

Enzyme-Linked Immunosorbent Assay (ELISA)

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Abstract

Food allergy is a public health concern especially after recognizing its constantly increased prevalence and severity. Despite careful reading of food ingredient statements, food allergic individuals may experience reactions caused by “hidden”, “masked”, or “contaminated” proteins that are known major allergens. Many techniques have been developed to detect even small traces of food allergens, for clinical or laboratory purposes. Enzyme-linked immunosorbent assay (ELISA) is one of the best validated and most routinely used immunoassay in allergy research, in allergy diagnosis in allergy-related quality control in various industries. Although as a technique it has been implemented for the last 45 years, the evolution in biochemistry allowed the development of ultrasensitive ELISA variations that are capable of measuring quantities in the scale of picograms, rendering ELISA attractive, robust, and very famous.

Key words Enzyme-linked immunosorbent assays (ELISA), Protocol, Method, Food allergy, Allergen

1 Introduction

1.1 Background

Food allergy is one of the major public health issues with prevalence between 5 and 10% that is constantly increasing [1]. It is not known what is causing this increase, but seems to be multifactorial with significant components being the genetic susceptibility, the interacting environment, cultural, behavioral, and dietary particularities [2–6].

The food allergens are proteins capable of stimulating a type-I hypersensitivity reaction in sensitized individuals through immunoglobulin E (IgE)-mediated reactions even at very small amounts [7, 8]. Till now there is no known etiological treatment for food allergy, therefore all efforts rely on prevention [9]. It can be relatively easy for an allergic individual to recognize food products made by ingredients they are allergic to, but it might be tricky to identify hidden, masked, or contaminated small amounts of allergens.

Prevention is based upon: (a) appropriate food labeling practices by food manufacturers to make it easy for food allergic

individuals to identify the presence of clinically important food allergens in their packed products [10], and (b) patients training to follow correct label reading practices to recognize and avoid everything they are allergic to. Teleologically, the identification of the traces that may exist in processed food is of major importance and for that reason the detection techniques that will be applied should be highly sensitive. The most commonly used analytical method for food allergen detection is based on the enzyme-linked immunosorbent assay (ELISA) technique.

1.2 ELISA Principles

The ELISA is a sensitive and specific analytic biochemistry assay utilized for detection and quantitative or qualitative analysis of an analyte without the requirement of sophisticated or expensive equipment [11–13]. The analyte could be any specific substance, either a specific protein or a more complex mixture of more than one protein (e.g. a biomolecular complex).

As a methodology, ELISA is based on a few important scientific advances the most important of which is the production of antigen-specific antibodies either monoclonal or polyclonal. Secondly, the development of radioimmunoassay techniques has been a milestone. With this technique the detection antibodies can be labeled with radioisotopes providing an indirect way of quantifying a protein by measuring the radioactivity. Alternatively, the indirect quantification can be performed by measuring the signal that is produced when utilizing the appropriate substrate, with antibodies that are chemically linked to biological enzymes. Similarly, fluorescence tag technology (a form of luminescence also known as a label or probe) allows the indirect quantification of protein chemically attached with a fluorescent dye by measuring fluorescence quantum yields and compare it with a standard. Generally, ELISA has good sensitivity with a limit of detection/limit of quantification (LOD/LOQ) down to lower nanogram scale [14, 15].

In ELISA, a liquid sample, that has specific binding properties, is added onto a stationary solid phase inside a reaction chamber or a microplate well. Then, different liquid reagents are sequentially incubated resulting in some optical change (e.g. color development by the product of an enzymatic reaction) in the final liquid. This change is integrated as a dynamic variable into mathematical formulas which compare the optical density with a known standard parameter, and are able to indirectly quantify the analyte of interest.

The detection of an antigen by ELISA relies on the recognition and specific binding of that antigen (potential allergen), which is known as ligand, to a specific antibody. Allergen-antibody binding depends on the solubility and integrity of the protein. These antigen-specific antibodies may recognize a particular epitope on an allergen (monoclonal antibodies), a mixture of allergens in the same source (e.g. Ara h 1, Ara h 2, Ara h 3, Ara h 6, etc. allergens in peanut) (polyclonal antibodies) or allergens with homolog

epitopes (cross reactive allergens). [16]. Cross-reactivity is the ability of an antibody to react with similar antigenic structures/epitopes on different proteins. This may occur on unrelated proteins from the same (e.g. Ara h 2 and Ara h 6 allergens in peanut) or different species (e.g. hevein-like protein in banana and latex, or LTP allergens in different foods of plant origin) and can be a result of lack of protein purification or measuring a protein in a mixture of unknown proteins with cross-reacting epitopes. Both cases may lead to false-positive results [17] rendering cross-reactivity the main shortcoming in protein quantification.

Some food products contain several major allergens (e.g. peanut contains Ara h 1, Ara h 2, Ara h 3, etc.) that may all cause an immunological response, although they may have dissimilar chemical and structural properties. This does not preclude the possibility of someone being allergic to only one protein (e.g. only Ara h 2, in peanut) [16].

1.3 Variations Between ELISA Protocols

There are two principle methods that vary in the way the antigen of interest is immobilized. In the *direct coating* approach the antigen is diluted usually in a carbonate/bicarbonate buffer with pH > 9 and directly attached to the inner surface of the wells of a microtiter plate by passive adsorption (Fig. 1a, b). In the capture, or *indirect coating*, also known as Sandwich ELISA approach, an antigen-specific antibody that is adsorbed onto the wells, immobilizes the antigen

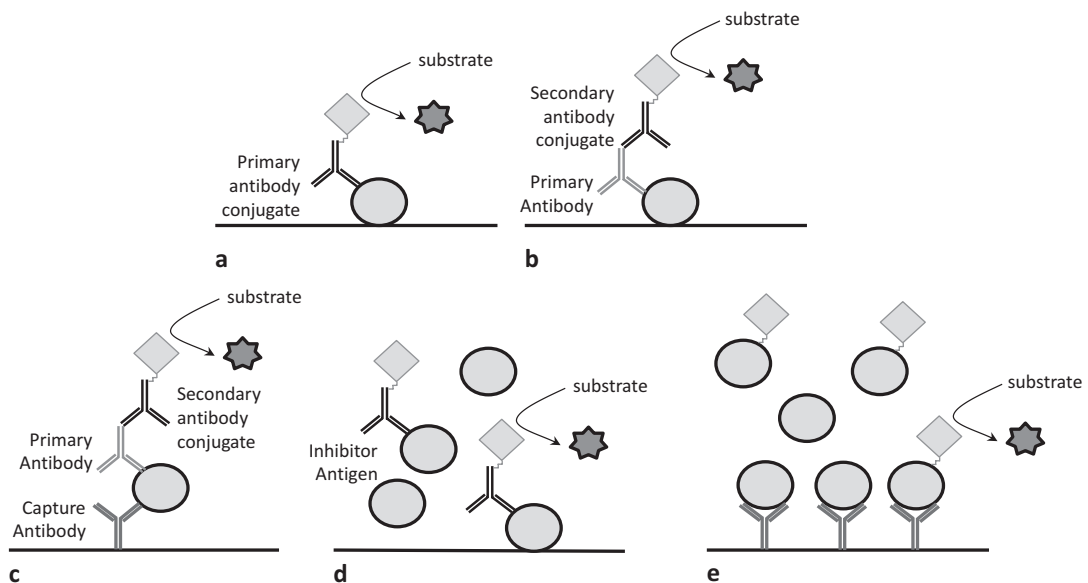


Fig. 1 Popular ELISA formats. (a) Direct ELISA, (b) Indirect ELISA, (c) Sandwich ELISA, (d) Competitive ELISA with labeled antibody, (e) Competitive ELISA with labeled antigen

of interest after incubation with the antigen sample (Fig. 1c). This approach is more sensitive and commonly used when the antigen to be detected is in small amounts, or its physicochemical properties do not allow sufficient adherence to the wells, or when the samples include more than one protein. With this approach only the antigen of interest is bound to that antigen-specific antibody.

There is a more complicated ELISA format that is called competition or competitive ELISA. The distinguishing feature of competitive ELISA is that the combination of the reference/standard analyte with an unknown amount of the same analyte (introduced from the unknown sample) competes for binding to a limited number of antibody binding sites. The competitive ELISA assay can be performed with either the analyte or the antibody absorbed to the solid phase. In the first variation of this format (Fig. 1d), the added sample analyte is competing with solid phase absorbed reference/standard analyte for binding to a limited amount of labeled antibody. In the second variation of this format (Fig. 1e), the labeled reference/standard analyte in solution is combined with the unknown sample analyte and they both compete for binding to a limited amount of solid phase absorbed antibody.

For the first variation, after the incubation period of the competitive mixture, any unbound antibody is washed off. The more antigen the sample has, the more conjugated antibody will be bound to the sample antigen and, consequently, the smaller the amount of the unbound antibody that will be available to bind to the coated antigen. In the second variation, the same approach applies for the conjugated antigen. Absence of color indicates the presence of antigen in the sample [18].

The selection of the format used depends mainly on the size of the target molecules. For instance, the sandwich format can be used to measure intact allergenic proteins or big fragments. On the other hand, the competitive format has to be used in certain circumstances like, for instance, in the case of small peptides which contain only one binding epitope that an antibody can recognize [19].

In all aforementioned approaches, the antigen of interest can be detected with two ways: in the *direct detection*, the primary antibody is labeled with an enzyme or a fluorescent chemical compound known as fluorophore (Fig. 1). The *indirect detection* method involves an additional step using another antibody (a secondary antibody that specifically binds to the primary antigen-specific antibody) conjugated with a detectable tag [e.g. horseradish peroxidase (HRP) or alkaline phosphatase (AP) enzymes], or biotin. In the case of a biotinylated antibody the detectable tag is bound to avidin or streptavidin, two molecules that bind very strongly with biotin molecule. The direct detection approach is faster and eliminates the potential extra background signal due to cross-reactivity between the secondary antibody and the coating antibody, however is less sensitive than the indirect detection

method which can provide signal amplification. This amplification can be explained either from the fact that biotinylated antibodies have multiple biotin tags per antibody molecule, thus allowing more than one avidin/streptavidin molecules to bind to each antibody molecule, or from the fact that avidin/streptavidin conjugation process with enzymes result in conjugates with more than one enzyme. Therefore, the indirect detection method is preferable when the target antigen is expected to be in low amounts.

Last but not least, a final chemical reaction is needed in order to generate a measurable signal from an enzyme. The catalysis of a specific substrate produces a fluorescent compound, chemiluminescence or, the most commonly used, colored compound. These signals can be measured with an appropriately filtered fluorometer, a luminometer or a spectrophotometric plate reader, respectively. Although chemiluminescent detection is considered more sensitive, the colorimetric approach is the most commonly used. Colorimetric substrates like 2-2'-azino-di-(3-ethylbenzthiazoline sulfonic acid (ABTS), 3,3',5,5'-Tetramethylbenzidine (TMB) and o-Phenyl-diamine-dihydrochloride for peroxidase (OPD) for HRP, and p-Nitrophenyl Phosphate for Alkaline Phosphatase (pNPP) for AP, form a soluble colored product that can be measured directly or halted using a stop solution for direct measurements.

All these different ELISA types may be implemented in different settings (laboratory-research or clinical-diagnostic) but they are all based on similar technical procedures [16]:

1. **Coating or Capture:** Direct or indirect (by the coated captured antibody) immobilization of the standards (e.g. allergens/antigens) to the inner surface of the wells of a polystyrene microtiter plate by adsorption.
2. **Blocking:** Addition of an irrelevant protein/molecule to cover all unsaturated surface-binding sites of the microtiter plate wells.
3. **Detection (or Probing):** Incubation with antigen-specific antibodies that are bound to the immobilized antigens.
4. **Signal Measurement:** Detection of the signal generated via the direct or secondary tag bound on a specific antibody.

1.4 Producing the Standards—Food Allergen Isolation

Regardless of the format that will be followed, there is always a need to obtain standardized extracts and pure allergens. Ideally, a purified recombinant standard can be used, but there might be a need to extract the standard protein directly from the examined food. Food allergens are denatured mixtures of proteins in complex matrices with different physicochemical properties, and therefore there is not a single, standard extraction method suitable for all foods [20].

Almost always the extraction starts with a tissue and cell disruption which is most of the times accomplished with homogenization (the process of reducing a substance to extremely small particles

and distributing it uniformly throughout a fluid). Homogenization is followed by different procedures aiming at solubilization and precipitation, fractionation and purification and allergen enrichment techniques (Fig. 2).

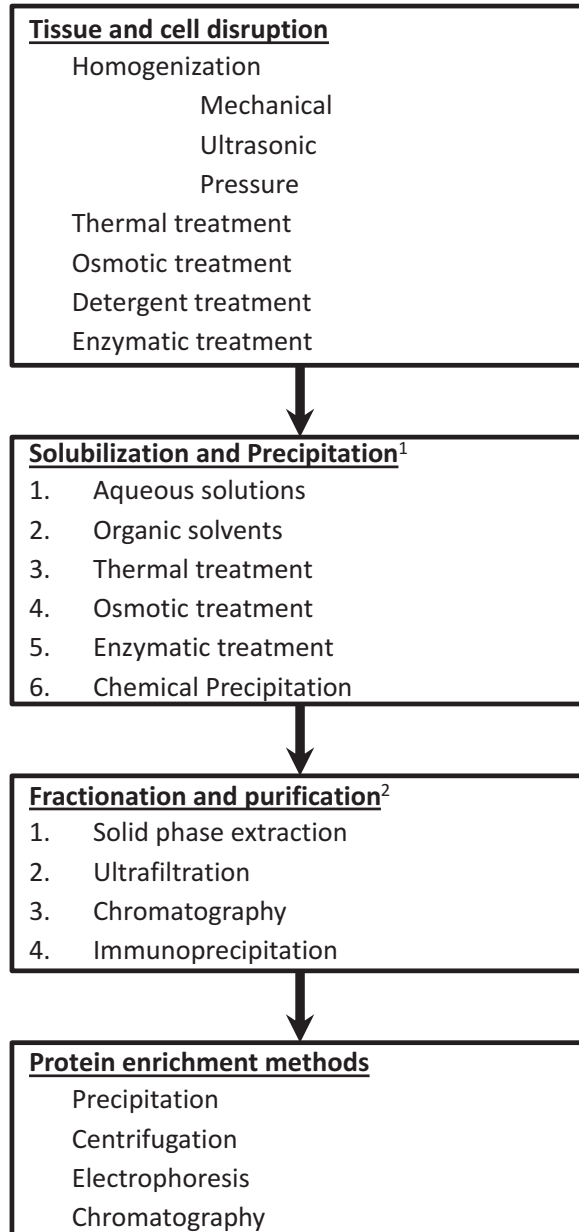


Fig. 2 Extraction and fractionation techniques for proteins from foods of plant or animal origin.

Superscript 1 protein separation from lipids, carbohydrates, nucleic acids, and mineral.

Superscript 2 sample cleanup and protein isolation

Protein extraction from foods of plant origin is generally more problematic because plant tissues are rich in proteases and other interfering compounds [21]. The method of choice of plant protein extraction (e.g. cereals, legumes, and fruits) is based on trichloroacetic acid (TCA)/acetone precipitation [22]. The combination of acetone with the negative charge of TCA and the extreme pH causes immediate denaturation and precipitation of the proteins, while it deactivates the proteolytic and, in general, modifying activity of the enzymes included in the treated mixture. One disadvantage of TCA-precipitated proteins is that they are difficult to redissolve [23].

Sample solubility can be improved by using an appropriate mixture of agents that exert chaotropic activity (agents that can disrupt the hydrogen bonding network between water molecules) like urea or thiourea, detergents like sodium dodecyl sulfate (SDS) or the phenol extraction procedure. Phenol exerts strong solvent action on proteins but only has a little predisposition to dissolve polysaccharides and nucleic acids, although as a procedure is time-consuming and toxic [24]. For storage proteins extraction (e.g. soybean proteins glycinin and β -conglycinin) ammonium sulfate may be a good precipitant [25, 26].

Aqueous alcohols (ethanol, isopropyl alcohol, butanol) are also used in the extraction process to remove oligosaccharides, phenolics, or inhibitors from defatted meals and seeds [24]. However, they may change the structure of the proteins, induce coagulation, reduce the number of available epitopes and, as a consequence, reduce the functional properties of the protein. To minimize this consequence, mechanical and thermal treatments can be applied, which might also interfere with protein functionality but to a much less degree [27, 28].

An example of proteins difficult to dissolve and isolate is gliadin. Gliadin is one of the main components of the gluten fraction in wheat, rye and barley seed and a member of the prolamins (a group of plant storage proteins). Gliadins are slightly soluble in ethanol but there are a few extraction solutions that are able to extract prolamins in a quantitative manner without interfering with the ELISA results [e.g. “cocktail solution” by Garcia et al. [29]. and the “RIDA[®] extraction solution” R 7099 (R-Biopharm AG)].

Another type of protein isolation and enrichment that is widely used in food allergy is immunoprecipitation. With this technique, an antibody is used to precipitate the protein of interest by forming a precipitating antigen–antibody complex [30].

1.5 Must Knows Before Using ELISA

1. *Homemade vs commercial ELISA kits*

Homemade ELISA kits may be sensitive and specific enough [31] but they require specialized, sometimes time-consuming extraction or isolation techniques and labor measurements of allergen concentration that can be affected by

several variables like food matrix and thermal processing [32, 33]. Thus the involvement of an expert is recommended at least at the initial steps of the development of such an ELISA kit, in order to be confident that the technique is accurate and applicable. This is not the case for commercially available ELISA kits that offer advantages such as good sensitivity, need for only limited technical equipment, simultaneous sample analysis and ease in execution without need for specialized personnel. This is the reason these kits are widely used by the food industry [34].

2. *Information about antibody specificities*

It is of crucial importance to know the clonality (monoclonal or polyclonal antibody), the host, the antibody isotype, the purification process and if possible which protein was used to generate the antibody (a fractionated and isolated protein from a natural source, a modified protein, a synthesized protein or a few specific peptides from that protein) [35].

3. *Information about cross-reactivity*

Knowing the exact protein utilized to produce an antibody could be enough to hypothesize which might be the cross-reactive epitopes recognized by the produced antibody, and where these epitopes can be found. Since epitope-specific antibody production cannot be customized when whole protein extracts are used, any potential cross-reactivity must be tested against a variety of food commodities or purified proteins that might be cross-reactive candidates.

4. *Information on matrices*

Epitope recognition is matrix-dependent as it has been shown for several foods with the most well examined paradigm being egg in which at least fat and gluten affect its allergenicity [36, 37]. Therefore, ELISA results are expected to be susceptible to matrix effects or perform differently in different matrices. Ideally, the antibody developer should have performed tests with different matrices and identify those that the antibody may have difficulties or may not be applicable for.

2 Materials

2.1 *Disposables and Equipments*

1. Clear ELISA microtiter 96-well plates (*see Note 1*).
2. Disposable plate sealers.
3. Assorted graduated cylinders.
4. Assorted volume, 8- or 12-channel multichannel precision pipettes.
5. Disposable plastic pipette tips.

6. Wash bottle and/or automatic plate washer.
7. ELISA plate reader or luminometer with appropriate software to detect the substrate (usually 405–650 nm).
8. ELISA plate shaker.
9. Incubator.
10. Vortex.
11. pH meter.
12. Adhesive plastic (Parafilm).
13. Small-volume bottles.
14. Timer.

2.2 Reagents

1. Coating buffer: 0.05 M sodium carbonate/bicarbonate buffer, pH 9.6.
2. Capture antibody: Diluted in Coating Buffer.
3. Wash buffer: phosphate-buffered saline containing 0.05% Tween-20 (PBST).
4. Blocking buffer: usually 2% (w/v) Bovine Serum Albumin (BSA) in Wash buffer. Alternatively, the following may be used: 1% human serum albumin (HSA) in PBS + 5% goat serum albumin (NGS), 1% human serum albumin (HSA) in PBS + 10% goat serum albumin (NGS), 1% HSA in PBST, 1% BSA in PBS + 10% NGS, 2% NGS in PBST, 1% normal sheep serum (NSS) in PBST, 2% NSS in PBST, 1% BSA in PBST, 2% BSA in PBST, etc. (*see Note 2*).
5. Standard: Purified antigen of interest. A standard curve span concentrations is dependent upon the predicted amount of antigen in the sample and the amount of standard protein available (usually from 0 to 3000 pg/mL). Dilution of the standard is performed with the blocking buffer.
6. Sample: The sample is diluted using the blocking buffer as many times needed to fit the standard curve concentrations span.
7. Conjugated detection antibody: dilute to the appropriate concentration in blocking buffer.
8. Enzyme conjugate: Streptavidin-HRP diluted to the appropriate concentration in blocking buffer.
9. Substrate: ABTS or TMB substrate.
10. Stop solution: 2 M sulfuric acid.
11. Control: As negative control use three wells coated with coating buffer and incubated in all following steps with blocking buffer.

3 Methods

3.1 ELISA Optimization

The following steps are needed for optimization. In all steps check for strong signal versus low background:

1. Use appropriate controls to account for any background signal/noise generated that cannot be attributed to the presence of the analyte under investigation. The best controls that can be used are wells coated according to the protocol but incubated in all other steps with the blocking buffer. In these wells, the conjugated detection antibody is added followed by the substrate, and in some others the conjugated antibody is omitted and only the substrate is added (*see Notes 3–5*).
2. In capture assay format, use two antibodies with different specificities. Titrate the primary antibody concentration between 500 ng/mL and 15 μ g/mL. The latter concentration will be sufficient to demonstrate saturation.

Optimize capture and detection antibody testing in at least three different concentrations. Check at the same plate different combination of capture versus detection antibody tested concentrations (*see Note 6*).

3. In direct assay format, titrate antigen concentration in the range of 1–20 μ g/mL. Optimize detection antibody testing in at least three different concentrations. Check at the same plate different combination of antigen versus detection antibody tested concentrations (*see Note 6*).
4. Optimizing blocking time. Optimal blocking requires 1–2 h, but 15 min may be sufficient in some arrays.
5. Optimizing Signal Detection. Select substrate according to the expected amount of antigen in unknown sample and ELISA reader sensitivity. If the antigen is below the threshold for detection then consider selecting a more sensitive substrate.
6. The ELISA titration data that are generated when increasing the concentrations of labeled analyte (or antibody) are typically plotted either linear-linear, log-linear, log-log, or log-logit. The most useful plot of the data is usually the log-log plot. It provides the most precise estimate of true values in the unsaturated region of the curve and it is easy to fit the data to a curve by linear regression (*see Note 7*).

3.2 ELISA Representative Protocols

3.2.1 Direct ELISA (Fig. 1a)

1. To coat the plate with antigen, add 50–100 μ L/well of different concentration of the antigen, seal the plate and incubate overnight at 4 °C.
2. Wash three times with wash buffer in an ELISA washer or for 3 $\times$ 5 min in a shaking platform. Wells should be filled and emptied to remove the solution by either aspiration or plate inversion (*see also Notes 8–10*).

3. Block (100–200 $\mu\text{L}/\text{well}$) the plate using blocking buffer, seal the plate and incubate for 60 min at 31 °C (*see* also **Note 11**).
4. Wash as **step 2**.
5. Add antibody conjugate solution (50–100 $\mu\text{L}/\text{well}$, same volume as used in **step 1** of the standard or sample), diluted in blocking buffer in a series of twofold dilutions (at least three). Apply in triplicates. Seal the plate and incubate for 1 h at 31 °C (*see* also **Note 12**).
6. Remove the solution and wash six times with wash buffer in an ELISA washer or for 6×5 min in a shaking platform.
7. Add substrate solution (same volume as used in **step 1** of the substrate), incubate for 30 min at 31 °C or until the desired color intensity is reached (expect a clear gradient for the standards) (*see* also **Notes 13–15**).
8. Stop the reaction, if necessary, by adding the same volume used in **step 1** of stop solution.
9. Measure the absorbance using an ELISA plate reader with the appropriate hardware (*see* also **Note 16**).
10. Analyze data and plot signal versus concentration of antigen.

3.2.2 Indirect ELISA (Fig. 1b)

Steps 1–4, same as **steps 1–4** in Subheading 3.2.1 direct ELISA.

5. Add primary antibody (same volume as used in **step 1** of the standard or sample), diluted in blocking buffer in a series of twofold dilutions (at least three). Apply in triplicates. Seal the plate and incubate for 1 h at 31 °C.

Then follow **steps 4–10** in Subheading 3.2.1 “Direct ELISA” for substrate addition, absorbance measurement, and data analysis.

3.2.3 Sandwich ELISA (Fig. 1c)

Sandwich ELISA (Fig. 1c) requires two different antibodies specific for the antigen to be detected. Each has to recognize different epitopes for this antigen. The first antibody (adsorbed to the plate) is called the capture or coating antibody, while the second antibody is called detection antibody. These antibodies must not compete for binding to the antigen. Not all combination of antibodies work properly and they have to be validated before used. Generally, monoclonal antibodies are used for coating and polyclonal for detection.

The recommended protocol steps are the following.

1. To coat the plate with capture antibody add 50–100 $\mu\text{L}/\text{well}$ of a prespecified concentration, seal the plate and incubate overnight at 4 °C or for 2 h at room temperature.

Steps 2–4, same as **steps 2–4** in Subheading 3.2.1 “Direct ELISA”.

5. Add standards and samples (50–100 μL /well, same volume as used in **step 1** of the standard or sample), diluted in blocking buffer in a series of twofold dilutions (at least three). Apply in triplicates. Seal the plate and incubate for 2 h at 31 °C.

Then follow **steps 4–10** in Subheading 3.2.1 “Direct ELISA” for substrate addition, absorbance measurement, and data analysis.

3.2.4 Competitive ELISA with Labeled Antibody (Fig. 1d)

Steps 1–4, same as **steps 1–4** in Subheading 3.2.1 “Direct ELISA”.

5. Competitive incubation:
Dilute the standard/samples in blocking buffer. Dilute the conjugated *antibody* in blocking buffer. Mix the standards/sample and the conjugated antibody together.
6. Add competitive mixture:
Add the mixture (50–100 μL /well, same volume as used in **step 1**), diluted in blocking buffer in a series of twofold dilutions (at least three). Apply in triplicates. Seal the plate and incubate for 1 h at 31 °C.

Then follow **steps 6–10** in Subheading 3.2.1 “Direct ELISA” for substrate addition, absorbance measurement, and data analysis.

3.2.5 Competitive ELISA with Labeled Antigen (Fig. 1e)

Steps 1–4, same as **steps 1–4** in Subheading 3.2.3 “Sandwich ELISA”.

5. Competitive Incubation:
Dilute the standard/samples in blocking buffer. Dilute the conjugated *antigen* in blocking buffer. Mix the standards/sample and the conjugated antigen together.
6. **Step 6** same as **step 6** in Subheading 3.2.4 “Competitive ELISA with Labeled Antibody”.

Then follow **steps 6–10** in Subheading 3.2.1 “Direct ELISA” for substrate addition, absorbance measurement, and data analysis.

3.3 Signal Amplification Using Biotin-Streptavidin System

In all previous protocols if the detection antibody is conjugated with biotin, stop before the step of adding antibody conjugate solution and continue with the following steps:

1. Add biotinylated detection antibody (50–100 μL /well, same volume as used in the detection antibody), diluted to the appropriate concentration in blocking buffer. Seal the plate and incubate for 2 h at 31 °C.
2. Remove the solution and wash three times with wash buffer in an ELISA washer or for 3×5 min in a shaking platform.

3. Add enzyme conjugates with streptavidin/avidin (the same volume used for the biotinylated detection antibody) diluted to the appropriate concentration in blocking buffer. Seal the plate and incubate for 2 h at 31 °C.
4. Remove the solution and wash six times with wash buffer in an ELISA washer or for 6 × 5 min in a shaking platform.

Then follow **steps 7–10** in Subheading **3.2.1** “Direct ELISA” for substrate addition, absorbance measurement, and data analysis.

4 Notes

1. The type of plate used is of major importance. Gamma-irradiated plates are preferable since this treatment enhances coating via increasing positive charge of the material they are made of. It is essential to use a flat bottomed plate with clear, transparent base since the absorbances of the colorimetric substrates are measured by shining a laser through the base of each well. For fluorescence, black plates with clear bottom are preferable to minimize background signal, and for chemiluminescence white plates with clear bottom for signal amplification.
2. The blocking buffer is usually based on mammalian protein solution and is used to cover all unsaturated surface-binding sites of the microtiter plate wells. The blocking buffer should be tested for cross-reactivity, too. In cases of cross-reactivity attributed to blocking buffer, a non-mammalian protein should be considered as an alternative (e.g. salmon serum or a protein-free blocking solution). It is advised to consider the addition of a surfactant like Tween®-20 in a final concentration of 0.05% (v/v) to the blocking buffer. Surfactants are capable of minimizing hydrophobic interactions between the antibody/antigens and the blocking protein preventing final weaker signals.
3. Background signal can be attributed to:
 - (a) nonspecific binding of analyte to the plate,
 - (b) insufficient blocking,
 - (c) presence of unexpected antibody reactivity in the sample,
 - (d) cross-reactivity of antibody to irrelevant antigens,
 - (e) nonspecific binding of detection reagent to the plate, and
 - (f) unstable substrate.
4. Use affinity purified antibodies (versus non-purified) for optimal signal:noise ratio.

5. Check for strong versus low background signal using the appropriate negative control wells. Check blank wells, too, to exclude unspecific signal from the type of plate used.
6. Once an optimal signal/noise ratio has been obtained, test several blocking and washing conditions to see if these will affect this ratio.
7. In homemade ELISAs it is advisable to first generate and optimize (sensitivity, range, and linearity) a standard curve for the analyte to be measured using a standard, the amount of which is known in advance, and afterward proceed to unknown samples testing.
8. Do not allow the plate to dry at any point.
9. Check washer alignment daily, ensure that the plate is leveled and examine the fill volume while wash (a slight dome should be observed at the top of the well) not allowing wells to overflow.
10. Examine the wells for complete aspiration of contents. Upon completion of wash cycle, blot to remove residual fluid.
11. To reassure constant condition, prefer incubation at constant known temperatures.
12. Prepare working dilution of conjugate just before you need it and do not leave it on the bench for excessive time. Excessively diluted conjugate should not be stored for future use.
13. Place plates in dark immediately after addition of light sensitive substrate solutions.
14. The commonly used substrates and their appropriate plate reader setting are
 - (a) 2-2'-azino-di-(3-ethylbenzthiazoline sulfonic acid (ABTS: 405–410 nm),
 - (b) 3,3',5,5'-Tetramethylbenzidine (TMB: non-stopped 620–650 nm, stopped 450 nm),
 - (c) o-Phenyl-diamine-dihydrochloride for peroxidase (OPD: non-stopped 450 nm, stopped 490 nm) and
 - (d) p-Nitrophenyl Phosphate for Alkaline Phosphatase (pNPP: 405–410 nm).
15. Do not hold substrate solution longer than 1 h. The temperature of solution is important because it affects rate of color reaction. Do not add fresh substrate to reagent bottle containing old substrate.
16. To remove fingerprints, clean bottom surface of plates with wash buffer. Wipe the bottom of the plate with a lint-free cloth/towel before reading.

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