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An overview of ELISA: a review and update on best laboratory practices for quantifying peptides and proteins in biological fluids

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Abstract

The enzyme-linked immunosorbent assay (ELISA) detects antigen-antibody interactions by using enzyme-labelled conjugates and enzyme substrates that generate colour changes. This review aims to provide an overview of ELISA, its various types, and its applications in detecting metabolites in biological fluids. The article discusses the history of the assay, its underlying principles and procedures, common ELISA protocols, and the most accurate and reliable techniques for measuring peptide molecules in biological fluids. Additionally, we emphasize best laboratory practices to achieve consistent, high-quality results and outline the essential materials for setting up an ELISA laboratory, drawing from our over 30 years of experience in the field.

Keywords

Biological fluids, ELISA, antibody, antigen, enzyme-linked immunosorbent assay

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Background

Based on MalaCards, the comprehensive human disease database, over 26,000 distinct illnesses are affecting people today.[1] In addition to water, the key chemicals that make up life are lipids, proteins, carbohydrates, and nucleic acids.[2] Imbalances in the synthesis and degradation of these macromolecules lead to diseases.[3] Accurate, rapid, and reliable testing of disorders in macromolecule synthesis and breakdown is essential for effective patient treatment.[4] For example, peptide molecules (fewer than 50 amino acids), synthesized and released in numerous tissues, have recently been linked to the development and progression of numerous disorders, including diabetes, inflammatory bowel disease, and polycystic ovary syndrome (PCOS).[4–7]

Laboratory analyses can be performed on a variety of biological fluids, including blood (plasma or serum), saliva, urine, milk, cerebrospinal fluid (CSF), amniotic fluid, gastric juice, semen, pleural fluid, peritoneal fluid, synovial fluid (joint fluid), bronchoalveolar lavage fluid (BAL), as well as cyst fluids (e.g., ovarian or hydatid) and fluids from various fistulas, in both research and diagnostic settings. These tests are performed to diagnose and differentiate diseases, assess disease severity, monitor treatment progress, and detect asymptomatic conditions.[8–12] Of the aforementioned biological fluids, venous blood is the most frequently used in both diagnostic and research studies.[13] Almost all peptide molecules can be analysed from venous blood without requiring any special conditions. The Enzyme-Linked Immunosorbent Assay (ELISA) method is widely used in laboratories to quantify peptide and protein molecules accurately, reliably, easily, and sensitively. This not only simplifies clinicians' tasks but is also crucial for ensuring that patients recover as quickly as possible.[14]

In this article, we have revised and expanded our previous publication on ELISA,[4] offering an updated perspective based on the latest information. We briefly discuss the history of the assay, the method's working principle and steps, common ELISA protocols, and the most accurate and reliable techniques for measuring peptide molecules in biological fluids. We also highlight laboratory practices that can help achieve universal ideal criteria, as well as the necessary materials for an ELISA laboratory, drawing from our over 30 years of laboratory experience.

Search strategy

For this narrative review, a comprehensive literature search of PubMed/Medline, Web of Science, Google Scholar, and Scopus was conducted to identify relevant studies. We used MeSH and free-text terms. Search terms included: enzyme linked immunosorbent assay OR ELISA OR ultra-high detection OR digital ELISA) OR ELISA History, OR Biological Fluids AND (serum, plasma, saliva, mucus, urine, amniotic fluid, vaginal fluid, semen, amniotic fluid, tears, pleural fluid, peritoneal fluid, bronchoalveolar lavage fluid, cerebrospinal fluid, interstitial fluid, milk, perspiration OR sweat fluid) AND (Analytical errors) AND (laboratory equipment and their uses). Relevant papers were included in a modified form of SANRA (Scale for the Assessment of Narrative Review Articles).[15]

In total, 69 articles were identified and formed the basis of this review.

History of ELISA

ELISA has evolved through multiple stages of development over the years and is now a commonly used diagnostic test in laboratories. In the 1960s, a few researchers pioneered a method that could chemically bind to biological enzymes and measure

them in the presence of an appropriate substrate, marking a significant milestone in the development of the ELISA method. This preliminary research paved the way for other researchers to further develop the ELISA method.[16] In 1971, an immunological method was introduced that involved conjugating antigens or antibodies with enzymes, replacing the use of radioisotopes.[16] By the early 1970s, several laboratories had adopted the ELISA test for diagnostic microbiology.[17] By 1980, the ELISA method had been established for diagnosing infections caused by influenza, parainfluenza, and mumps viruses.[18–20] In 1980, the ELISA test was modified and evolved into the current new generation of ELISA tests, that use microtiter plates.[20]

Principles of ELISA

ELISA is an immunological biochemical assay used to detect and measure antibodies, antigens, peptides, proteins, glycoproteins, and hormones in biological samples.[4,21] The method is based on the principle of detecting the antigen-antibody interaction and the enzymatic activity linked to the antibodies.[4,22] In the ELISA test, the antigen or antibody being targeted adheres to plastic surfaces, referred to as the 'sorbent.' The antigen recognized by the specific antibody (such as the immunoglobulin (Ig) G fraction of serum or monoclonal antibodies) is called the 'immuno.' When this antibody binds to a second antibody (also 'immuno'), it becomes 'enzyme-linked.' The enzyme then reacts with a substrate, producing a measurable coloured product.[4,21,23]

Patient samples such as serum, plasma, urine, saliva, milk, or tears, are accepted as antigens.[4] Specially prepared reagents are combined with the patient sample.[24–26] The final mixture consists of free labelled antibody, labelled antigen, and the antigen-antibody complex. After the antigen-antibody binding, the complex is firmly attached to the inner wall of the test tube, while any unbound components in the medium are washed away. At the end of the experiment, the target substance, bound to the inner wall, is measured.[21]

ELISA methodology

The key components essential for ELISA include:

a. Solid phase (Matrix): 96-well microplates (Figure 1(a)), where analytes (standards, antigens, or antibodies) are attached (Figure 1(b)). Rigid polystyrene,

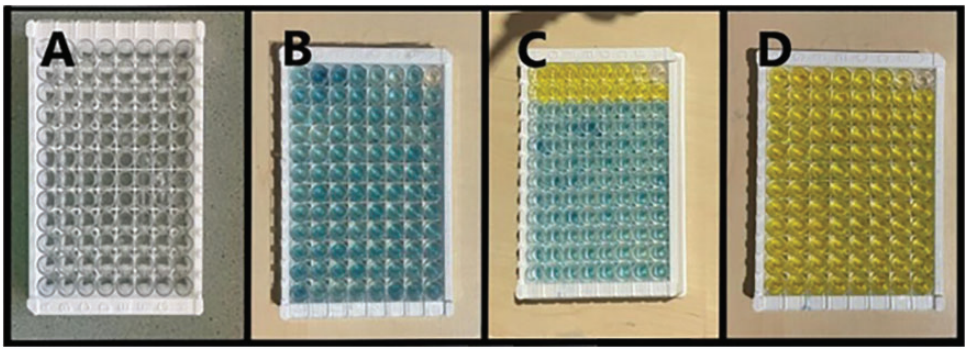

Figure 1. ELISA kit contents. (a) Microplate; (b) Microplate loaded with analytes; (c) Yellow colour occurs when stopping solution is added and (d) Yellow colour occurs when stopping solution is added.

polyvinyl, and polypropylene tubes or microplates are preferred as solid phases.

b. Conjugate: Enzyme-labelled antibodies specific to the molecule and primary antibody being detected. For instance, if the aim is to detect the presence of an antibody in human plasma, the conjugate will contain anti-human IgG.[21,27] The most commonly used enzymes for labelling the conjugate include alkaline phosphatase (AP) substrate BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium), horse radish peroxidase (HRP, MA: 44,000, containing 4 lysine residues and acting as a glycoprotein), and substrate tetramethylbenzidine (TMB), which produces a blue colour in the presence of peroxidase.[4,21,28] When HCl is added as a stop solution, a yellow colour is produced (Figure 1(c), (d)). A colour change occurs when these enzymes (enzyme conjugates bound to the antibody) interact with their chromogenic substrate.

c. Substrate: It is the substance that reacts with the enzyme in the conjugate to produce the formation of antigen-antibody complexes. Depending on the structure of the chromogen in the substrate the reaction produces green, yellow, or blue products (Figure 1(c), (d)).

d. Wash: Rinsing with phosphate-buffered solution (PBS) or wash buffer between each step is essential.

e. Stop: The final step of ELISA, known as 'stopping the reaction,' involves using acidic (H_2SO, HCl) or basic (NaOH) solutions to stop the enzyme-substrate reaction at the desired time.[28] Typically, the enzyme-substrate reaction is completed within 30–60 minutes.

f. Reading: The intensity of the colour produced at the end of the reaction is measured spectrophotometrically on an ELISA reader at a wavelength of 400–600 nm, depending on the substrate used, with 450 nm being the most commonly used wavelength. The relationship between optical density in the wells and analyte concentration in the sample can be direct (as in sandwich formats of ELISA). A standard curve is generated from serial dilution data, with concentration plotted on the x-axis (using a log scale) and absorbance on the y-axis (using a linear scale).[22] Additional equipment for the ELISA reader includes a computer and a printer.

ELISA protocols

Direct ELISA

This method is used to detect antibodies. It requires pure or semi-pure antigen, a test solution containing antibodies, and an enzyme conjugate that binds to Ig in immunized samples. In other words, it involves testing for the presence of an antigen by using antibodies specific to the suspected antigen (Figure 2(a)). The test is performed as follows a) The plate (made of polystyrene, polyvinyl, etc.) is coated with a known antibody (i.e., an antigen-specific enzyme-bound antibody). b) The suspected material (antigen) is added, binding to the inner surface of the test tube. c) After each step, a wash is performed to remove any unbound substances following incubation for the appropriate duration. d) The substrate is then added to produce a colour change in the antibody-bound enzyme. e) Finally, after adding the stop solution, the molecule of interest is measured using the ELISA reader.[4,29,30]

Indirect ELISA

This method is used to detect and identify soluble antigens. The difference between indirect and direct ELISA is that, in indirect ELISA, it is not the primary antibody that identifies and captures the antigen to be measured, but a secondary antibody in the

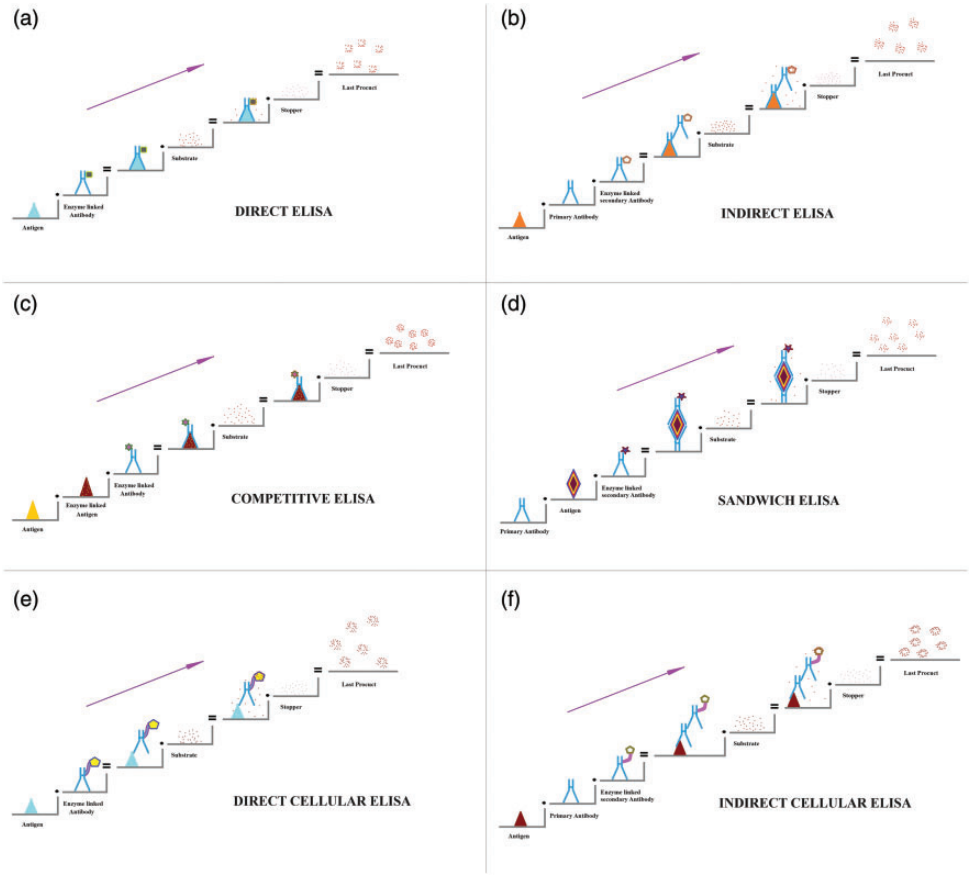

Figure 2. Schematic drawings of: (a) Direct ELISA; (b) Indirect ELISA; (c) Competitive ELISA; (d) Sandwich ELISA; (e) Direct cellular ELISA and (f) Indirect cellular ELISA.

medium that binds with the primary enzyme. This type of ELISA is used to detect antibodies in suspected biological fluids and measure their titers. The required chemicals include pure or semi-pure antigen, a test solution containing the antigen, and the enzyme-antibody conjugate specific to the antigen. The test is performed as follows: a) The plate is coated with the known antigen. b) The suspected biological sample (e.g., serum, plasma, etc.) is added. c) The conjugate (anti-antibody) is added. d) The substrate is added. After each step, the plate is washed following the appropriate incubation period. In direct ELISA, the conjugate is prepared against the antigen, while in indirect ELISA, it is prepared against the primary antibody (Figure 2(b)).[4,29]

Competitive ELISA

This method is commonly used to measure antibodies. In this method, the inner surface of the measuring tube is coated with antigen, which is tagged with a marker enzyme. Essentially, it operates on the principle that the patient's antigen and the labelled antigen compete for binding to the antibody, similar to the approach used in RIA (radioimmunoassay) with radioactive labelling agents. For this type of ELISA, the medium must contain the patient's antigen

(X), a known quantity of labelled antigen (X*), and a known quantity of antibody. For example, if 20 antibody molecules and 20 labelled antigen molecules (X*) are used, and 5 labelled X* antibodies bind to 5 X* antigens, the remaining 15 antibodies are bound by the patient's antigen X. Thus, the greater the binding of the labelled X* antigen to the antibody, the lower the amount of the patient's antigen that remains bound. (Figure 2(c)).[4,23]

Sandwich ELISA

The amount of antigen in unknown samples is determined. This method requires two antibodies that must recognize different epitopes. The first antibody, known as the capture antibody, binds to the antigen, while the second antibody is a biotinylated antibody that detects the captured antibody. After washing away the unbound antigen, another antigen-specific antibody is added and incubated again. The enzymes typically used in this method are HRP and ALP, with TMB serving as the chromogenic substrate. This method is the most sensitive of all ELISA types, offering 2–5 times more sensitivity. As a result, it is the preferred measurement option when only small amounts of specific antibodies are available and no purified antigen is present. (Figure 2(d)).[4,22]

Direct Cellular ELISA

This method is used to detect cell surface antigens and receptors. Cells are incubated with enzyme-linked antibodies specific to cell surface molecules. After the bound conjugate is washed, the enzyme's substrate is added, allowing the antigen level in the cells to be determined (Figure 2(e)).[31]

Indirect Cellular ELISA

This technique measures the amount of antibodies specific to cell surface antigens. After incubation with the samples, unbound antibodies are removed. The cells are then incubated with enzyme-conjugated antibodies specific to the primary antibody. Any unbound enzyme conjugates are washed away, and the substrate is added (Figure 2(f)).[31]

Limitations of ELISA

Direct ELISA tests are the simplest and fastest to perform among all ELISA types, but they have some limitations. These limitations should be considered when deciding if ELISA is the most suitable analysis format for a specific research or diagnostic need. The previously published review article provides a detailed discussion of the detection limits, advantages, and drawbacks of various ELISA types.[4] A summary of these limitations is provided below.

Sensitivity

Compared to indirect or sandwich ELISA formats, direct ELISA tends to be the least sensitive. The use of only one antibody for detection limits signal amplification, reducing its sensitivity for detecting antigens present in low quantities

Antibody availability

Direct ELISA requires high-quality, specific antibodies against the target antigen to function effectively. For certain antigens, obtaining or producing these antibodies can be difficult, which limits the applicability of direct ELISA.

Single analyte detection

Direct ELISA can detect and quantify only one antigen at a time. It is not suitable for multiplexing, which involves the simultaneous detection of multiple antigens in a single sample.

Background noise

Nonspecific binding of antibodies or other sample components to the solid surface can lead to background noise in direct ELISA, even after the blocking step. This can amplify background signals, reducing the signal-to-noise ratio and potentially affecting the accuracy of the test.

Restricted dynamic range

The quantitative dynamic range of direct ELISA may be limited. Compared to other ELISA formats, the test typically has a smaller linear range, making it less suitable for measuring a wide range of antigen concentrations.

Cross-reactivity

The potential for cross-reactivity with related or comparable antigens can result in interference or false-positive results.

Time consuming

When processing a large number of samples, the multiple incubation and washing steps required by direct ELISA can make the test time-consuming. This may limit its use in high-throughput settings.

Chromogens used in ELISA

P-Nitrophenyl-phosphate (PNPP)

PNPP ($C_6H_4NO_6P$-$_2$) measures AP. Dissolution in water produces a yellow colour. Activity is based on the ability of phosphatase to catalyse the hydrolysis of PNPP to p-nitrophenol, a chromogenic product with maximum absorption at 405 nm.[32] It is an important chemical used both for ELISA and for molecules analysed spectrophometrically. The substrate concentration is much higher than Km (the Michaelis constant), giving it an advantage over a radioactive analysis. PNPP is used because of its low cost and since the substrate concentration is not a limiting factor in the reaction, the rate of reaction can be measured over a wide range of substrate concentrations. This molecule is light-sensitive and should be stored away from light. It should also be kept at −20°C when not in use, as it degrades at higher temperatures.[32,33]

2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS)

The chromogenic peroxidase substrate ABTS (C18H18N4O6S4) is widely used in ELISA procedures. In ELISA, a soluble end product is formed that is green in colour and can be read spectrophotometrically at 405 nm. This compound is also used in the food industry to measure the antioxidant capacity of foods. Because ABTS radical is not affected by + ionic strength and can be used to determine both hydrophilic and hydrophobic antioxidants. In this case, the ABTS radical + radical cation, a molecule that gives maximum absorbance at wavelengths of 645, 734, and 815 nm, can react with most antioxidants, including phenolic compounds, and be converted back to its colourless neutral form (ABTS).[34,35]

O-phenylenediamine dihydrochloride (OPD)

OPD ($C_6H_4(NH_2)2$, an aromatic diamine, is a water-soluble substrate for HRP that produces a yellow-orange product detectable at 492 nm by ELISA plate readers. Although OPD is a white compound, it appears dark due to oxidation in air. OPD can also condense with ketones and aldehydes to form industrially valuable products. For example, it reacts with formic acids to produce benzimidazole. OPD has been reported to be potentially carcinogenic in some studies but results are conflicting. ELISA kit

manufacturers should therefore take this property of OPD into account.[36,37]

3.3′,5.5′-tetramethylbenzidine (TMB)

TMP ([-$C_6H_2(CH_3)$2-4-NH_2]2) produces a blue colour, allowing the measurement of HRP. It gives maximum absorbance between 370 and 652 nm wavelengths and is highly sensitive. As the concentration of the molecule to be measured increases, the oxidation of TMB, a coloured product, also increases. Due to this feature, it may allow the measurement of some molecules through biosensors to be installed on smartphones in the future. This molecule is degraded by sunlight and fluorescent lights. Therefore, it should be stored in amber bottles and kept away from sunlight and fluorescent lights. In addition, although TMB is white in colour, it turns pale blue-green when dissolved in ethyl acetate. In addition, if the sample used has haematuria, the blue colour becomes darker (when in contact with haemoglobin). This results in higher concentrations of the samples to be measured in the ELISA. Therefore, haematuria should be excluded. To measure accurate concentrations in the presence of haematuria, ELISA kits containing other chromogens should be used.[38] This molecule is a crucial chromogenic molecule and is also used in immunohistochemistry procedures.[39]

Examination of pre-analytical stage errors

The period from the collection of samples for research to their analysis is known as the pre-analytical phase (the process before the analysis of the test), and in peptide analysis and other clinical studies, almost two-thirds of all errors occur in this stage. The most significant errors made during this stage stem from mistakes made before sampling. For example, errors arising from medications and foods consumed by the individual are included in this category. Therefore, questioning which types of medications patients are taking and which foods they are eating is extremely important in terms of good laboratory practice. Additionally, not every peptide is expected to be involved in the pathophysiology of every disease. Therefore, a solid hypothesis should be established before conducting peptide research.[40] For a measurement to be precise and trustworthy, these sources of error should be removed.[4] Additionally, some patients may be using multiple protease inhibitors, such as amprenavir, atazanavir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir, saquinavir, and tipranavir, as an essential part of retroviral therapy.[41] This should be addressed during the patient's medical history inquiries since these protease inhibitors may affect the concentration of peptides to be measured in biological fluids.[4]

Protease inhibitors and their role in accurately measuring specific peptides

In the fields of medicine, molecular biology, and biotechnology, peptidases, substrates, and peptidase inhibitors are key factors that impact the accurate measurement of peptide concentrations in biological fluids.[4] Over 39,000 cleavage sites in proteins, peptides, and synthetic substrates have been identified. According to the MEROPS database, more than 700 proteases are synthesized by cells.[42] Since peptides are small molecules that are rapidly degraded by proteases, it is advisable to use biochemistry tubes containing protease inhibitors to prevent this degradation.[4]

Aprotinin (Trasylol). Aprotinin is an irreversible protease inhibitor with a molecular weight of 6512.[43] This molecule was once the most commonly used antifibrinolytic agent in cardiac surgery and was derived

from bovine pancreases until it was discontinued in 2007.[44] Today, it is commonly added to tubes containing aprotinin-EDTA to prevent peptide degradation by proteases (Note: Protease inhibitors should be avoided in enzymatic studies as they can inhibit enzyme activity). Studies suggest that 500 kallikrein inhibitor units of aprotinin are enough to safeguard peptides from proteases in one millilitre of biological fluid. Aprotinin remains the preferred choice for protecting peptides from proteases; biochemistry tubes containing aprotinin-EDTA are available in several medical markets.[45]

4-(2-Aminoethyl)-benzenesulfonyl fluoreide (AEBSF). This water-soluble protease inhibitor has a molecular weight of 239.5 Daltons and functions as an irreversible serine protease inhibitor. It targets and inhibits proteases such as chymotrypsin, kallikrein, plasmin, thrombin, and trypsin. Even at low pH levels, it effectively protects peptides from protease degradation. If biological samples are being analysed at low pH, this protease inhibitor should be the preferred choice. For one millilitre of biological fluid, 500 kallikrein inhibitor units of AEBSF are sufficient to prevent protease activity on peptides.[4,46,47]

Phenylmethylsulfonyl fluoride (PMSF). This molecule, a serine protease inhibitor, is commonly used in the preparation of cell lysates. PMSF (molecular weight: 174.19 Da) does not inactivate all serine proteases but specifically targets the active site of the serine residue in hydrolase enzymes, without binding to other serine residues in proteins or peptides. The recommended concentration of PMSF ranges from 0.1 to 1 mM, though it has a short half-life in aqueous solutions. Unless otherwise specified, AEBSF is generally preferred over PMSF.[48]

Kispeptins, ghrelins and salusins. The protease cocktail is the most widely used inhibitor mix for kispeptins. It protects peptides and proteins from degradation by aminopeptidases, metalloproteases, and serine, cysteine, and aspartic acid proteases. This cocktail is particularly recommended for molecules that are quickly degraded by proteases, such as kispeptins. To ensure reliable peptide measurements in biological fluids, samples should be collected in tubes containing the proper protease inhibitors; otherwise, the results may not be accurate.[49]

Ghrelin is a highly fragile peptide composed of 28 amino acids, with or without a fatty acid attachment. It primarily exists in four different forms in biological fluids. Secreted mainly by the X/A cells in the gastric fundus, ghrelin is resistant to acidic conditions. When collecting biological samples, it is important to add 500 kallikrein units of aprotinin and store the samples with a 1/10 ratio of 1 normal HCl to maintain its stability.[50,51]

Salusins are also highly fragile peptides, existing in both alpha and beta forms. To ensure accurate measurements, it is crucial to use protease inhibitors when collecting biological samples. Additionally, these peptides have a tendency to adhere to the walls of collection tubes, which can be prevented by adding Tween-20 or NP-40 to the tubes. Without these precautions, the detection of salusin alpha and beta peptides may be unreliable or impossible.[52,53]

Optimal conditions for ELISA measurements

The antigen and antibody should be present at optimal concentrations on the solid phase coating. It is essential to ensure that the incubation period, pH, and temperature are all within ideal ranges. While fresh serum or plasma typically has a neutral pH (approximately 7), these biological

samples quickly lose CO_2, which raises the pH to alkaline levels (pH >8). Alkaline pH can interfere with antigen-antibody interactions, leading to inaccurate results. If needed, the samples should be adjusted to the optimal pH conditions before use.[54]. The appropriate substrate should also be carefully selected. Determining the optimal concentration of antigen and antibody is crucial for establishing reference samples and control points, which can be done using the checkerboard method (where concentrations are diluted stepwise from at least twice the highest value down to a quarter of the concentration to find the minimal measurable range).[55] Additionally, it is important that the same technician conducts the entire ELISA study to maintain consistency, and only ELISA kits from a single manufacturer should be used within the same study.[4]

Ensuring the accuracy of ELISA measurements – insights from our lab experience

A negative control, such as PBS added to the well, must be included in the experiment. If the negative control produces a positive result, it could be due to inadequate washing of the plate, poor blocking, or contamination of the substrate solution (commonly from contamination with the secondary antibody). If there is no colour in the positive controls or samples, possible sources of error include improper storage or expiration of reagents, incorrect coating of the microwell plate, incorrect order of reagent addition, or missed steps in the procedure. Additionally, enzyme conjugates may be defective or contaminated.

If the positive control and samples show minimal colour development, it may be due to an incorrect dilution of the enzyme-labelled antibody. Insufficient incubation time could also be a factor. Incomplete resuspension of the washing buffer during the washing steps may contribute to the issue. Additionally, the substrate concentration could be incorrect, or the enzyme conjugates and substrate may be defective or contaminated. Poor sealing of the microplate may also lead to reduced signal.

If colour develops in the samples but not in the positive controls, the error could stem from improper storage or expiration of the positive controls. If sufficient colour is formed but the sample absorbance is lower than expected, the issue may lie in the instrument being set to an incorrect wavelength.

If the standard curve is not smooth, the error could have occurred during the preparation of dilutions from the original standard solutions. If the standard curve appears flat, it may indicate that the standards were not added. In general, if there is little or no colour development at the end of the experiment, it could be because the standard or sample was not added, or the reagents required for colour formation were either not added or incorrect ones were used. Additionally, improper storage of the kits or excessive washing of the plate wells could be contributing factors.

Common errors

Even after addressing the potential errors mentioned above, other issues may still arise when performing the ELISA method. These additional potential errors include:[4,56,57]

Preparation of standards. Standards should be prepared through serial dilution, starting from the highest concentration down to the lowest. Any errors in this process can lead to inaccurate results for the entire study. It is crucial to mix the solution thoroughly during dilution. Additionally, separate controls should be prepared for each individual study.

Preparation of the binding solution. The pH of the binding solution is critical: 0.1 M Na

HPO4 should be adjusted to pH 9.0 or pH 6.0 using 0.1 M NaH2PO4. Incorrect pH will prevent proper binding. Additionally, it is important to note that certain antibodies may require pH 6.0.

Washing. Washing is a crucial step in ELISA procedures. All washing steps should be carried out using PBS, and it is recommended to use an automated washing device. Proper washing removes non-specific binding without impacting specific binding. Inadequate washing can lead to false positives

Reaction stop. Failing to stop the reaction promptly, or delaying the process, can cause spontaneous (non-specific) colour changes in the chromogenic substrate over time, leading to inaccurate results. It is also important to carefully monitor the incubation times.

Readings. Typical wavelengths used for readings are 405, 450, and 630 nm. A common mistake is reading at wavelengths outside those recommended by the kit manufacturers. Using too small a wavelength can lead to high absorbance from the molecules in the biological fluid, while using too large a wavelength can result in underestimating the concentration of the target substance. It is also essential to read the samples immediately after adding the stop solution to avoid erroneous results. A modern reading device that can store data is also beneficial for ensuring measurement accuracy. Other factors, such as the conformation and stability of the target antigen, the type and concentration of the enzyme conjugate, the quality of the substrate, and potential human or reader errors, can also interfere with ELISA results.[21,28] Errors in background (blank) values when using serum or plasma can be minimized by increasing the concentration of the blocking solution (for example, from 1% to 2% BSA w/v) or by adding a small amount of a non-ionic detergent like Tween-20 (e.g., 0.05% v/v). Alternatively, using a plate shaker and extending the blocking incubation time can help reduce high background (blank) readings during ELISA measurements.[58,59]

Advantages of ELISA

ELISA is often preferred due to the wide availability of the necessary instruments in nearly all laboratories, its simple and straightforward procedures, and its high specificity and sensitivity (able to detect levels as low as 1 pg/ml).[60] Additional advantages include the low cost, easy accessibility, and long shelf life of the reagents, as well as its ability to measure hormones, peptides, and a wide range of other molecules in biological fluids such as serum, plasma, saliva, and cell extracts. ELISA is also widely used for detecting various infections, including COVID-19.[61]

Recent advancements in ELISA technology aim to improve the limit of detection. One such innovation is digital ELISA, along with its combination with thio-NAD cycling. Digital ELISA, developed about 15 years ago, enables single-molecule detection by performing the enzymatic reaction in femtoliter-sized microwells, allowing detection of extremely low target concentrations. Over time, digital ELISA has undergone significant improvements. For example, a digital homogeneous non-enzymatic immunosorbent assay was developed; this is a highly sensitive technique derived from digital ELISA that eliminates the need for washing or signal amplification steps.[62] While a commercially available highly sensitive ELISA detected 0.84 pg/ml of prostate-specific antigen, digital ELISA could detect as low as 0.055 pg/ml.[63]

To further enhance sensitivity, a modified ELISA using thio-NAD cycling has been explored. Traditional sandwich

ELISA provides measurable signals by colour change of the substrate, but its sensitivity is limited because the signal increases linearly over time. To overcome this limitation and achieve ultrasensitive, some researchers have tried to implement an ultrasensitive ELISA by combining thio-NAD cycling with a sandwich ELISA.[64] This modification helps amplify the signal, allowing detection of ALP at zeptomole levels (i.e., 10^{-21} moles) thus significantly improving the assay's sensitivity.[65]

Disadvantages of ELISA

As mentioned previously, the ELISA method is a straightforward procedure with many advantages, making it a common choice in laboratories. However, the primary drawback of this method is the hook effect (also called antibody excess or the prozone phenomenon), where an unexpected decrease in signal or inaccurately low results occur even when the analyte concentration is high.[66] To mitigate the hook effect, two approaches can be considered:[67] (a). *Sample Dilution*: Preparing different dilutions of the sample to ensure it falls within the assay's linear range. (b). *Two-step Assay Protocol*: By separating the capture and detection stages, competing reactions can be minimized. To effectively use ELISA for accurate detection and quantification of target analytes, careful experimental design and validation are crucial.[66,67]

Manufacturers of ELISA kits

Today, numerous manufacturers worldwide provide various ELISA protocols. The contact information for these well-known ELISA kit suppliers has been updated from our previous publication.[4]

ELISA kits available on the market are typically designed to quantify molecules in serum or plasma samples. However, for proper laboratory practice, in addition to validation by the ELISA kit manufacturers, each laboratory should re-validate the kits themselves. This is especially important if the kits are intended for use in measuring molecules in other biological fluids, such as saliva, milk, or synovial fluid. In such cases, validation studies (including tests for validity and accuracy) are essential. While the general principles of validation are consistent, they may vary depending on the specific application. For instance, a pregnancy test may require a sensitivity of 95%, while a test for myocardial infarction must have a sensitivity greater than 99%. Typical validation studies include assessments of sensitivity, selectivity, threshold control, intra-day and inter-day variability, inter-user variability, and stability.[68] The steps involved in validating an ELISA kit are generally as follows.[4,68,69]

Validation of ELISA kits

Linearity. After diluting the samples, it is important to verify whether the measured concentrations are linear. Specifically, one validation test to assess whether an ELISA kit designed for serum works with the same sensitivity in saliva samples is a linearity assay. To conduct this, both serum and saliva samples should be diluted in phosphate buffer at $1/2$, $1/4$, $1/8$, and $1/16$ concentrations. These samples should then be analysed using the ELISA method, with their concentrations measured to check for linearity. In essence, the samples are tested twice at 3–4 different dilutions, and the results are averaged. These measured concentrations are then compared with the expected standard values to determine if they are linear.

Recovery experiment. This experiment assesses whether the target molecule interferes with measurements in different biological fluids. For instance, an ELISA kit designed for serum is tested with serum

and saliva samples containing specific concentrations of the target molecule (e.g., 10, 20, 40, 80 ng/ml) to determine if there is interference in saliva. These concentrations are then analysed using the ELISA method to measure how much of the molecule is recovered. Before conducting the recovery assay, the baseline concentration of the target molecule in the biological fluids should be established through ELISA (e.g., if saliva naturally contains 20 ng/ml of the target peptide, and 80 ng/ml of the pure peptide is added, the expected reading should be 100 ng/ml if there is no interference).

Precision (reproducibility) assay. In ELISA precision testing, the results should consistently be the same, ensuring reproducibility. Precision testing encompasses intra-assay (within the same assay), inter-assay (between different assays), and inter-laboratory (across different laboratories) variations.

In the intra-assay (or intra-day variation) test, control samples are run 15–20 times consecutively and measured at least five times throughout the day at regular intervals using the ELISA device to assess day-to-day variability. The mean and standard deviation (SD) are then calculated, and the coefficient of variation (% CV) is determined using the formula % CV = (SD/mean) $\times 100$. A lower % CV indicates better reproducibility (% CV$\downarrow \geq$ precision $\uparrow$). An intra-assay CV of 10% or less is typically considered acceptable and reflects the reliability of the ELISA kit.

In the inter-assay (day-to-day variation) test, samples are run 15–20 times consecutively and measured at least five times on different days using the ELISA device to assess variation between days. Similar to the intra-assay, the mean and standard deviation (SD) are calculated. The coefficient of variation (% CV, inter-assay) is then determined using the formula % CV = (SD/mean) $\times 100$. A lower % CV indicates better repeatability (% CV$\downarrow \geq$ precision $\uparrow$). It is important to calculate CV values based on concentration data, not optical density. Typically, intra-assay CV values should be below 10%, while inter-assay CV values should be below 15%. Higher than expected CV values may result from errors such as pipetting issues, high sample viscosity (e.g., saliva), repeated freezing and thawing, vortexing or centrifugation mistakes, uncalibrated pipettes, or improper pipette tips. Additionally, inter-assay variability should be assessed by having a sufficient number of users—representative of those who will actually use the test—perform the test. An inter-assay CV of 15% or less is generally considered acceptable, indicating good measurement reliability of the ELISA kit. A summary of the main steps in the ELISA kit validation check is provided in Table 1.

Limit of detection (LOD). Each assay type has a detection limit, which refers to the smallest concentration of molecules that can be reliably detected. This limit is the lowest point at which the test can consistently identify a substance, and values below this threshold will not yield accurate results. To establish the limit of detection (LOD), a molecule of known concentration is diluted and measured. If any molecules in a biological sample fall below the detectable level, a pure standard molecule is introduced. The amount of this added standard is subtracted to determine how much of the target molecule is undetectable. It is important to note that the LOD is not directly comparable between different assay types, such as ELISA and protein measurement systems, and suggesting that protein measurement systems have a lower LOD than polymerase chain reaction (PCR) is both misleading and incorrect. Furthermore, ELISA kits from different manufacturers may have varying minimum detection ranges.

Sensitivity refers to how effectively a test identifies positive samples as positive. For

Table 1. Main Steps in the ELISA Kit Validation Check.

Validation parameters	Validity	Minimum number of samples required	
		Qualitative tests	Quantitative tests
Accuracy	Positive	3	3
	Low positive	3	3
	Negative	3	3
Sensitivity	Positive	8	8
	Low positive	8	8
Specifity	Negative	15	8
Precision (intra-assay)	Positive	1	4
	Low positive	1	3
Precision (inter-assay)	Positive	2	2
	Negative	2	2
Linearity	Positive	0	2 (at least 3 dilutions containing 10-fold dilutions)

example, if a new test analyses 100 positive samples and correctly identifies 96 of them, the sensitivity is 96%. Selectivity, on the other hand, is the ability of a test to identify negative samples correctly as negative. For example, if the same test analyses 100 negative samples and correctly identifies 95 as negative, the selectivity is 95%. These measures reflect the test's performance when used by the end-user and are distinct from the metrics used by the manufacturer to monitor the consistency of the assay during production,[4,68,69]

Equipment needed in an ELISA laboratory

The materials shown in this section are the ones currently in use in our laboratory. There are many different types of laboratory materials available globally that can perform the same functions. By obtaining the materials listed here, it is possible to establish a fully equipped ELISA laboratory.

a. ELISA kit and microwell plate

The microwell plate is a flat, polystyrene plate with 96 pre-coated, stabilised wells, each capable of holding up to 350 µl of liquid (Figure 1). ELISA kit manufacturers provide precoated, stabilized 96-well microtiter plates, along with standard and control samples (positive and negative controls), dilution buffer, conjugated detection antibody, TMB substrate (or other chromogens mentioned above), and stop solution in a single package. This eliminates the need to purchase these materials separately. We frequently use kits from the reputable *SunRed* company. These kits are popular because they are user-friendly and help researchers save time. We have conducted comparisons of various manufacturers' kits assess their reliability as part of our internal quality control processes.

b. Microplate reader

The microplate reader, also known as a microtiter reader or microplate photometer, is a device used to detect and measure biological, chemical, or physical processes in samples placed in microtiter plates. These devices are produced by various manufacturers around the world and are capable of delivering highly accurate measurements.

The system is equipped with microplate reader software, typically controlled by a PC. This software allows for the recording of up to 100,000 test results and the ability to transfer them to a printer.

c. Microplate washer

This device comes in different formats, including strip washers, full plate washers, and washer-dispenser combinations. It is used during the washing stages of ELISA experiments to remove unbound antigens, antibodies, and contaminants.

d. Homogenizer

This device rapidly breaks down biological tissue samples at high speeds and rotations to homogenize and suspend them. It is an essential tool when studying supernatants from biological tissues in the ELISA method. Biological tissues must be prepared in consistent amounts by weighing them with a precise balance. The ELISA test can then be conducted by homogenizing the tissues. Zirconium iron beads are commonly used for tissue homogenization. While biological tissue can also be prepared by crushing it in an Eppendorf tube with an iron bar in the absence of a homogenizer, this method is not recommended due to its lack of accuracy.

e. Other equipment required in an ELISA laboratory

These may include: a precision balance; refrigerator and freezer (kept at $-70^{\circ}C$); vortex mixer; incubator; distilled water production device; deionised water; automatic pipettes; pipette tips; timers; surgical gloves; paraffin; labelling pens; scissors; rubber bulbs; beakers; conical flasks; Eppendorf tubes.

Conclusions

Today, ELISA techniques are widely used to diagnose and monitor a variety of diseases, including those related to endocrine, metabolic, cardiovascular, and microbiological conditions. The ELISA test is one of the most sensitive methods for measuring substances like peptides, proteins, and hormones, capable of detecting even the smallest concentrations, ranging from 5 pg/ml to 10 pg/ml. The sensitivity of ELISA assays is determined by the specifics of the antibody-antigen interaction. To ensure accurate and high-quality results for diagnosing and monitoring conditions, standardized ELISA procedures and well-trained staff are crucial. This includes proper sample collection, transport, analysis, and capacity. Regular in-service training helps ensure consistent processes, criteria, and results. By improving laboratory conditions and addressing potential error sources, we can enhance patient comfort, reduce strain on national healthcare systems, and produce more reliable results.

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Supplementary material

Supplemental material for this article is available online.

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