usually visible light, caused by excitation of atoms in of that compound after absorption shorter wavelength of electromagnetic radiation within about 10^{-8} seconds.

[2] **Electrochemiluminescence** (also called as electrogenerated chemiluminescence or **ECL**) is a phenomenon where a light emission arises from a high-energy electron transfer reaction between electrogenerated species, which is usually accompanied with the regeneration of emitting species.

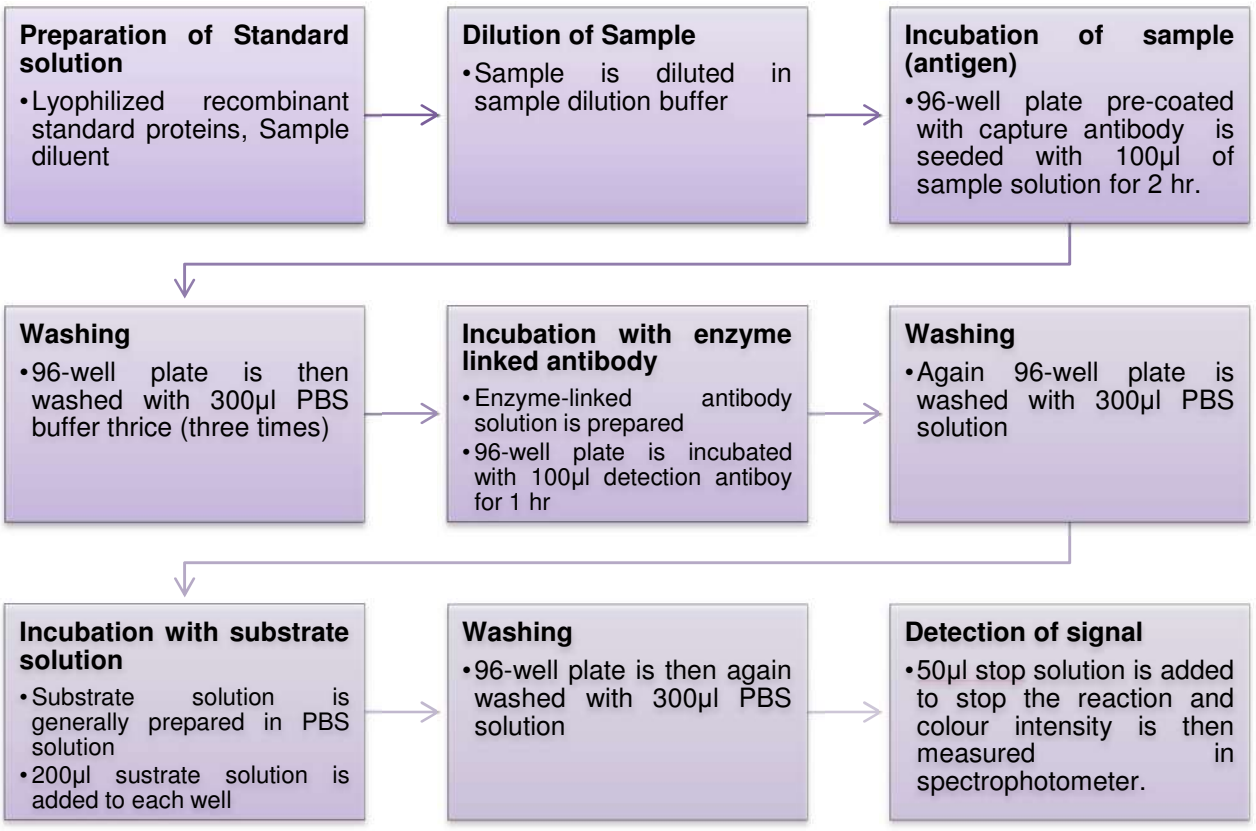
[3] **Reflectometry** is laboratory technique of measuring the scatter of energized particles from the layered surface for analyzing thin layer of objects such as biological membrane or layered metallic surface performed by.

of signal during the analytic reaction, which can be enhanced by coating the plate with high affinity antibodies. There are two main variations of ELISA method, which are;

- A variation of ELISA which can be used to detect the presence of antigens that are recognized by an antibody.
- Another variation of ELISA which can be used to test for antibodies that recognize an antigen.

Procedure of ELISA

The ELISA process begins with coating the multi-well plate with either antigen or antibody which usually takes 30 min to overnight incubation. The incubation of plated sample is followed by primary antibody reaction by incubating 96 well plate-coated samples in primary antibody solution for 2 hours after blocking of sites that are not of interest or unbound sites. Then the application of secondary antibody is carried out with further incubation for 2 hours by secondary antibody solution, thereafter substrate preparation is generally done freshly just before the reaction and finally ready for observation after about 30 min.



Basic steps in enzyme-linked immunosorbent assay (ELISA)

The components in the ELISA are immobilized which makes the process easy to perform. But as the assay requires the use of surface binding for appropriate separation, washing is carried out several times repeatedly after each step in order to ensure removal of unbound entities. The excess fluid is to be necessarily removed during the course of the reaction in the interest to

prevent the unwanted dilution of the reagents that will be added in the subsequent steps. For ensuring the desired uniformity dedicated plate washers are implemented in this process. A color producing substrate, like tetramethylbenzidine (TMB), in staining is then used to develop colour. It produces the color based on an enzymatic reaction. The substrate can be chosen based on the instrument available like in case of colorimetric reactions spectrophotometers are used and in case of a fluorometric tag fluorimeters are implemented.

Types of ELISA

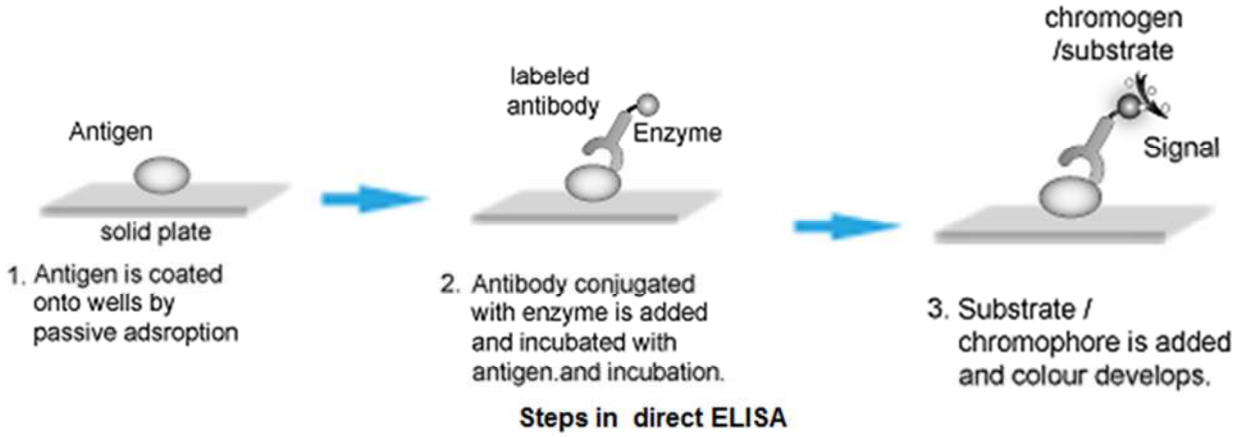
On the basis of purpose of ELISA, it is of two types;

Qualitative ELISA: This type of method of ELISA is intended to know the quality of test and result is represented by positive or negative results.

Quantitative ELISA: This type of method of ELISA is intended to measure the quantity of antigens or antibody in the sample. The result is based on the optical density or fluorescent units of the sample are interpolated into a standard curve, which is typically a serial dilution of the target.

On the basis of methods, ELISA can be divided into four types direct ELISA, indirect ELISA, sandwich ELISA and competitive ELISA or ELISPOT.

Direct ELISA: Direct ELISA is suitable for the detection of proteinaceous antigens and may require pre-purification of sample. Direct ELISA can be performed when desired antibody is available in a pre-conjugated state i.e. fluorometric, colourimetric or enzymatic. It is much faster as compared to other ELISA techniques due to limited steps followed. This technique allows for coating of antigen directly to wells of microtitre plate which is followed by the addition of enzyme labelled primary antibody that detects the antigen that is complementary. There is no requisite of cross-reacting secondary antibody in this technique. But this technique poses a few disadvantages with regards to its specificity. Since specificity of antigen immobilization is low, there is an occurrence of higher background noise as compared to other ELISA techniques. This is due to the non-specific interaction of sample proteins along with target protein in a microtitre plate.



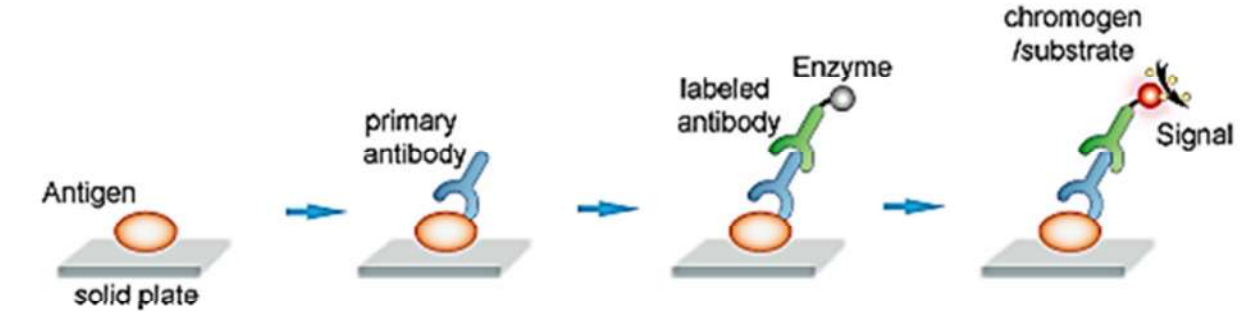
Steps in direct ELISA

The flexibility of direct ELISA is minimum since each target protein is required to be bound by enzyme labelled primary antibody. The labelling procedure for each primary antibody is laborious and time consuming which would adversely affect its immunoreactivity. The lack of secondary antibody minimises signal amplification that reduces the sensitivity of the assay. Therefore, the direct ELISA technique is preferred mostly to analyse the immune response towards an antigen.

- **Advantages of direct ELISA**
  - Fast and minimal steps involved in the procedure
  - Eliminates possible non-specific binding of secondary antibody.

- **Disadvantages of direct ELISA**
  - The immobilization of the antigen is not specific due to which background related issues are seen.
  - Offers less flexibility in terms of primary antibody.
  - The absence of signal amplification reduces sensitivity.

Indirect ELISA: The indirect detection method, secondary antibody is added for detection on the basis of direct ELISA in addition to primary antibody. It is the most popular ELISA format. Antigen is passively attached to wells by incubation. After washing, antibodies specific for the antigen are incubated with the antigen. Wells are washed and all bound antibodies are detected by the addition of anti-species antibodies targeted to the isotype such as mouse IgG1, goat IgM, rabbit IgG1,k, chicken IgY, etc., that is covalently linked to an enzyme. Such antibodies are specific for the species in which the first antibody added were produced. After incubation and washing, the test is developed and can be read under spectrophotometer at the wavelength according to reaction.



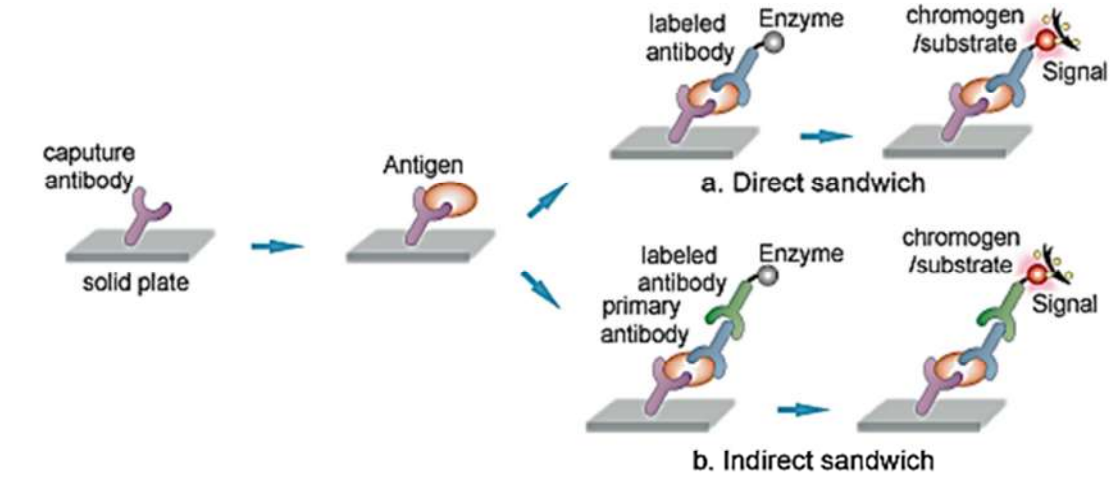
Steps in indirect ELISA

- **Procedure for indirect ELISA**
  - Coat the micro titer plate wells with antigen.
  - Block all unbound sites to prevent false positive results.

- Add sample containing antibody (e.g. rabbit monoclonal antibody) to the wells and incubate the plate at 37°C.
- Wash the plate, so that unbound antibody is removed.
- Add secondary antibody conjugated to an enzyme (e.g. anti- mouse IgG).
- Wash the plate, so that unbound enzyme-linked antibodies are removed.
- Add substrate which is converted by the enzyme to produce a colored product.
- Reaction of a substrate with the enzyme to produce a colored product.

– **Advantages of indirect ELISA**
  - Wide varieties of secondary conjugates are available for detection.
  - Immunoreactivity of the primary antibody is not compromised.
  - Multiple binding of the secondary antibody affords some signal amplification.
  - Different visualization markers can be used with the same primary antibody.
  - Cost savings, since fewer labeled antibodies are required.
– **Disadvantages of indirect ELISA**
  - It needs extra step in the protocol.
  - Some non-specific binding of the secondary may cause background noise.

Sandwich ELISA: In this method of ELISA, antigen is measured between the two layers of antibodies i.e. capture antibody and detection antibody. First, antibody is coated on the microtiter well. A sample containing antigen is added to the well and allowed to react with the antibody attached to the well, forming antigen-antibody complex. After the well is washed, a second enzyme-linked antibody specific for a different epitope on the antigen is added and allowed to react with the bound antigen. Thereafter, unbound secondary antibody is removed by washing with PBS. Finally substrate is added to the plate which is hydrolyzed by enzyme to form colored products. It is of two types; direct sandwich ELISA and indirect sandwich ELISA.



Steps in Sandwich ELISA

Direct Sandwich: In this method of sandwich assay, capture antibody is attached on the solid phase. After washing away excess unbound antibody, antigen is added and is specifically captured. The antigen is then detected by a second enzyme labeled antibody directly against the antigen. This type of assay is useful where a single species anti-serum is available and where antigen does not attach well to plates.

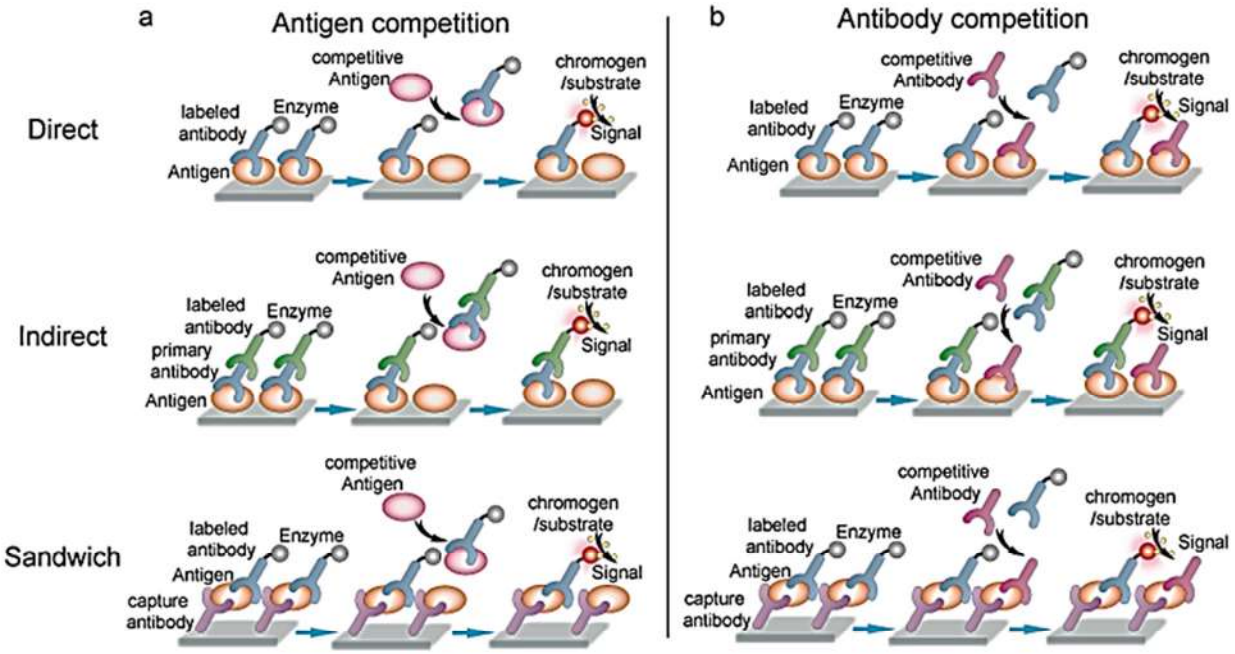
Indirect Sandwich: In this method of sandwich assay, the antigen is detected with a second unlabeled antibody. This antibody is in turn detected using an anti-species enzyme labeled conjugate. It is essential that the anti-species conjugate does not bind to the capture antibody; therefore the species in which the capture antibody is produced must be different. The same considerations about the need for that at least two antigenic sites allowing the "sandwich" are relevant. The advantage of this system is that a single anti-species conjugate can be used to evaluate the binding of antibodies from any number of samples.

- ***Procedure of sandwich ELISA***
    - Prepare a surface to which a known quantity of antibody is bound.
    - Add the antigen-containing sample to the plate and incubate the plate at 37°c.
    - Wash the plate, so that unbound antigen is removed.
    - Add the enzyme-linked antibodies which are also specific to the antigen and then incubate at 37°C.
    - Wash the plate, so that unbound enzyme-linked antibodies are removed.
    - Add substrate which is converted by the enzyme to produce a colored product.
    - Reaction of a substrate with the enzyme to produce a colored product.
- ***Advantages of sandwich ELISA***
    - High specificity, since two antibodies are used the antigen is specifically captured and detected.
    - Suitable for complex samples, since the antigen does not require purification prior to measurement.
    - Flexibility and sensitivity, since both direct and indirect detection methods can be used.

Competitive ELISA: The competitive ELISA test is used to measure the concentration of an antigen in a sample. In this test, antibody is first incubated in solution with a sample containing antigen. The antigen-antibody mixture is then added to the microtitre well which is coated with antigen. *The more the antigen present in the sample, the less free antibody will be available to bind to the antigen-coated well.* After the well is washed, enzyme conjugated secondary antibody specific for isotype of the primary antibody is added to determine the amount of primary antibody bound to the well. The higher the concentration of antigen in the sample, the lower the absorbance is detected. Detection in this method of ELISA can be achieved by applying all other methods of ELISA, for example direct, indirect and sandwich ELISA through antigen or antibody competition.

- **Procedure of competitive ELISA**
    - Antibody is incubated with sample containing antigen.
    - Antigen-antibody complex are added to the microtitre well which are pre-coated with the antigen.

- Wash the plate to remove unbound antibody.
- Enzyme linked secondary antibody which is specific to the primary antibody is added.
- Wash the plate, so that unbound enzyme-linked antibodies are removed.
- Add substrate which is converted by the enzyme into a fluorescent signal.



Steps in Competitive ELISA

– **Advantages of competitive ELISA**
  - High specificity, since two antibodies are used.
  - High sensitivity, since both direct and indirect detection methods can be used.
  - Suitable for complex samples, since the antigen does not require purification prior to measurement.

Buffers of ELISA: There are usually three types of buffer used in the ELISA, which can be made in house or can be sourced from a variety of commercial antibody and reagent suppliers directly. These buffers are following;

Coating buffers: This is the first step in ELISA where an antigen or antibody in required dilution is incubated until adsorbed to the surface of the well (generally overnight). Due to hydrophobic interactions between the amino acids side chains on the antibody or antigen and the plastic surface passive adsorption occurs. Adsorption is also dependent on few other factors such as time, temperature, pH of the coating buffer and concentration of the coating agent. Typical coating conditions involve adding 50-100 µl of coating buffer, containing antigen or antibody at a concentration of 1-10 µg/ml, and incubating overnight at 4°C or for 1-3 hours at 37°C. During coating, it is important to maintain a moist environment in the well to minimize evaporation; plate sealers are generally used to achieve this. Higher concentrations of antibody/antigen may actually have a negative effect on coating, leading to oversaturation of the wells, which can

inhibit antibody binding due to steric hindrance. The two most common coating buffers are bicarbonate buffer at pH 9.6 or PBS.

Blocking buffers: Blocking is a necessary step in ELISA to prevent the non-specific binding of detection antibodies to the multiwell plate surface itself. Proteins and detergents are two main types of blocking agents. Proteins are classified as permanent blocking agents and hence added after the capture antibody has adsorbed to the well surface. Detergents only block temporarily, meaning their blocking function disappears during washing steps. Identifying a blocking buffer is completely user's choice which has two main considerations namely reducing the nonspecific binding i.e. background and desired sensitivity. Blocking agent can consist of an unrelated protein or a protein derivative that does not react with any of the antibodies used in the detection step. When a plate is fully blocked, assay sensitivity will be enhanced since additional non-specific signal will be reduced. The most commonly used blocking buffer contains 1% BSA or milk proteins dissolved in PBS. Usually 150 – 250 µl of blocking buffer is added to the well to incubate for one hour at 37°C in order to fully block the plate.

Washing buffers: ELISA wash steps remove unbound nonspecific materials and are very important component of ELISA protocol as ELISA uses surface binding for separation. The wash buffer is generally PBS, with a small concentration of a non-ionic detergent such as Tween-20. Washing is typically repeated 3-5 times between each step in the ELISA, with 30 second incubation for each wash step to thoroughly remove unbound nonspecific material. It has been observed that more number of wash is of significance than more time of wash incubation. In the final wash step excess of wash solution must be removed to prevent the dilution of the reagents added in the subsequent stage. Simply tapping the washed plate upside down on an absorbent paper helps to remove excess liquid. It is crucial not to let the plate dry out.

Test control samples of ELISA

Controls are very important for performing ELISA as they help in several ways. Different controls have different functions to play. Based on the function controls are named as negative, positive and standard.

Blank control: In an ELISA experiment blank wells are the ones where only primary antibody is excluded and other components are added. Blank wells don't show any color. In some cases blank and negative control are same.

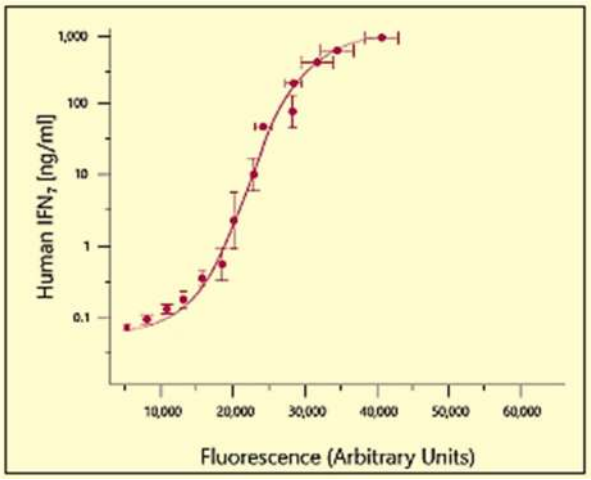
Negative control: The negative control in an ELISA test means a sample known not to express the protein of interest. This helps to check for non-specific binding and false positive results. Each experiment should contain a negative control sample in order to validate the results.

Positive control: In an ELISA test, positive control has an important role to play. A positive test for positive control indicates that the protocol is working fine. It will verify that any negative results are valid. A positive control can be an endogenous soluble sample known to contain the protein being detected. A purified protein or peptide known to contain the immunogen sequence for the antibody can also be a positive control. Any tissues, cells or lysates that have been used successfully can be considered a suitable positive control.

Standard: A sample containing a known concentration of a protein from which the standard curve can be obtained. Then this standard curve can be extrapolated in order to calculate the unknown concentration of the protein.

Data interpretation of ELISA

Data gathered from ELISA is typically graphed with optical density (or fluorescence) vs concentration of antigen. To know the concentration of analytes in the sample, a standard curve is generated with standard protein solution. Roughly, the data can be interpreted in three steps, which are as follows;

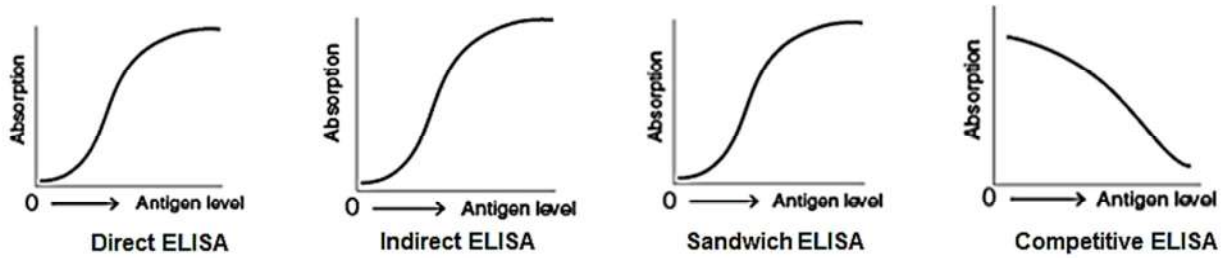


Qualitative: ELISAs can also be used to achieve a yes or no answer indicating whether a particular antigen is present in a sample, as compared to a blank well containing no antigen or an unrelated control antigen.

Quantitative: ELISA data can be interpreted in comparison to a standard curve (a serial dilution of a known, purified antigen) in order to precisely calculate the concentrations of antigen in various samples.

Semi-Quantitative: ELISAs can be used to compare the relative levels of antigen in assay samples, since the intensity of signal will vary directly with antigen concentration.

Generally, a sigmoidal curve is observed in all ELISA methods except competitive ELISA or ELISPOT where a regression line is observed. In ELISPOT, a regression line is observed due to the fact that on increasing the concentration of antigen, absorbance is lowered.

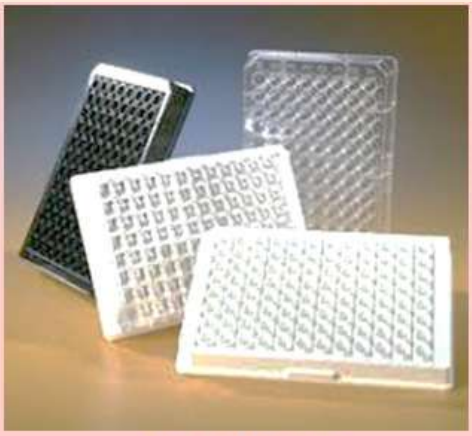


Application of ELISA

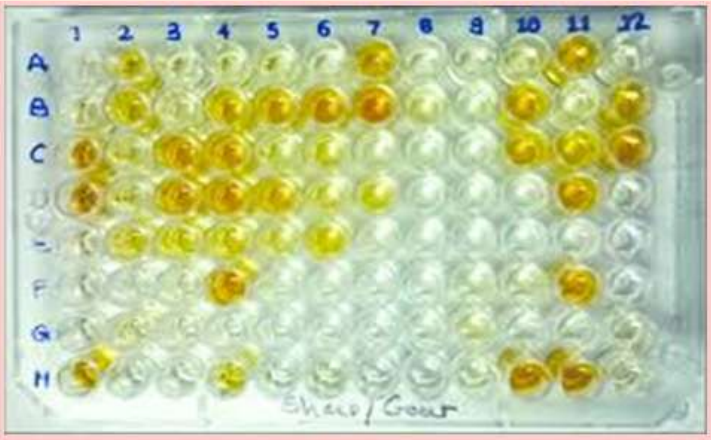
- Presence of antigen or the presence of antibody in a sample can be evaluated.
- Determination of serum antibody concentrations in a virus test.
- Used in food industry when detecting potential food allergens.
- Applied in disease outbreaks- tracking the spread of disease e.g. HIV, bird flu, common, colds, cholera, STD etc.

ELISA Plates

Usual dimension of 96-well plat is 8 cm × 12 cm that contains an 8 × 12 matrix of 96 well, each of which are about 1 cm high and 0.7 cm in diameter. It is made up of plastic.



Blank ELISA Plate



ELISA Plate in procedure

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