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ELISA technical guide and protocols

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Introduction

The enzyme linked immunosorbent assay (ELISA) is a powerful method for detecting and quantifying a specific protein in a complex mixture. Originally described by Engvall and Perlmann (1971), the method enables analysis of protein samples immobilized in microplate wells using specific antibodies. The technique has revolutionized immunology and is commonly used in medical research laboratories. ELISA also has commercial applications, including the detection of disease markers and allergens in the diagnostic and food industries.

The ELISA method was made possible because of scientific advances in a number of related fields. Technology enabling the production of antigen-specific monoclonal antibodies by Kohler and Milstein (1975) led to their use as probes for detecting individual molecules in complex protein mixtures or tissue samples. Initially, detection was achieved by radioimmunoassay using antibodies labeled with radioisotopes, but because of health risks alternatives were sought. Avramais (1966, 1969) and Pierce (1967) developed methods to chemically link antibodies to biological enzymes whose activities produce a measurable signal with solutions containing appropriate substrates. With the development of fluorescence technology, signal generation using fluorophore-labeled antibodies has also become prevalent, especially in multiplex arrays.

Although many variants of ELISA have been developed and used in different situations, they all depend on the same basic elements:

1. **Coating/Capture:** direct or indirect immobilization of antigens to the surface of polystyrene microplate wells.

2. **Plate Blocking:** addition of irrelevant protein or other molecule to cover all unsaturated surface-binding sites of the microplate wells.

3. **Probing/Detection**: incubation with antigen-specific antibodies that affinity-bind to the antigens.

4. **Signal Measurement:** detection of the signal generated via the direct or secondary tag on the specific antibody.

In a typical assay designed to detect an antigen in a complex protein mixture, the antigen is immobilized either by direct adsorption or via an antibody adsorbed to the wells of a microplate. The plate is blocked and the antigen is probed with a specific detection antibody. The detection antibody may be directly labeled with a signal-generating enzyme or fluorophore or it may be secondarily probed with an enzyme- or fluor-labeled secondary antibody (or avidin-biotin chemistry, see below). For enzymatic detection, the appropriate enzyme substrate is added. The signal observed is proportional to the amount of antigen in the sample. Washing between steps ensures that only specific (high-affinity) binding events are maintained to cause signal at the final step.
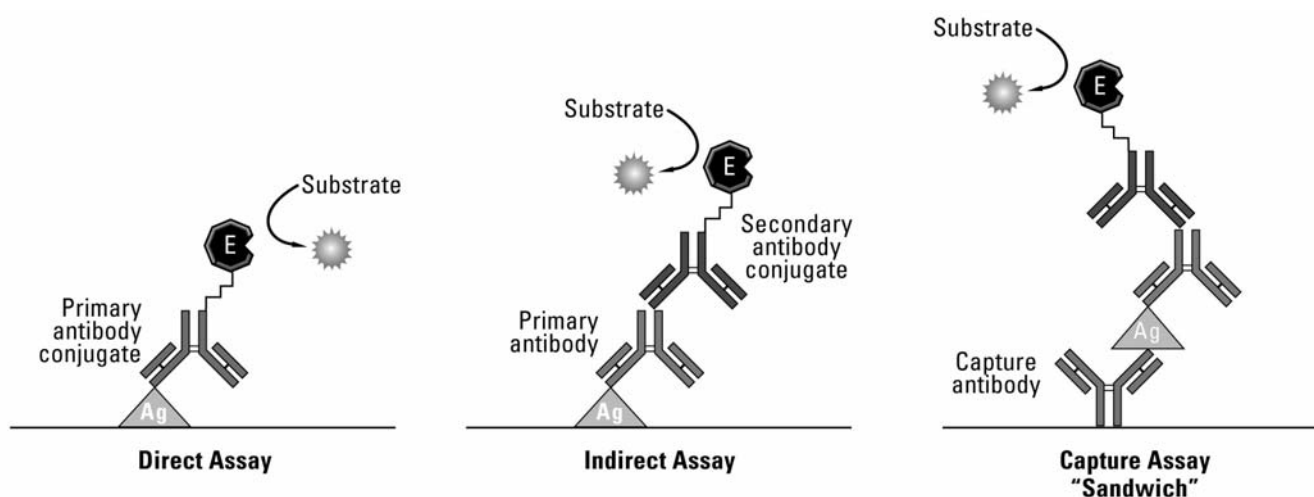

Diagram of popular ELISA formats.

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Variations between ELISA protocols

A. Antigen Immobilization

Antigen immobilization varies between two principle techniques. In a traditional (direct coating) ELISA, antigens are directly attached to the plate by passive adsorption, usually using a carbonate/bicarbonate buffer at pH >9. Most but not all proteins bind tightly to the polystyrene surface of microplates in alkaline conditions. However, if antigen is present at low levels or does not adhere well to the plastic, then the alternative sandwich or capture ELISA may be used. In capture (indirect coating) ELISA, antigen-specific antibody is adsorbed onto the plastic, which in turn binds and immobilizes the antigen upon incubation with the antigen sample. Attachment of the antibody is typically achieved using the same carbonate/bicarbonate buffer at pH >9, or in rare instances pre-activated plates are used for a more directed attachment approach.

Sandwich ELISAs have become very popular when using complex protein samples because only the specific antigen becomes immobilized rather than the entire sample of proteins. The more antigen that is immobilized, the higher is the potential sensitivity of the assay. Sandwich ELISAs require two different antibodies that bind specifically to the antigen (each reacting with a different epitope). The first antibody (bound to the plate) is called the capture antibody or coating antibody, whereas the second antibody detects the immobilized antigen and is called the detection antibody. Such antibodies are known as "matched pairs"; they must be validated to work in combination, as they must not compete for binding to the antigen for accurate results to be possible. Combinations of monoclonal and polyclonal antibodies can be used; it is more common to use the monoclonal as the coating antibody and the polyclonal as the detection antibody. Sandwich ELISAs sometimes require more optimization than traditional ELISAs but usually the signal-to-noise (S:N) ratios are higher.

B. Direct vs. Indirect Detection of Antigen

Direct detection involves labeling of the detection antibody with an enzyme or an alternative signaling molecule such as a fluorophore. Indirect detection involves an additional probing step using another antibody or streptavidin that is labeled with a detectable tag. This additional probe is called the secondary antibody, as its sole purpose is to deliver the measurable signal tag by binding to the detection (primary) antibody. Direct detection is generally faster than indirect detection and potential background signal from secondary antibody cross-reactivity with the coating antibody is also eliminated. Even in the best of circumstances however, direct detection cannot provide the signal amplification gained from the use of a secondary antibody or avidin/biotin systems. Consequently direct detection is generally less sensitive than indirect detection and is best used only when the target is relatively abundant.

C. Biotin Signal Amplification

Biotin is a small (MW 244) vitamin molecule that is easily modified so that it can be chemically attached to proteins, antibodies and other biomolecular probes of interest. Avidin and streptavidin are proteins that originate from different sources but have nearly identical functions in binding very strongly and specifically to the biotin molecule. Thermo Scientific NeutrAvidin™ Protein is a specially modified form of avidin. Hereafter in this document, "avidin" and "streptavidin" will be used interchangeably to mean any one of these biotin-binding proteins. The avidin-biotin affinity system is frequently employed in the design of tagging and detecting systems for ELISA.

Avidin-Biotin Complex

Diagram of the signal amplification that is possible with the ABC system.

A common adaptation of indirect detection is to amplify the signal using avidin-biotin chemistry. There are two approaches. The first method involves using a biotinylated detection antibody, which is probed using avidin or streptavidin protein conjugated to either horseradish peroxidase (HRP) or alkaline phosphatase (AP) enzymes. The second approach also employs a biotinylated detection antibody, but it is probed with a pre-incubated mixture of avidin and biotinylated enzyme, a process known as "avidin-biotin complex" (ABC) signal amplification.

Signal amplification occurs through two mechanisms with these approaches. First, biotinylation (biotin-labeling) typically results in multiple biotin tags per antibody molecule, thus allowing more than one streptavidin molecule to bind to each antibody. Binding is aided by the fact that avidin-type proteins are tetrameric and have four biotin-binding sites per molecule. Second, the process of either labeling the streptavidin molecule with enzymes or using a pre-incubated mixture of streptavidin plus biotinylated enzyme results in conjugates having more than one enzyme. The combined effect of this
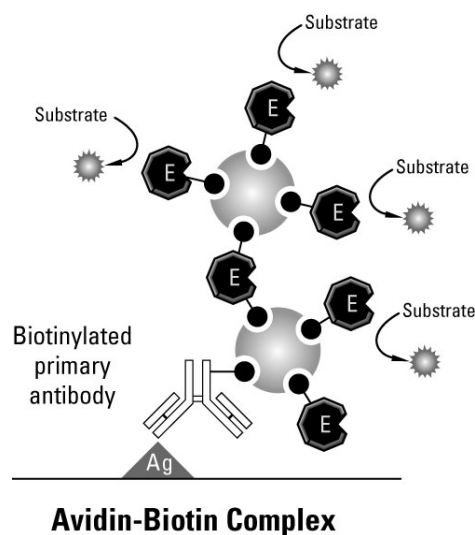
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multiple labeling is to increase the number of enzyme molecules in the final immune complex. This increases the catalysis of appropriate substrate and gives a stronger signal compared to a conventional enzyme-labeled secondary antibody.

D. Fluorescence

The relatively recent expansion in the number of stable fluorophores in the visible and infrared ranges has made fluorescent signal detection an attractive option for ELISA applications. This kind of approach is common when performing multiplex arrays, as more than one antigen can be detected simultaneously with antibodies conjugated to different fluorophores. In fluorescence assays, the detection antibody is either labeled directly or the secondary antibody (or occasionally avidin) is labeled for indirect detection. When multiplexing using labeled secondary antibodies it is essential to use detection antibodies from different species in order to distinguish the separate signals. The detection limit is typically around 100pg/well which is less sensitive than colorimetric or chemiluminescent detection.
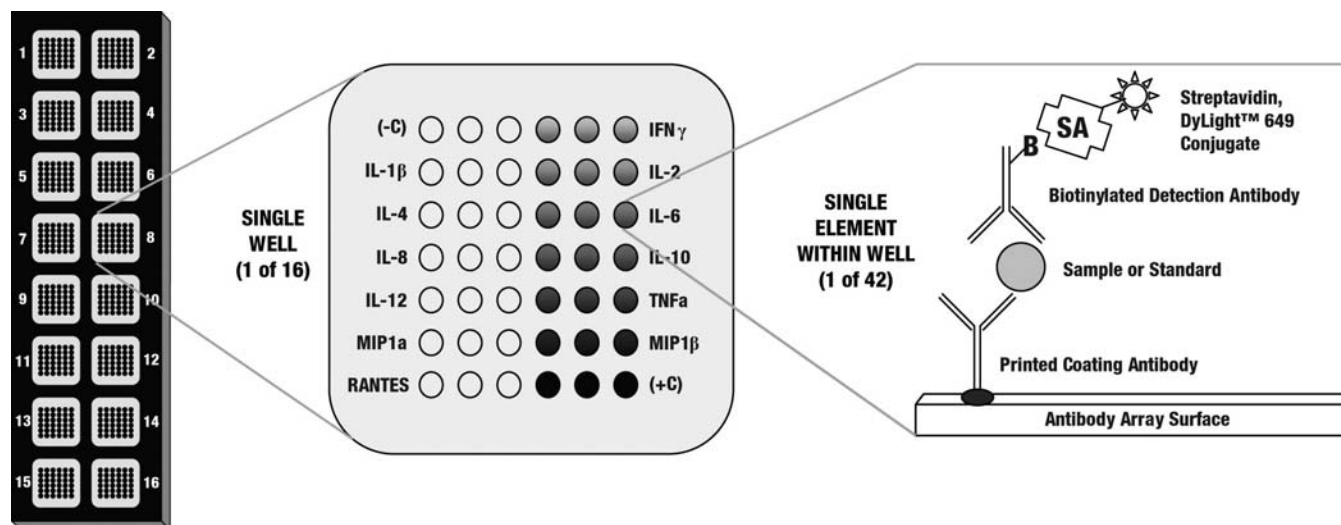

Diagram of a multiplex array ELISA made made possible by using fluorescence. In this case, twelve different capture antibodies are coated as an array of printed spots on a glass slide. Each antibody captures a different analyte and is detected by its matched detection antibody, which is biotinylated. Finally, all spots are detected through a fluor-labeled streptavidin conjugate (in this case, the Thermo Scientific DyLight™ 649 Fluorophore).

E. Enzymatic Detection

Two enzymes are commonly used in ELISA applications. Alkaline Phosphatase (AP) is a large enzyme used in a minority of assays. Its size (140 KDa) makes it difficult to conjugate more than one or two molecules of the enzyme to each molecule of an antibody or avidin, and this limits the amount of signal that can be generated. AP is also prone to stability issues unless stored and handled correctly.

Horseradish Peroxidase (HRP) is a more commonly used enzyme. Its small size (40KDa) allows more molecules to be coupled to antibodies or avidin, and this can boost signal generation. HRP is the enzyme of choice for most researchers performing ELISAs and can be used with a variety of substrates (see below), most of which are more sensitive than AP equivalents.

F. Substrates

Enzymatic signal generation requires the catalysis of a substrate to produce a colored or fluorescent compound or chemiluminescence (visible light). The signal is measured using a spectrophotometric plate reader, a fluorometer with the appropriate filters or a luminometer set to read total light output. Each type of substrate is discussed below; more information about specific products can be found at our website.

Colorimetric substrates form a soluble, colored product that accumulates over time relative to the amount of enzyme present in each well. When the desired color intensity is reached, the product absorbance is either measured directly or in some cases a stop solution is added to provide a fixed end point for the assay. Colorimetric substrates are available for both horseradish peroxidase (TMB, OPD, ABTS) and alkaline phosphatase (PNPP).

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Chemifluorescent detection is also enzyme-based but the generated product is fluorescent rather than colorimetric. The signal is measured using a fluorometer with the appropriate excitation and emission filters. Chemifluorescence reactions are either measured over time in kinetic assays or halted using a stop solution for direct measurement. Examples of chemifluorescent substrates for HRP are Thermo Scientific QuantaRed™ and QuantaBlu™ Substrates.

Chemiluminescence is a chemical reaction that generates energy released in the form of light. Most chemiluminescent substrates are HRP-dependent although some AP equivalents are available. The most common approach is to use luminol in the presence of HRP and a peroxide buffer. The luminol is oxidized and forms an excited state product that emits light as it decays to the ground state. Light emission occurs only during the enzyme-substrate reaction, therefore when the substrate becomes exhausted the signal ceases. Chemiluminescent detection is generally considered to be more sensitive than colorimeteric detection. Chemiluminescent substrates for HRP include Thermo Scientific SuperSignal™ ELISA Pico and ELISA Femto Substrates.

Factors affecting assembly of the immune complex

Although ELISA is a powerful and well-characterized application, attempting to develop and optimize a specific assay can be difficult. The method involves the assembly of a large immune complex with multiple components, therefore failure to capture signal can potentially be caused by any of these factors and optimization is essential. A list of such factors and associated variables is described in Table 1, followed by a discussion of several pertinent issues. The protocol section at the end of this guide includes specific instructions and suggestions about optimization procedures.

When setting up an ELISA it is advisable to first generate and optimize a standard curve for the analyte of interest before analyzing multiple samples of unknown composition. If the standard curve displays the correct sensitivity, range and linearity, the researcher can proceed with confidence to process the samples.

Table 1. Factors that affect ELISA signal generation.

Factor	Variable Characteristic
Assay Plate	material, well shape, pre-activation
Coupling Buffer	composition, pH
Capture Antibody	specificity, titer, affinity, incubation time and temperature
Blocking Buffer	composition, concentration, cross-reactivity
Target Antigen	conformation, stability, available epitope(s), matrix effects
Detection Antibody	specificity, titer, affinity, incubation time & temperature, cross-reactivity
Enzyme Conjugate	type of enzyme, type of conjugate, activity, concentration, cross-reactivity
Washes	buffer composition, volume, duration, frequency
Substrate	sensitivity, manufacturer lot, age
Signal Detection	filters, imaging instrument, exposure time

A. Types of Plate and Coupling Options

Plates used in conventional ELISA applications are typically made of polystyrene. Other materials such as polypropylene, polycarbonate and in some instances nylon are occasionally used; many plates are now gamma-irradiated to impart a positive charge, which aids coating procedures. The absorbances of colorimetric substrates are measured by shining a laser up through the base of each well, so it is essential to use a flat bottomed plate with a clear base. Fluorescent detection requires the use of an opaque black or white plate, and fluorometric plate readers measure either from above or below the plate. Chemiluminescent detection requires the use of a black or white opaque plate and may also be measured from the top or the bottom. For this reason both fluorescent and chemiluminescent assays must be performed in plates with a clear bottom. Although black plates are preferred for fluorescence (as background is lower) and white plates are preferred for chemiluminescence (as it magnifies the signal), the plates can be used interchangeably.

The most common technique for attachment of proteins or peptides to a plate is passive adsorption. This is mediated primarily by hydrophobic interactions, but some electrostatic forces may also contribute. A popular coating buffer is carbonate-bicarbonate buffer (0.2 M sodium carbonate/bicarbonate pH 9.4). The high pH aids solubility of many proteins and peptides and ensures that most proteins are unprotonated with an overall negative charge, which helps when binding to a

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positively charged plate. Other buffered solutions such as Tris-buffered saline (TBS) or phosphate-buffered saline (PBS) at physiological pH are sometimes used but coupling is generally not quite as efficient.

In some instances researchers require a more directed approach for attachment of the coating antibody or protein sample to the plate, and several pre-activated plates exist for this purpose. For specific, orientated binding of the coating antibody, plates that are pre-coated with Protein A or Protein G are available (not advised for sandwich ELISAs because of potential cross-reactivity with detection and/or secondary antibodies). For biotinylated samples or coating antibodies, plates that are pre-coated with streptavidin are ideal. Some assays require direct immobilization of a histidine-tagged protein, in which case nickel- or copper-coated plates are suitable (these plates can also be used to bind and orientate capture antibodies as IgG molecules have a histidine rich sequence in their Fc domain). For group-specific attachment of molecules via amines or thiols to form a covalent bond, maleic-anhydride or maleimide-activated plates can be used. (These are especially useful for direct attachment of peptide antigens, which do not coat well by passive adsorption because of their small size.) Please visit our website for a complete listing of our many Thermo Scientific Pierce™ Coated Microplates.

B.   Antibodies

Not all antibodies can be used successfully in ELISA applications, and individual antibodies must be evaluated. During the adsorption process, the three-dimensional structure of an antigen might be altered and such that it can no longer bind its target epitope. Some antibodies are raised against peptides; if the peptide represents an internal sequence of the antigen, then the antibody might not bind if the whole antigen is immobilized on the plate. Furthermore, for an antibody to work successfully in ELISA, it should react specifically with the antigen but not cross-react with a component of the blocking buffer. For sandwich assays where two different antibodies are required, it is essential that the two antibodies react with different epitopes on the antigen or an epitope that appears several times on the antigen. For example, if the antigen is immobilized on the plate through the capture antibody, then the detection antibody must be able to interact with its own epitope without steric hindrance from the first antibody or the plate. In ELISA applications where a secondary antibody is used as part of the detection complex it is also essential that the capture and detection antibodies be raised in different animal species so that the secondary antibody does not react with the coating antibody. Antibodies that work in combination with each other are generally known as "matched pairs".

Another important factor to consider when setting up an ELISA is the concentration of the antibodies. Each will require optimization, the optimal range being partially determined by the form and origin and also by the substrate used for signal generation. Detection antibody and enzyme conjugate working solutions should ideally be prepared in blocking solution to reduce non-specific interactions. For recommended antibody concentrations see the Appendix at the end of this document.

C.   Blocking Buffer

Blocking buffers usually consist of formulations of proteins designed to prevent non-specific binding of proteins to the plate. An optimal blocking buffer maximizes the signal-to-noise ratio and does not react with the antibodies or target protein. If cross-reactivity is observed then a different blocker should be tested and if repeated cross-reactivity is observed it may be advisable to switch to a non-mammalian protein blocker such as salmon serum or a protein free blocking solution. Visit our website for more information on our wide selection of ready-to-use blocking buffers.

Some systems may benefit from the addition of a surfactant such as Tween®-20 (a gentle non-ionic detergent) to the blocking solution. Surfactants can help to minimize hydrophobic interactions between the blocking protein(s) and the antigen or the antibodies. Typically a final concentration of 0.05% (v/v) Tween-20 is used. In addition, blocking buffers should be used in sufficient volumes to completely coat the wells; for example 300μl should be used for each well of a typical 96-well plate.

D.   Target Antigen

The target antigen should be present in a buffer or matrix that allows it to interact with a pre-coated capture antibody or be coupled to the plate directly. Direct coupling may require that the antigen be exchanged into a suitable coupling buffer. In rare instances the three-dimensional structure of an antigen may be altered during the adsorption process such that it no longer binds its target epitope. In such cases, the use of a plate pre-coated with a binding protein (such as a capture antibody) may eliminate this problem. If the antigen is present in the form of a biological sample, then the effects of the matrix (i.e. serum, plasma components) should be controlled by performing spike-and-recovery and linearity-of-dilution experiments. For more information on this topic, see the related Tech Tip #58: Linearity and spike-and-recovery experiments for ELISA.

If performing a quantitative ELISA it is essential to have an equivalent standard protein (generally a purified recombinant) where the amount of specific protein is known in advance. The standard is used to prepare a series of solutions of known

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concentration by serial dilution of a protein stock solution followed by construction of a standard curve plotting concentration versus absorbance. Absorbance values for samples of unknown concentration are extrapolated from this curve to determine the actual amount of specific protein in the sample.

E.   Enzyme Conjugate

The concentration of the enzyme conjugate is one of the most crucial parameters in the optimization process. The amount of enzyme that binds directly influences the amount of signal that is generated. Too little enzyme and the signal may be very weak with a poor signal-to-noise ratio. Too much and the background may be too high, again resulting in a poor signal-to-noise ratio and little distinction between standards of different concentrations. For recommended enzyme conjugate concentrations see the Appendix at the end of this document.

F.   Washing the Plate

The two most commonly used wash buffers in ELISA applications are Tris-buffered saline (TBS) and phosphate-buffered saline (PBS) containing 0.05% (v/v) Tween®-20. To wash a plate, wells should be repeatedly filled and emptied by either aspiration or plate inversion (i.e., dumping and flicking solution into a suitable receptacle). Generally at least 3 x 5 minute washes should be applied after the incubation of coating antibody, sample and detection antibody; 6 x 5 minute washes should be given after incubation with the enzyme conjugate. It is not necessary to wash after the blocking step although this is not detrimental to the assay.

G.   Substrates and Signal Detection

The choice of a particular substrate will depend on the equipment available but also on the degree of sensitivity required. Chemiluminescent substrates are the most sensitive, with antigen detection possible in the sub-picogram per well range. Colorimetric and chemifluorescent substrates are typically able to detect low- to mid-picogram levels of antigen per well. Precipitating substrates are not used with plate assays as the precipitate settles in the wells and prevents the measurement of absorbance.

Colorimetric substrates are measured using a standard plate reader with the appropriate filters. Chemifluorescence is measured using a fluorometer with the appropriate excitation and emission filters. Chemiluminescence is most commonly measured using a luminometer although some plate readers have an option to read chemiluminescence or can be adapted to measure total light output. If a wavelength must be selected, measurement at 425nm gives a crude indication of chemiluminescence. Some fluorescent plate readers can also be used if excitation is turned off.

Common Occurrences and Explanations

A.   Low Signal-to-Noise Ratio

<u>Low or absent signal</u>

A weak signal indicates an ELISA system that requires optimization. There may be a number of causes summarized briefly as follows: 1) One of the components of the assay may not have been correctly optimized and may be present at a limiting concentration causing the overall signal to be low. 2) One of the reagents may have degraded or been contamination, in which case it should be replaced. 3) The antibodies used may not bind effectively to the antigen or may not be an efficient matched pair (antibodies may not work in combination with each other). In such cases alternative antibodies must be tested. 4) The detection system (substrate) may not be sensitive enough to give the desired signal, or the standard curve may not be in the dynamic range appropriate for the sample. It may be necessary to concentrate the sample, or switch to a more sensitive substrate.

<u>High background levels</u>

High background signal is most commonly the result of either insufficient washing or blocking, sample components or antibodies cross-reacting with the blocking buffer or the use of too much enzyme conjugate. A common misconception is that a particular substrate can cause or increased background. However, the substrate itself cannot generate signal without activity of the pertinent enzyme. For this reason, it is important to balance the amount of enzyme giving specific signal versus that giving background signal and this is best controlled by optimizing the enzyme conjugate, antibodies and blocking solution. It is also important to note that optimal conditions for one antigen may not be optimal for another. If one component of the

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detection system is altered (for example a different antibody or substrate), then some of the other components may also need to be re-optimized.

B. Signal Fades Quickly (generally seen with chemiluminescence only)

When a particular ELISA system produces a chemiluminescent signal that fades quickly the system requires optimization as described in the preceding paragraph. This is because the most likely causes of fading signal are similar to the causes of high background – namely an excess of HRP. Excessive HRP can exhaust the substrate and this prevents the emission of light. It is advisable to check the concentration of enzyme conjugate and ensure it is appropriate for the particular chemiluminescent substrate used in the assay. In some instances it may also be necessary to optimize the antibody concentrations, blocking solution and wash steps.

C. Substrate Forms a Precipitate or Plate Visibly Glows in the Dark

If a colorimetric substrate forms a precipitate in the wells of the plate or if a chemiluminescent substrate visibly glows when added to the plate, there is too much enzyme present. Further dilution of the enzyme conjugate is most likely required although optimization of antibody concentrations, better blocking and additional washing may also be necessary.

D. Fluorescent Signal is Low

Overexposure of fluorescent reagents to intense light can cause photo-bleaching, which is caused by decomposition of the fluorophore. Protection of the fluorophore from strong light is essential for effective signal generation at the end of the assay. An alternative cause of low signal is photo-quenching, which is characterized by fluorophores present in close proximity to one another quenching the signal of neighboring molecules by dispersion of energy. In such instances, relabeling may be required to reduce the degree of labeling or in some cases the use of a soluble chemifluorescent substrate may be more appropriate.

E. Poor Standard Curve Linearity and Dynamic Range

The dynamic range of an ELISA refers to the range of antigen concentrations that can be measured accurately in a particular assay. It is a reflection of the quality of the standard curve, which should have low variation between replicates and a defined linear portion. If the standard curve has a low dynamic range, then samples must fall within a tight concentration range in order to deemed accurate. Factors that contribute to poor dynamic range are discussed in the "low signal-to-noise ratio" section.

If the curve has good linearity but poor variation between replicates (i.e., standard error) there might be technical problems, such as inconsistent pipetting between samples or individual users. To highlight pipetting errors and create more reproducible results, all samples and standards should be measured at least in duplicate or triplicate. In addition, pipettors should be calibrated so that they always deliver the same volume from assay to assay.

General Capture/Sandwich ELISA Protocol

This protocol represents an example of a standard capture or sandwich ELISA using a biotinylated detection antibody and streptavidin-HRP indirect detection system with commonly used reagents and TMB (tetramethyl benzene) substrate. For other ELISA variations, see the section entitled Modifications to the ELISA Protocol at the end of this procedure.

1. Add 50-100 μl coating antibody to each well

2. Cover plate, incubate for 2 hours at room temp to overnight at 4°C

3. Wash plate three times, 5 minutes each

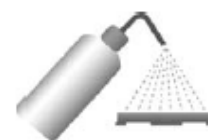**4.** Add 300 μl blocking buffer to each well

5. Cover plate and incubate at room temp for 1 hour to overnight at 4°C.

6. Remove blocker, add sample or standards to each well in duplicate or triplicate

7. Cover plate and incubate at room temp for 1 hour

8. Wash plate three times, 5 minutes each

9. Add biotinylated detection antibody to each well

10. Cover plate and incubate at room temperature for 1 hour

11. Wash plate three times, 5 minutes each

12. Add enzyme conjugate to each well

13. Cover plate and incubate at room temp for 1 hour

14. Wash plate six times, 5 minutes each

15. Add substrate solution to each well

16. Develop at room temp. for 30 minutes, stop reaction if necessary

17. Measure absorbance using appropriate hardware

18. Analyze data and plot signal vs. concentration of antigen

A. Materials Required

Hardware

- Clear 96 well plate
- Multi-channel precision pipettor with disposable plastic tips
- Plate reader or luminometer equipped to detect the substrate

Reagents

Coating buffer:	0.2 M sodium carbonate/bicarbonate, pH 9.4
Capture antibody:	Diluted in Coating Buffer (see Appendix for appropriate concentration range).
Wash buffer:	0.1 M phosphate, 0.15 M sodium chloride, pH 7.2 containing 0.05% Tween 20
	Note: 0.1M Phosphate can be replaced by 25 mM Tris, pH 7.2
Blocking buffer:	2% (w/v) Bovine Serum Albumin (BSA) in Wash Buffer
	Note: alternative buffers are listed in the product list at the end of this document
Standard diluent:	2% (w/v) BSA in Wash Buffer. Note: Ideally the standard diluent composition would be as close as possible to the sample matrix. For example if measuring the concentration of an antigen in culture supernatant, culture medium should be used as the standard diluent. However biological sample matrices such as serum are impossible to replicate, therefore BSA is commonly used in these instances. Often the blocking buffer is also used as the standard diluent
Samples/standards:	See preparation sections below.
Detection antibody (biotinylated):	Diluted in 1/5 strength standard diluent (see Appendix for appropriate concentration range)
Enzyme conjugate:	Streptavidin-HRP diluted in 1/5 strength standard diluent (see Appendix for appropriate concentration)
Substrate:	TMB substrate (see product list for a list of alternative substrates)
Stop solution:	2M sulfuric acid

B. Preparation of Standards

Typically a standard curve may span concentrations from 0 to 1000 pg/ml but may go as high as 3000 pg/ml depending on the predicted amount of antigen in the sample and the amount of standard protein available. Typically two-fold or three-fold dilutions of the standard protein are prepared from the stock solution using the standard diluent. When preparing serial dilutions of a protein standard, use fresh tips after each dilution.

C. Preparation of Samples

If the concentration of antigen in the sample potentially exceeds the highest point of the standard curve (i.e. $\geq 1,000$ pg/ml) prepare one or more dilutions of the sample using the standard diluent.

D. Procedure

Important: Do not allow the plate to dry at any point.

1. Dilute the capture antibody to the appropriate concentration allowing sufficient volume for 50-100 µl per well.

2. Add the diluted capture antibody to the plate, cover and incubate for 2 hours at room temperature (RT).

3. Remove the solution and wash the plate with 200 µl per well wash buffer for 3 x 5 minutes on a shaking platform.

4. Add 300 µl blocking buffer per well, cover the plate and incubate for 1 hour at room temperature. Alternatively block overnight at 4°C.

5. Prepare the samples and standards. The volume per well should be the same as the capture antibody used in step 1.

6. Remove the blocking buffer and add the samples and standards. Cover the plate and incubate for 1 hour at RT.

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7. Remove the solution and wash the plate with 200 μl per well wash buffer for 3 x 5 minutes on a shaking platform.

8. Dilute the biotinylated detection antibody to the appropriate concentration. The volume per well should be the same as the capture antibody used in step 1.

9. Add the diluted detection antibody to the plate, cover and incubate for 1 hour at RT.

10. Remove the solution and wash the plate with 200 μl per well wash buffer for 3 x 5 minutes on a shaking platform.

11. Dilute the enzyme conjugate to the appropriate concentration. The volume per well should be the same as the capture antibody used in step 1.

12. Add the diluted enzyme conjugate to the plate, cover and incubate for 1 hour at RT.

13. Remove the solution and wash the plate with 200 μl per well wash buffer for 6 x 5 minutes on a shaking platform

14. Add substrate solution to the plate. The volume per well should be the same as the capture antibody used in step 1.

15. Incubate the plate at RT until the desired color intensity is reached. Ideally a clear gradient will result for the standards.

16. Stop the reaction by adding an equal amount of stop solution.

17. If using TMB, measure the absorbance at 450 nm. For other substrates use the appropriate detection technique.

Modifications to the ELISA protocol

A. Performing Direct Antigen Immobilization

When immobilizing the antigen-containing sample directly to the plate, there is obviously no need for a capture antibody. Different concentrations of the sample should be prepared in coating buffer and identical volumes added directly to the plate. The rest of the protocol should be performed as described using a detection antibody and enzyme conjugate plus substrate.

B. Using an Enzyme Labeled Secondary Antibody

When using a non-biotinylated detection antibody followed by an enzyme-labeled secondary antibody, there will be slightly less amplification of enzyme compared to using a biotinylated detection antibody with streptavidin-HRP. Therefore it may be necessary to use a slightly higher concentration of secondary antibody-enzyme than one would normally use for a streptavidin-enzyme conjugate.

C. Using an Alkaline Phosphatase System

If alkaline phosphatase is to be used instead of HRP for the enzyme conjugate an AP-specific substrate must be used. The most common alkaline phosphatase substrate used in ELISA applications is *p*-nitrophenyl phosphate (PNPP). PNPP has a detection limit of around 10 ng per well, and enzyme conjugates are typically used at a working concentration range of 100-200 ng/ml.

D. Using Fluorescence

With fluorescence, no enzyme conjugate or substrate is required. Typically, the fluor is attached directly to a secondary antibody or in some cases the detection antibody or avidin. The working concentration of labeled antibody or protein is typically 2-4 μg/ml. Signal is measured using a plate reader equipped to measure fluorescence, or by using a fluorometer.

E. Using Chemifluorescence

Unlike fluorescence, chemifluorescence detection systems require the use of HRP as described in the main protocol. Typically the final concentration of the HRP enzyme conjugate should fall within the range of 25-50 ng/ml. Chemifluorescence is measured using a fluorometer with the appropriate filters.

### F. Using Chemiluminescence

Chemiluminescence is generally considered to be the most sensitive detection technique for ELISA applications with detection of sub-picogram amounts of antigen possible. For this reason, low amounts of enzyme conjugate are recommended for chemiluminescent detection. Typically the working concentration of the enzyme conjugate can vary anywhere between 10-100 ng/ml depending upon the sensitivity of the substrate and the other parameters in the assay. Check the substrate instructions for detailed recommendations.

G. Competition ELISAs

In a competition ELISA, the assay is based on competition between the antigen in a standard/sample and an enzyme conjugated version of the same antigen for a limited amount of antibody bound to a pre-coated plate. Both are mixed together in the same well. As the concentration of antigen in the sample increases, the amount of labeled antigen captured by the coating antibody decreases. Therefore there is an inverse relationship between optical density (OD) and the amount of analyte in the sample. Amounts of labeled and non-labeled antigen to use in the assay should be determined empirically.

ELISA Optimization

This section describes optimization steps for each component of the assay starting at the capture antibody (for sandwich ELISAs) right through the enzyme conjugate and choice of substrate. Although each component is described separately, in many instances it is possible to optimize two components simultaneously by performing a checkerboard titration as shown in the layout below.
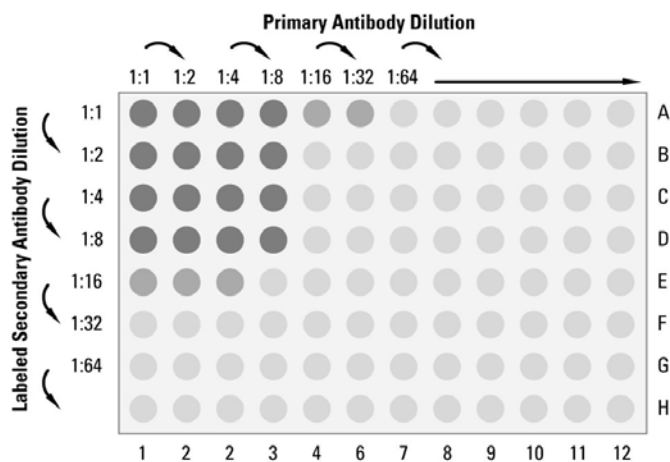
Example of a checkerboard titration experiment to optimize two ELISA parameters at once. This example shows primary antibody versus detection antibody with all other reagents constant. Researchers developing an ELISA using the format described in the protocol (using affinity purified monoclonal antibodies) will typically use starting concentrations of 12 μg/ml and 5 μg/ml for capture and detection antibodies respectively, with the enzyme conjugate at a constant concentration of 100 ng/ml. After the capture and detection antibodies have been optimized, the enzyme conjugate can then be subjected to titration, but 100 ng/ml is often appropriate.

A. Optimizing Capture Antibody Concentration

1. Prepare different concentrations of the capture antibody in coating buffer (see ranges described in the Appendix).

2. Apply an equal volume of each concentration to the plate and proceed from step 2 of the protocol.

3. Check for strong signal versus low background.

B. Optimizing the Blocking Buffer

1. Prepare different blocking solutions. If the blocking solution is not pre-formulated (i.e., it is a single protein, such as BSA), try different concentrations of the protein.

2. Apply an equal volume of each to the plate and proceed from step 4 of the protocol.

3. Check for strong signal versus low background.

C. Optimizing the Standard Diluent

1. Try to match the standard diluent as close as possible to the matrix of the sample. If the matrix itself cannot be exactly duplicated then test different standard diluent solutions.

2. Apply an equal volume of each to the plate and proceed from step 5 of the protocol.

3. Check for good dynamic range for the standard curve and linearity-of-dilution for the sample.

4. If the standard curve has a poor dynamic range, then it may be necessary to choose a different diluent. If the sample has poor linearity-of-dilution when diluted in the diluent there may be an imbalance between the sample matrix and the standard diluent. In such cases spike-and-recovery or linearity-of-dilution experiments should be performed. For more information on this topic, see the related Tech Tip #58: Linearity and spike-and-recovery experiments for ELISA.

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D. Optimizing Sample Concentration

1. Prepare different concentrations of the sample using standard diluent. Test a wide range of sample concentrations, keeping in mind the detection limit of the substrate being used.

2. Apply an equal volume of each concentration to the plate and proceed from step 6 of the protocol.

3. Check for strong signal versus low background.

4. To confirm that the biological sample matrix is not masking or enhancing the signal, spike-and-recovery and linearity-of-dilution experiments should be performed. For more information on this topic, see the related Tech Tip #58: Linearity and spike-and-recovery experiments for ELISA.

E. Optimizing the Detection Antibody Concentration

1. Prepare different concentrations of the detection antibody in standard diluent (see ranges described in the Appendix).

2. Apply an equal volume of each concentration to the plate and proceed from step 9 of the protocol.

3. Check for strong signal versus low background.

F. Optimizing the Enzyme Conjugate Concentration

1. Prepare different concentrations of the enzyme conjugate in standard diluent according to the range described in the Appendix. Ensure the concentration is in accordance with the range described for the substrate.

2. Apply an equal volume of each concentration to the plate and proceed from step 12 of the protocol.

3. Check for strong signal versus low background.

G. Optimizing Signal Detection

1. Select substrate(s) based on likely amount of antigen in sample and ability to detect with appropriate instrument.

2. Apply the working solution to the plate and proceed from step 14 of the protocol.

3. If antigen can clearly be detected over a dynamic range then the substrate is appropriate. If the antigen is below the threshold for detection then select a more sensitive substrate.

Appendix

The following tables provide recommended ranges for different ELISA components. Concentrations are guidelines only; for best results optimize each component individually.

Recommended starting concentration ranges for coating and detection antibodies for ELISA optimization. The use of non-purified antibodies will work but may result in higher background. It is generally recommended to use affinity purified antibodies for optimal signal:noise ratio.

Source	Coating Antibody	Detection Antibody
Polyclonal serum	5-15 μg/ml*	1-10 μg/ml*
Crude ascites	5-15 μg/ml*	1-10 μg/ml*
Affinity purified polyclonal	1-12 μg/ml	0.5-5 μg/ml
Affinity purified monoclonal	1-12 μg/ml	0.5-5 μg/ml

Recommended secondary antibody concentrations for ELISA in different systems. Check the instructions for the substrate as they may recommend a more defined concentration range for the enzyme conjugate.

Enzyme	System	Concentration
HRP	Colorimetric system	20-200 ng/ml
	Chemifluorescent system	25-50 ng/ml
	Chemiluminescent system	10-100 ng/ml
AP	Colorimetric system	100-200 ng/ml
	Chemiluminescent system	40-200 ng/ml

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