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An Overview of ELISA Biochemistry: A Brief Evaluation

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Abstract

ELISA, known as the enzyme-linked immunosorbent assay, is widely recognized as the most reliable immunoassay method. This technique is highly sensitive and serves the purpose of identifying and measuring various substances such as hormones, antibodies, antigens, glycoproteins, allergens, and proteins. The process involves the formation of antibody–antigen complexes that generate a detectable response, enabling quantification. In response to encountering foreign substances called "antigens," the immune system of an individual produces antibodies as a protective response. An antigen is a protein of foreign origin that, when coupled with an antibody, causes the body to mount an immune reaction. Antibodies have specific regions that bind to antigens. This interaction is used in ELISA testing and enables the identification of particular protein antigens and antibodies with very little sample volume. ELISA kits are widely used in the diagnosis of Ebola, pernicious anaemia, acquired immunodeficiency syndrome (AIDS), rotavirus, Lyme disease, syphilis, toxoplasmosis, Zika virus, carcinoma of the epithelial cells, and many other pathologies and therefore play an important role in life science research.

Keywords: Sandwich ELISA kit, competitive ELISA kit, immunoassay, diagnostic applications, antibody–antigen complexes

INTRODUCTION

ELISA, or enzyme-linked immunosorbent assay, is a fundamental technique used to detect and quantify hormones, proteins, antibodies, and peptides present in biological samples such as tissue, blood, plasma, and serum [1]. Antibodies are the proteins produced in blood in response to the immunogenicity illicit by the specific antigens. It helps in determining the presence of antibodies in response to a specific infection in the body. When compared to other antibody assays, ELISA has unique properties as it provides qualitative and quantitative results and separates specific and non-specific interactions through serial binding to solid surfaces, which is a polystyrene microtiter plate [2].

PRINCIPLE OF ELISA

The ELISA assay was developed for the purpose of detecting antigens or antibodies. The technique employs a specific antigen or antibody that is firmly adhered to a solid phase, such as the plastic surface of a polyvinyl plate or polystyrene tube; hence, it is also known as solid-phase immunosorbent assay [3]. When a test sample is put on a microtitre plate, antigen–antibody (Ag–Ab) reactions will occur if antigen or antibody is present in the sample (with immobilised Ab or Ag). Enzyme-labeled antibodies are additionally added to the reaction mixture; these antibodies will eventually bind with either the test antibody's Fc region or the test antigen [4].

The components of the enzyme system are as follows:

1. An enzyme, such as alkaline phosphatase or horse radish peroxidase, that has been labeled or coupled to a particular antibody [5];

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2. An appropriate substrate [6]:
   - o-Phenylenediamine dihydrochloride for peroxidase;
   - Alkaline phosphatases for P Nitrophenyl Phosphate (PNPP).

At the end, the substrate is added to the Ag–Ab reaction, which is further catalysed to produce a coloured end point (for instance, a yellow-coloured compound in the case of alkaline phosphatase). The concentration of antigen or antibody in the test sample is quantified via an ELISA reader, and the intensity of colour is directly proportional to the concentration or amount of the same [7].

CHARACTERISTICS OF ELISA

The ELISA technique is more frequently used in the identification of antibodies or antigens due to its higher sensitivity and specificity. It uses only microliter volumes of test reagents and is just as sensitive as radioimmunoassay (RIA). The detection of several antibodies and antigens, including hormones, poisons, and viruses, has now been widely used around the globe [8–10]. The main characteristics of ELISA are as follows:

1. The excellent specificity and sensitivity of the ELISA test;
2. It is possible to read the results of quantitative ELISA tests visually;
3. A lot of tests can be performed simultaneously. ELISAs are appropriate for use in monitoring and centralised blood transfusion programmes since they are developed specifically for screening large numbers of specimens at once;
4. ELISA reagents are stable and can be distributed in district and rural laboratories, but their usage is restricted to specific optimised conditions as more complex instruments and qualified workers are required to perform the test.

CLASSIFICATION OF ELISA

ELISA tests can be classified into three different categories depending upon the methods used for binding interactions between antibodies and antigens, namely:

- *Indirect ELISA:* Antigen captured microtiter well plate [11].
- *Sandwich ELISA:* Antibody captured microtiter well [12].
- *Competitive ELISA:* Antigen-coated microtiter well plate filled with Ag–Ab complex mixture [13].

Indirect ELISA

The two-step indirect ELISA process involves the binding of a primary antibody and an enzyme-tagged secondary antibody. The primary antibody is first incubated with antigen, followed by incubation with a tagged secondary antibody. However, secondary antibodies can induce a cross-reaction, which might result in nonspecific signals [14]. In the indirect ELISA test, the sample antibody is sandwiched between the antigen coated on the plate and an enzyme-labeled anti-species globulin conjugate. A chromogenic reagent and an enzyme substrate are added to develop colour. The amount of bound sample antibody is directly and inversely related to the colour production. The colour development in the test wells increases with the amount of antibody in the sample [15]. Indirect ELISA is useful for quantifying total antibody levels in samples [16–18] (Figure 1).

Protocol

1. Coat the microtiter plate with antigen: Pour 50 µl of antigen solution (a coating agent) into each well.
2. Seal the coated plates with plastic wrap and incubate them in an incubator for 2 h at 37°C.
3. After incubation, uncover the microtiter plate and then transfer the solution to a container.
4. Washing step: After removing the coating solution, wash the plate twice by adding 200 µl of phosphate buffer saline (PBS) to each well. Remove the last few drops by patting the dish with a piece of paper towel.
5. Blocking step: Add 200 µl of blocking buffer to the coated wells and incubate for 30 min at room temperature to block the last of the protein-binding sites.

6. Dispose of the solution and carry out the washing procedure. Flip the microplate gently onto the paper towel. Further, add 50 µl of the antibody solution via micropipette to the wells.
7. Place plastic wrap over the plate and let it sit at room temperature for 2 h. Discard the liquid, then place dry absorbent paper on the plate's bottom.
8. Repeat the washing step.
9. Repeat the blocking step.
10. Repeat the washing step.
11. Add 50 µl of the secondary antibody reagent to each well.
12. The microtiter plate should be covered with plastic wrap and let dry for 2 h at room temperature.
13. Repeat the washing process.
14. Add 75 µl of the substrate solution to each well of the microtiter plate.
15. Place plastic wrap over the microtiter well plates and let them dry at room temperature for an hour. Fill the wells on the microtiter plates with 25 µl of the stop solution (0.5 M NaOH) in the vial.
16. Use a 405-nm filter with a microtiter plate reader to measure p-nitrophenyl phosphate (NPP) hydrolysis.

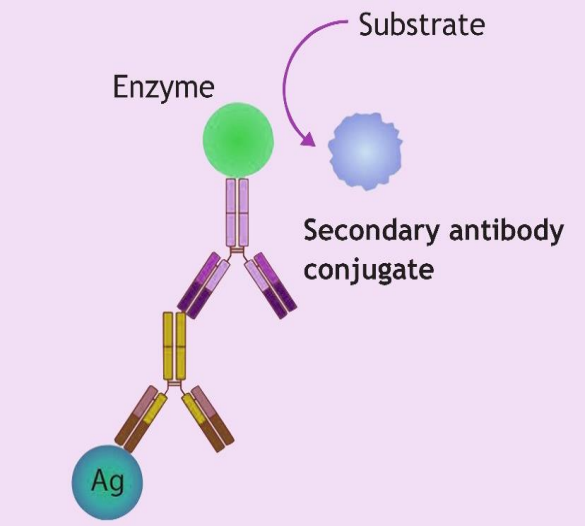

Figure 1. Indirect ELISA assay.

Sandwich ELISA

A sandwich ELISA measures the amount of antigen present between the two layers of capture and detection antibodies. At least two antigenic sites must be present on the target antigen to optimise its binding to antibodies. In this type of ELISA, both monoclonal and polyclonal antibodies can be used for detection purposes. Monoclonal antibodies can quantify even a minute difference in antigen by recognising a single epitope. A polyclonal antibody is typically used as the capture antibody to extract as much antigen as feasible [19]. Sandwich ELISAs increase sensitivity by skipping the sample purification step before analysis, about two to four times more sensitive than other ELISA technique. Sandwich ELISAs increase sensitivity by skipping the sample purification step before analysis (2–5 times more sensitive than direct or indirect) [20] (Figure 2).

Coating with Capture Antibody

1. Use carbonate/bicarbonate buffer (pH 9.6) to coat the capture antibody at a concentration of 1–10 g/ml in the wells of a PVC microtiter plate.
2. Seal the plate with adhesive plastic and incubate at 4°C overnight.
3. Each well of the microtiter plate is washed twice with 200 µl of PBS with the removal of coating solution. Flip the plate over a sink to discard the solution.
4. Remove the remaining drops by patting the dish with a piece of paper towel [21].

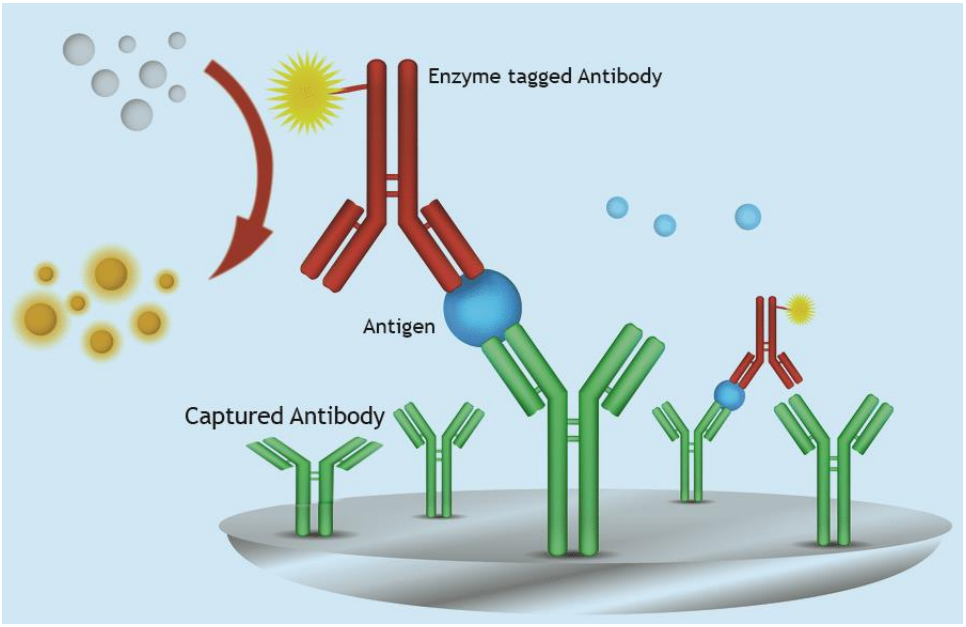

Figure 2. Sandwich ELISA assay.

Incubation and Detection with Secondary Antibody

1. Fill each well with 100 µl of diluted detection antibody from the microtiter well plate.
2. Wrap the plate with adhesive plastic and let it dry at room temperature for 2 h.
3. Use a PBS buffer to clean the plate four times.
4. Add 100 µl of labeled secondary antibody to the blocking before use.
5. Place adhesive plastic over the microtitre well plate and let it dry at room temperature for a few hours.
6. Wash the plate with PBS buffer four times [22].

Detection

The two most used enzymes for ELISA assays are alkaline phosphatase (ALP) and horse radish peroxidase (HRP) [23].

ALP Substrate

P-nitrophenyl-phosphate (pNPP) is the most often employed substrate for the majority of applications. After 15–30 min of incubation at room temperature, check the nitrophenol's 405 nm yellow colour, and halt the reaction by adding an equal volume of 0.75 M NaOH [24].

HRP Chromogens

Hydrogen peroxide is the substrate for the HRP enzyme. The oxidation of a hydrogen donor is coupled by the cleavage of hydrogen peroxide, which changes colour with the execution of the reaction [25].

TMB (3,3',5,5'-tetramethylbenzidine)

Each well should first be filled with the TMB solution. After 15–30 min, add an equal volume of the stopping solution (2 M H_2SO_4). The optical density should be observed at 450 nm.

Calculations

Always run ELISA tests on duplicate or triple samples. This will help in providing adequate data for the results' statistical validation. There are now many computer programmes that help in ELISA result processing. Make a calculation of the average absorbance values for each set of comparable standards and samples. Duplicates should lie within 20% of mean [26].

Standard Curve Plot

Plot the mean absorbance (y axis) versus the protein concentration to create a standard curve for the target protein (x axis). Obtain a best-fit curve between the graph's points (there are many suitable computer software programmes that help in graph plotting). Each sample's target protein concentration can be calculated using the standard curve. This is normally done via curve-plotting software. This will further provide you with an equation to determine the concentration (x) from an absorbance (y) in the standard curve's range. The samples should be diluted or concentrated prior to the ELSA test in order to get accurate results. In order to evaluate the results for the samples, the concentration derived from the standard curve must be multiplied by the dilution factor [27, 28].

Coefficient of Variation

The coefficient variation (CV) is given by,

$$C_v = \sigma/\mu$$

Where, σ = standard deviation
μ(mean).

The results are expressed as % age of deviation from the mean and hence identify the errors.

Larger errors are indicated by a larger variance. Several computer programmes can determine the CV values using the ELISA data [29].

High CV May Result From

Pipette tips should be sealed to the pipette before use to prevent inaccurate pipetting and guarantee that the proper volume of liquid is drawn up [30].

Reagent Splashing Between Wells

Contamination of reagents or screen samples by bacteria or fungi.

Reagent Contamination

Variations in temperature across the plate; make sure the plates are incubated in a temperature-controlled space away from draughts.

Make sure the plates are always covered during the incubation steps if some of the wells are drying out.

Spike Recovery

The impact of sample components on antibody detection of the antigen is assessed by spike recovery. For instance, many proteins included in tissue culture supernatant may prevent antibody binding and raise the signal-to-noise ratio, which will cause the target concentration to be underestimated [31]. Protein spikes at predetermined concentrations are added to both the sample matrix and a reference diluent. The technique is used to measure the spiked protein. The sample matrix is regarded as valid for the assay process if the findings are identical. If the recovery is different, something in the sample matrix is preventing the analyte from being detected [32].

Direct Competitive ELISA Assay

In competitive ELISA, the purified antigens compete with antigens present in the sample for binding with antibodies that have been immobilised in microtiter plate wells. It is also known as "inhibition ELISA." The same concept works with the antibodies present in the test sample and the purified antibodies for the immobilised antigen. A direct ELISA assay system employs a labeled antibody or antigen, whereas an indirect assay configuration use reported tagged secondary antibodies. Competitive ELISA can be used to quantify the concentration of small molecules or antigens present in a sample [33].

In the direct ELISA assay, antigen-specific capture antibodies are adsorbed onto the microtiter plate prior to incubating with either known standards or unidentified test samples. Labeled antigen can only attach to the capture antibody when neither the antigen standard nor the antigen in the test samples have occupied the antibody's binding site. Unbound labeled or unlabeled antigens are removed and substrate is added. The concentration of antigen in the test sample or standard determines the amount of labeled antigen bound to the antibody by producing a signal that is inversely proportional to the concentration of antigen in the sample. Higher antigen concentrations in the test sample result in weaker signals due to the less-tagged antigen linked to the capture antibody. (Fig. 3).

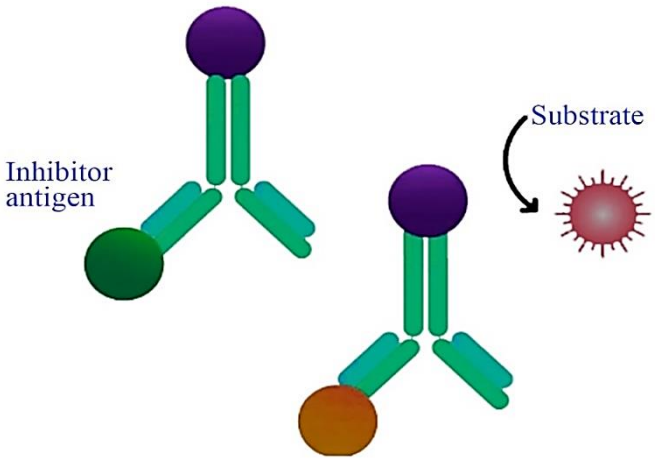

Figure 3. Direct Competitive ELISA Assay.

ADVANTAGES OF ELISA ASSAY

1. ELISA results provide an accurate diagnosis of a specific disease as they involve the use of two antibodies.
2. Results are highly responsive, as both direct and indirect methods are available.
3. Provide quick and rapid results.
4. The possible detection range of ELISA includes quantitative, semi-quantitative, standard curve, qualitative, calibration curve, etc.
5. It is easy to perform and does not require the presence of radioactive material [34–36].

APPLICATIONS OF ELISA

1. Detection of antigens and antibodies in the sample;
2. Identification of any food allergens present in the food sample;
3. To calculate the serum antibody concentration for a viral test;
4. During a COVID-19 outbreak, rapid kits are used to identify the presence of antibodies in a blood sample to assess the spread of the disease.
5. ELISA kits are extensively used in the detection of AIDS, syphilis, pernicious anaemia, rotavirus, Ebola, Lyme disease, epithelial cell carcinoma, toxoplasmosis, and many other pathologies [37, 38].

ELISA TROUBLESHOOTING

High Background Signals [38–40]

1. Fresh 3,3',5,5'-Tetramethylbenzidine (TMB) substrate should be used**,** which must be transparent and colourless before being added to wells.
2. Incubation of the substrate must be done in complete darkness. Make sure the substrate is not exposed to light by keeping it in a dark area. While doing the experiment, minimise exposure to light.
3. Raising salt concentrations may lessen weak or non-specific interactions off-target.

4. Reusing plate sealers can result in the existence of leftover HRP, which can cause TMB to change colour without warning. Use new plate sealers and reagent reservoirs on each stage to prevent this.
5. Increase the sample's dilution factor or reduce the substrate's concentration.
6. Prepare fresh buffer every time.
7. Make sure the reagent has been adequately diluted or reduce the concentration of detection reagent.
8. Try to use different blocking reagents and/or add blocking reagents to the wash buffer.

Out of Range Absorbance [41–44]

1. Appropriate washing procedures should be used. To completely drain the plate after each washing process, flip it onto absorbent tissue and give it a firm tap if necessary to get rid of any leftover liquid.
2. Plate sealers should be used to cover assay plates during incubations. Every time the plate is opened, apply a new sealer, which prevents wells from contaminating each other.
3. Mix the substrate solution thoroughly before using it.
4. Always use fresh buffers.

High Variation [45–48]

1. Make sure the pipettes are calibrated and functioning properly. Ensure the pipette tips are pressed on sufficiently to form a tight seal. While carefully diluting the plate, ensure that each pipette tip is picking up and releasing the appropriate volume of liquid.
2. Verify that no reagent comes in contact with the plate sealer before using it again. While adding reagents, it is important to use different pipette tips.
3. Refrain from stacking plates as it prevents an even dispersion of temperature among the wells.
4. With an ELISA plate shaker, the plate should be stirred throughout the incubation process at a constant pace to keep the fluids in the wells moving continuously without splashing.
5. When pipetting, try to keep your hands away from the well's bottom. To prevent disturbing the well's bottom, direct the pipette's tip towards the well's side.
6. Use internal controls, verify calculations, and make new standard curves.
7. Make sure the plates' lids are always on during incubation. Put a humidifying water tray on the bottom of the incubator.
8. Prior to reading the plate, make sure there are no bubbles.
9. All the pipettes should be calibrated.

Weak Reproducibility [49–53]

1. Make sure that the incubations are conducted at the correct temperature. Before proceeding further, make sure that all the reagents, including the plate, are at room temperature.
2. Ensure that the automated plate washer is under proper pressure. If washing is done manually, pipette the wash buffer gently.
3. Do not allow the wells to become dry after the assay has started. For all incubations, seal the plate with sealing film.
4. Change the assay to one that is more sensitive (for example, direct ELISA to sandwich ELISA). Extend the incubation period or increase the temperature, which may alter the detection technique.
5. Ensure that the target of interest and the assay buffer are compatible (for example, enzymatic activity and protein interactions are retained).
6. Decrease dilution factor or increase sample concentration.
7. Try different antibody concentrations or dilutions.
8. Prior to the starting the experiment, all reagents should be kept on the work bench at room temperature for about 30 min.
9. Make sure that all the reagents are used before their expiration date.

Poor Standard Curve [54–56]

1. The preferred coating buffers are PBS with a pH of 7.4 or carbonate bicarbonate buffer with a pH of 9.6.
2. Extend the incubation period if possible or use different plates.
3. Examine the pipetting technique and adjust the reagent volume.
4. Check dilution, titrate if necessary.
5. Plot using several scales, such as log-log or a 5-parameter logistic curve fit.

CONCLUSION

ELISA technique has several unique features in comparison to other traditional available antibody assays, as it helps in quantitative and qualitative analysis all together with the separation of specific and non-specific reactions taking place through serial binding to solid surfaces, which is commonly a polystyrene microtiter well plate. BTL Biotechno Labs Pvt. Ltd. is committed to advancing life science research in India by providing a comprehensive selection of ELISA kits. These kits are designed to identify a wide array of proteins and molecules, including growth factors, drugs, cytokines, biomarkers associated with conditions such as diabetes, infectious diseases, cancer, as well as small molecules and various other targets. ELISA kits serve as valuable research tools, offering researchers a quick, straightforward, and accurate means of detecting and quantifying their study targets in biological samples and cell cultures. BTL Biotechno Labs Pvt. Ltd. also provides multiplex ELISA kits, competitive ELISA kits, sandwich ELISA kits, and cell-based ELISA kits for many biological targets. The kits include standards, buffers, pre-coated plates with antibodies for capture and detection, and additional reagents.

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