ELISA Basics Guide

BIO·RAD

Table of Contents

1 **Introduction**

The basic enzyme-linked immunosorbent assay (ELISA), or enzyme immunoassay (EIA), is distinguished from other antibody-based assays because separation of specific and nonspecific interactions occurs via serial binding to a solid surface, usually a polystyrene multiwell plate, and because quantitative results can be achieved. The ELISA procedure results in an enzyme-catalyzed change in color, fluorescence, or luminescence which correlates to the amount of analyte present in the original sample. ELISAs are quick and simple to carry out, and since they are designed to rapidly handle a large number of samples in parallel, they are a very popular choice for the evaluation of various research and diagnostic targets. Figure 1 shows a typical ELISA result.
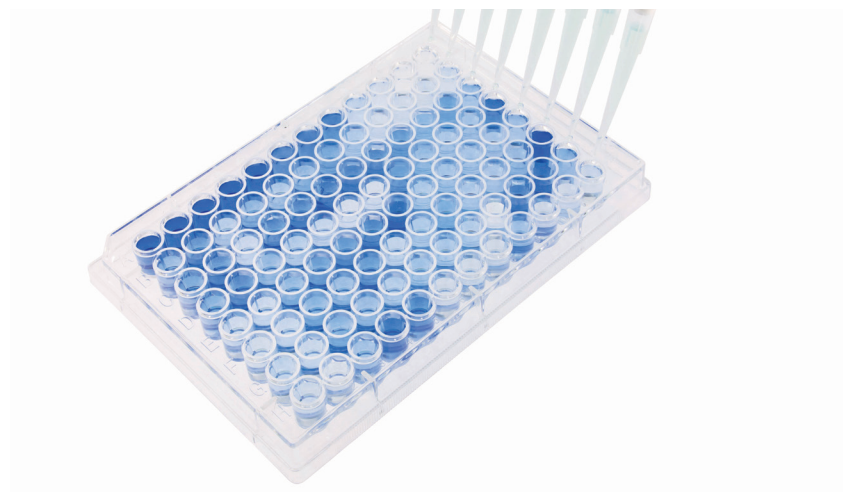
Fig. 1. Typical ELISA output. Darker wells indicate higher levels of analyte in the original sample.

ELISAs were first developed in the early 1970s as a replacement for radioimmunoassays. They remain in wide use in their original format and in expanded formats with modifications that allow for multiple analytes per well, highly sensitive readouts, and direct cell-based output.

ELISAs begin with a coating step, where the first layer, either an antigen or an antibody, is adsorbed to a well in an ELISA plate. Coating is followed by blocking and detection steps as shown in the simple schematic diagram on page 4.

Since the assay uses surface binding for separation, several washes are repeated between each ELISA step to remove unbound materials. During this process it is essential that excess liquid is removed in order to prevent the dilution of the solutions added in the next stage. For greatest consistency, specialized plate washers are used.

ELISAs can be quite complex, including various intervening steps and the ability to measure antigen concentrations in heterogeneous samples such as blood. The most complex and varying step in the overall process is detection, where multiple layers of antibodies can be used to amplify signal.

Coating
Antigen is adsorbed onto well in ELISA plate in coating buffer

Remove buffer and wash plate

Blocking
A buffer containing unrelated protein is used to block free sites in the wells

Remove buffer and wash plate

Detection
Enzyme conjugated detection antibody binds antigen

Remove buffer and wash plate

Readout
Substrate is catalyzed by enzyme to generate colored readout

Key

Analyte/Antigen

Enzyme

Directly conjugated primary antibody

Conjugated secondary antibody

Capture antibody

2 ELISA Technology

ELISA Formats

The first step in an ELISA experiment is the immobilization of the antigen in a sample to the wall of the wells of a microtiter plate. This can be achieved by direct adsorption to the plate's surface or by using a "capture antibody". The capture antibody has to be specific to the target antigen and is mainly used in a specific ELISA type called "sandwich ELISA". After immobilization, a detection antibody is added, which binds to the adsorbed antigen thereby leading to the formation of an antigen-antibody complex. The detection antibody is either directly conjugated to an enzyme, such as horseradish peroxidase (HRP), or provides a binding site for a secondary conjugate. In general, ELISAs can be grouped into the four main categories: direct, indirect, sandwich, and competitive ELISAs.

Direct ELISA

Figure 2 illustrates the setup of direct ELISA; an antigen is immobilized in the well of an ELISA plate. The antigen is then detected by an antibody directly conjugated to an enzyme such as HRP.
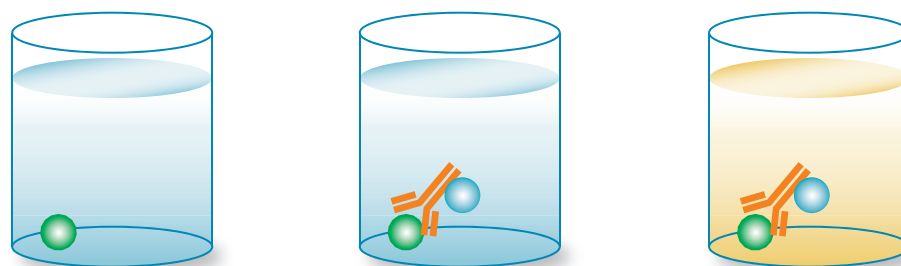

Fig. 2. Overview of direct ELISA.

Direct ELISA detection is much faster than other ELISA techniques as fewer steps are required. The assay is also less prone to error since fewer reagents and steps are needed, i.e. no potentially cross-reacting secondary antibody needed. However, there are some disadvantages to this method. As the antigen immobilization is not specific, higher background noise may be observed in comparison to indirect ELISA (see below). This is primarily because all proteins in the sample, including the target protein, will bind to the plate. Direct ELISA is less flexible since a specific conjugated primary antibody is needed for each target protein. As no secondary antibody is used, there is no signal amplification, which reduces assay sensitivity. Finally, the direct ELISA technique is typically used when the immune response to an antigen needs to be analyzed.

Advantages	Disadvantages
Faster than other ELISA – the technique has fewer steps Less prone to error – as less reagents and fewer steps are required	Antigen immobilization is not specific – may cause higher background noise than indirect ELISA. Mainly because all proteins in the sample, including the target protein, will bind to the plate
	Less flexible – each target protein needs a specific conjugated primary antibody
	No signal amplification – reduces assay sensitivity
Best for: when analyzing the immune response to an antigen.	

Indirect ELISA

Figure 3 demonstrates how an indirect ELISA is set up; antigen is adsorbed to a well in an ELISA plate. Detection is a two-step process. First, an unlabeled primary antibody binds to the specific antigen. Second, an enzyme conjugated secondary antibody that is directed against the host species of the primary antibody is applied.
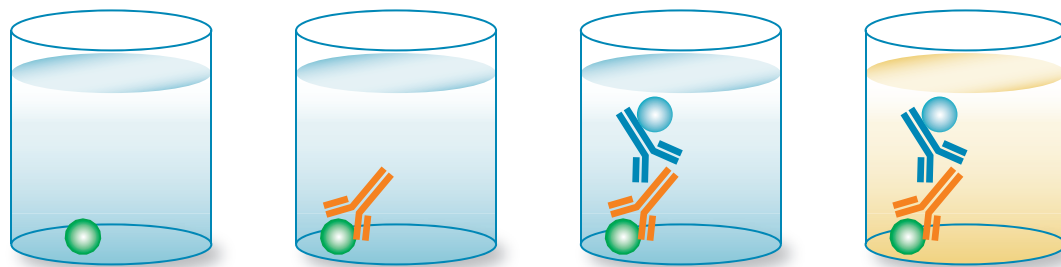

Fig. 3. Overview of indirect ELISA.

The indirect ELISA method is more sensitive than the direct ELISA method as more than one labeled secondary antibody can bind the primary antibody. It is also more economical than the direct ELISA, as fewer labeled antibodies are needed. Indirect ELISA delivers greater flexibility since different primary antibodies can be used with a single labeled secondary antibody. Among its disadvantages is the possibility of cross-reactivity of secondary antibody to the adsorbed antigen, which could increase background noise. Also, indirect ELISA assays take longer to run than direct ELISAs, since an additional incubation step for the secondary antibody is required. The indirect ELISA is most suitable for determining total antibody concentration in samples.

Advantages	Disadvantages
High sensitivity – more than one labeled secondary antibody can bind the primary antibody	Possibility of background noise – secondary antibody may be cross-reactive
Economical – fewer labeled antibodies are needed	Longer procedure than direct ELISA technique – additional incubation step for secondary antibody needed
Greater flexibility – different primary antibodies can be used with a single labeled secondary antibody	
Best for: determining total antibody concentration in samples.	

Sandwich ELISA

Sandwich ELISAs require the use of matched antibody pairs (capture and detection antibodies) as shown in Figure 4. Each antibody is therefore specific for a different and nonoverlapping region or epitope of the antigen. It is important that matched antibody pairs are tested specifically in sandwich ELISA to ensure that they detect different epitopes, to achieve accurate results. The capture antibody, as its name implies, binds the antigen which can then be detected in a direct ELISA or in an indirect ELISA configuration.
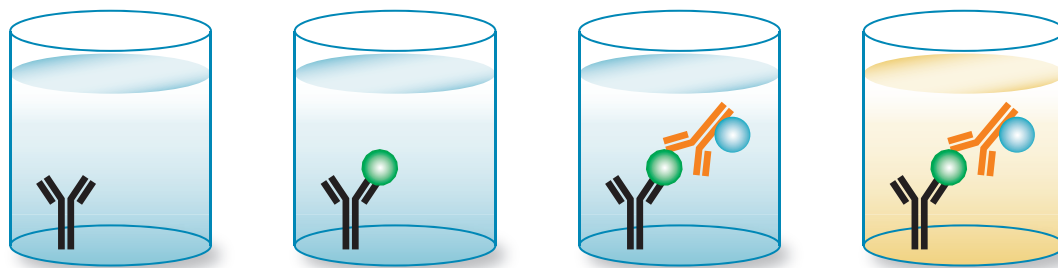

Fig. 4. Overview of direct sandwich ELISA.

The procedure for a sandwich ELISA firstly requires the well of an ELISA plate to be coated with a capture antibody. The analyte or sample is then added, followed by a detection antibody. The detection antibody can be enzyme conjugated, in which case this is referred to as a direct sandwich ELISA. If the detection antibody used is unlabeled, a secondary enzyme-conjugated detection antibody is required. This is known as an indirect sandwich ELISA. The key advantage of a sandwich ELISA is its high sensitivity; it is 2-5 times more sensitive than direct or indirect ELISAs. It also delivers high specificity, as two antibodies are used to detect the antigen, and offers flexibility since both direct and indirect methods can be used. The advantages bring with them a few disadvantages; if a standardized ELISA kit or tested antibody pair is not available, antibody optimization will be required to choose a pair of antibodies that bind to different epitopes on the antigen.

If a secondary antibody is used, it is important to ensure that the capture and detection antibodies have different host species to avoid cross-reactivity from the secondary antibody. The secondary antibody selected should also ideally have been tested for cross-reactivity with IgG from the host species of the capture antibody. It is possible to use capture and detection antibodies that have the same host species, as long as they are of different IgG isotypes. However a secondary antibody that specifically targets the isotype of the detection antibody would be required.

Sandwich ELISAs are particularly suited to the analysis of complex samples, since the antigen does not need to be purified prior to the assay, yet still delivers high sensitivity and specificity (e.g. measuring cytokine levels in an immune response).

Advantages	Disadvantages
High sensitivity – 2-5 times more sensitive than direct or indirect ELISA	Antibody optimization can be difficult – cross-reactivity may occur between the capture and detection antibodies. Needs a standardized ELISA kit or tested antibody pair
High specificity – two antibodies are involved in capture and detection	
Flexibility – both direct and indirect detection can be used	
Best for: analysis of complex samples, since the antigen does not need to be purified prior to measurement.	

Competition/Inhibition ELISA

How it works: the competition/inhibition ELISA, also known as a blocking ELISA, is perhaps the most complex of all the ELISA techniques. However, each of the above assay types can be adapted to a competitive format. The competitive/inhibition ELISA is predominantly used to measure the concentration of an antigen or antibody in a sample by detecting interference in an expected signal output. Essentially, sample antigen or antibody competes with a reference for binding to a limited amount of labeled antibody or antigen, respectively. The higher the sample antigen concentration, the weaker the output signal, indicating that the signal output inversely correlates with the amount of antigen in the sample.

Coating

Control antigen is absorbed onto well in ELISA plate in coating buffer

Remove liquid and wash plate

Blocking

A buffer containing unrelated protein is used to block free sites in the wells

Remove liquid and wash plate

Prepare sample (unknown antigen) and detection antibody mix

Sample

Add test sample mix to wells

Remove liquid and wash plate

Detection Antibody

Add enzyme conjugated secondary detection antibody

Remove liquid and wash plate

Readout

Substrate is catalyzed by enzyme to generate colored readout

In this example the antigen concentration in the sample was low. The antibodies bound the control antigen that had been absorbed to the plate in the coating step
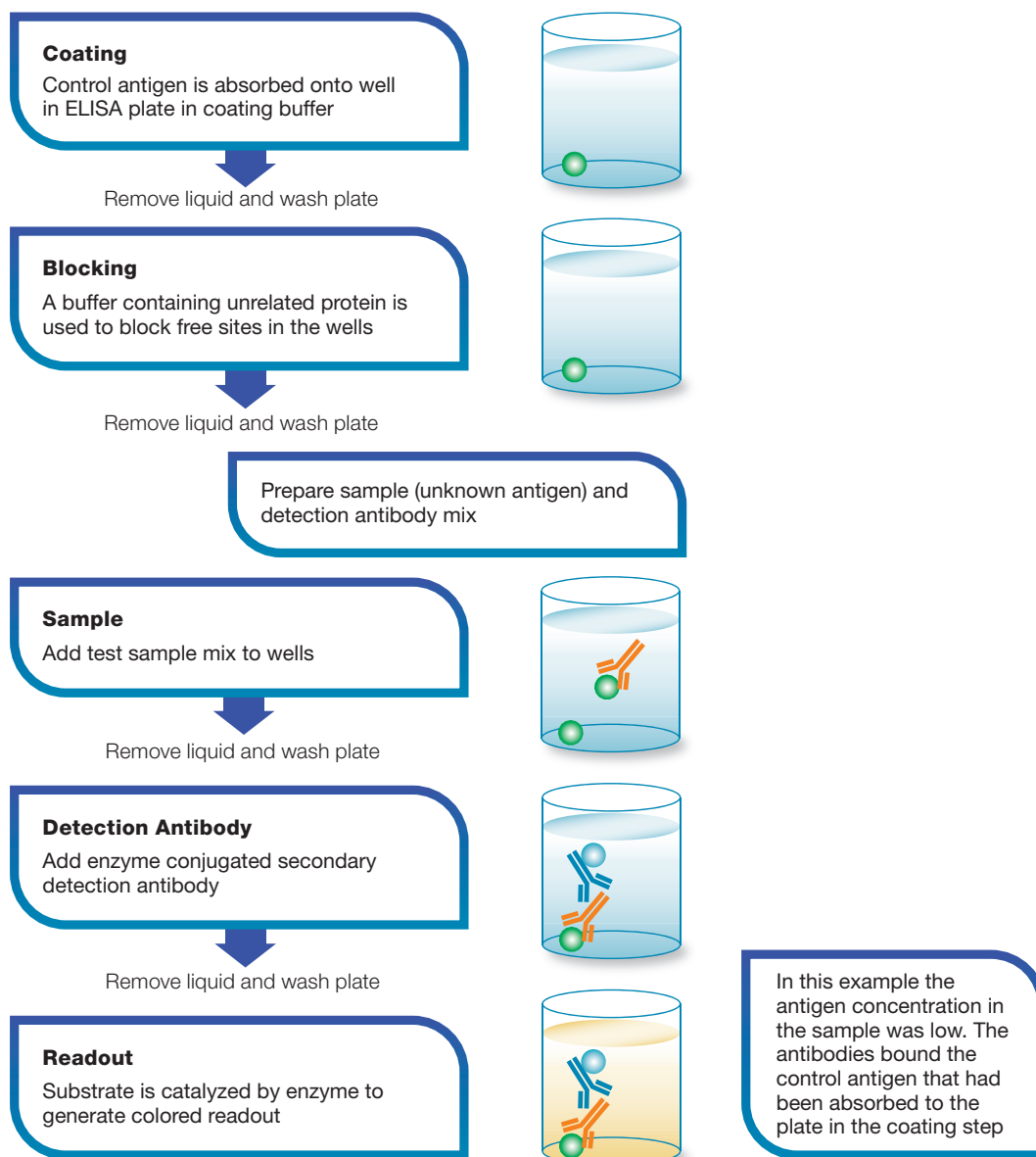
Fig. 5. Competition ELISA.

An example of a competition ELISA to test for antigen based on the direct detection method is shown in Figure 5.

In this example, a known antigen is used to coat a multiwell plate. Following standard blocking and washing steps, samples containing unknown antigen are added. Labeled detection antibody is then applied for detection using relevant substrates (e.g. 3,3',5,5'-Tetramethylbenzidine or TMB). If there is a high concentration of antigen in the sample, a significant reduction in signal output will be observed. In contrast, if there is very little antigen in the sample, there will be very little reduction in the expected signal output. In the example shown in Figure 5, there would be a reduction in signal output.

Advantages	Disadvantages
Main advantage – no sample processing is required and crude or impure samples can be used	Same limitations as base ELISA – as each ELISA technique can be adapted to a competitive format
More robust – less sensitive to sample dilution and sample matrix effects than the sandwich ELISA	
More consistent – less variability between duplicate samples and assays	
Maximum flexibility – it can be based on direct, indirect, or sandwich ELISA	
Best for: commonly used when only one antibody is available for the antigen of interest. It is also suitable for detecting small antigens that cannot be bound by two different antibodies such as in the sandwich ELISA technique.	

Principles of Multiplex Immunoassays

Multiplexed immunoassays are comprised of fluorescently dyed magnetic microspheres (also called beads), each with a distinct color code or spectral address, to allow for simultaneous detection of multiple biomolecules in a single well of a 96-well microplate. The assays are read on dedicated flow cytometry-based instruments with two lasers and associated optics, to identify the unique target assayed and measure the different molecules bound to the surface of the beads. A high-speed digital signal processor efficiently manages the fluorescence data, which is then output in a software and analyzed.

Multiplex immunoassays formats are similar to ELISA and can be a sandwich, competitive, indirect, or direct.

Visit **bio-rad.com/multipleximmunoassays** to learn more on the technique.

3 ELISA Detection Options

Direct Detection and Indirect Detection

ELISAs, by definition, take advantage of an enzymatic label to produce a detectible signal that is directly correlated to the binding of an antibody to an antigen. A few different types of enzymes and enzyme substrates (covered in Section 6) are typically used for ELISAs. Several methods for incorporating the enzyme step into the process can also be applied. The final assay signal is measured with a spectrophotometric, fluorescence, or luminescence plate reader (depending upon the substrate chosen).

One aspect of ELISA terminology that often leads to confusion is the variability in the way the terms direct and indirect are applied. We will adhere to the use of these terms as they apply to the detection portion of the assay as indicated below.

Direct Detection

Antibodies are directly labeled with alkaline phosphatase (AP) or HRP; this is the most common ELISA detection strategy. HRP and AP substrates typically produce a colorimetric output that is read by a spectrophotometer. Detection can also occur by fluorescently-labeled antibodies; here the assay is usually termed a fluorescence-linked immunosorbent assay (FLISA).

Indirect Detection

Antibodies are coupled to biotin, followed by a streptavidin-conjugated enzyme step. Alternatively, it is possible to use unlabeled primary antibodies followed by enzyme-coupled or biotinylated secondary antibodies. If the secondary antibody is biotinylated, then a tertiary step is required for detection. In this case, treatment with the streptavidin-enzyme conjugate is followed by an appropriate substrate.

4 ELISA Results

The ELISA assay yields three different types of data output:

Quantitative

ELISA data can be interpreted in comparison to a standard curve (a serial dilution of a known, purified antigen) in order to precisely calculate the concentrations of antigen in various samples (Figure 6).

Qualitative

ELISAs can also be used to achieve a yes or no answer indicating whether a particular antigen is present in a sample, as compared to a blank well containing no antigen or an unrelated control antigen. The inclusion of a single fixed level positive control can be used to give a yes/no criteria for minimum antigen level.

Semi-quantitative

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

Standard Curve

ELISA data is typically graphed with optical density vs log concentration to produce a sigmoidal curve as shown in Figure 6. Known concentrations of antigen are used to produce a standard curve, and then this data is used to measure the concentration of unknown samples by comparison to the linear portion of the standard curve. This can be done directly on the graph or with curve fitting software which is typically found on ELISA plate readers.
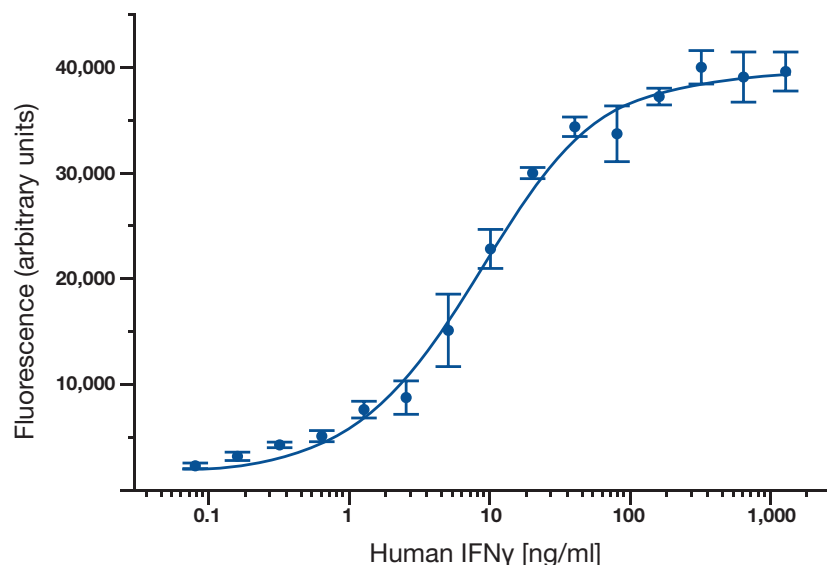
Fig. 6. A typical ELISA standard curve.

Calibration Curve Models

If a quantitative result is needed, the simplest way to proceed is to average the triplicate of the standards readings and deduct the reading of the blank control sample. Next, plot the standard curve, find the line of best fit or at least draw a point to point curve so that the concentration of the samples can be determined. Any dilutions made need to be adjusted for at this stage. This is generally the practical extent to which manual calculation can be taken.

A variation is to plot the data using semi-log, log/log, log/logit and its derivatives – the 4 or 5 parameter logistic models. Using software based/automated solutions makes it possible to consider more sophisticated graphing approaches. Using linear regression within a software package adds several more checking possibilities; it is possible to check the R2 value to determine overall goodness of fit. For that portion of the curve where the relationship of concentration to readout has a linear relationship, R2 values >0.99 represent a very good fit. Accuracy can then be further enhanced by using further standard concentrations in that range. One aspect of the linear plot is that it compresses the data points on the lower concentrations of the standard curve, hence making that the most accurate range (area most likely to achieve the required R2 value). To counteract this compression a semi-log chart can be used; here the log of the concentration value (on x-axis) is plotted against the readout (on y-axis). This method gives an S-shaped data curve that distributes more of the data points into the more user friendly sigmoidal pattern. The log/log (log of concentration against log of readout) plot type manages to linearize more of the data curve. The low to medium standard concentration range is generally linear in this model, only the higher end of the range tends to slope off. The log/logit and its derivatives, the 4 or 5 parameter logistic models, are more sophisticated requiring more complex calculations and estimations of max, min, EC50, and slope values. The 5 parameter model additionally requires the asymmetry value.

While these calibration curve models can deliver improved performance, a good starting point would be using the log-log plot with a check on the recovery percentage (analyte recovery from spiked samples). Alternatively, at least 'back-fitting' the standard curve readout values, is frequently 'a good enough' approach. The simplest way to check is to back calculate the calibration standards and check that they fall within 20% of the nominal readout value. One caveat is not to rely on 'good' R2 values and find that calibration curve model that delivers the best recovery values for the standards.

Sensitivity

ELISAs are one of the most sensitive immunoassays available. The typical detection range for an ELISA is 0.1 to 1 fmole or 0.01 ng to 0.1 ng, with sensitivity dependent upon the particular characteristics of the antibody-antigen interaction. In addition, some substrates such as those yielding enhanced chemiluminescent or fluorescent signal, can be used to improve results.

As mentioned earlier, indirect detection will produce higher levels of signal and should therefore be more sensitive. However, it can also cause higher background signal thus reducing net specific signal levels.

5 Controls

The previous section covered the need for standards to obtain quantitative ELISA results. It should be noted that standards should be acquired in sufficient amounts (or a reliable supply needs to be found) not only for the development phase but also for the expected service life of the assay. Additional to standards, controls should be included in ELISAs.

The most basic control is the blank sample control. Additional controls are needed to provide a comparison to real world physiological conditions and a control mechanism for assurance that the assay continues to provide accurate results. Hence control samples that have had their analyte concentration validated by another method are employed. These can be set up as positive, but also as negative controls. A control subcategory is spiked samples; here a known amount of standard has been added to the matrix used for the ELISA. Spiked controls can indicate assay performance by calculating percent recovery from the ELISA readout. When recombinant proteins are used, their equivalent functionality to the endogenous wild type versions of the protein needs to be checked.

6 ELISA Components and Considerations

ELISA Plates

Plate Format

Flat-bottomed, 96-well plates, made from polystyrene or polyvinyl chloride, are used in the vast majority of ELISA assays. Alternatively a strip well plate can be used. This is a frame in the size of a 96-well plate that is populated with as many 8-well or 12-well strips as the experiment requires. Further variants are 384-well and 1536-well plates; these have the same footprint as the traditional 96-well plates but obviously are able to process more samples per plate. For optimum use they require automated handling and hence are near exclusively used in high throughput screening. Some enzyme substrates, such as those that produce fluorescent or chemiluminescent signals may require opaque plates for optimal results.

Plate Characteristics

It is important to use plates designed for ELISAs because they are manufactured to maintain consistency, minimizing edge effects, and providing optimal optical conditions for data collection. It is a good idea to test plates from several manufacturers for batch-to-batch and plate-to-plate variability, especially if an assay is being developed for commercial, diagnostic, or quality control uses. The usual expectation is a 5% or lower variation in common controls across 2 plates. Standard polystyrene ELISA plates fall into the low to medium binding type, meaning that they will capture around 100–200 ng of IgG/cm^2. Modification of the polystyrene yields binding capacities of 400–500 ng of IgG/cm^2, these are commercially available as high-binding plates. Finally, antigen or antibody pre-coated plates are commercially available although most often as part of an optimized ELISA kit with all components included.

Buffers

Standard Buffers

Several different buffers are used during an ELISA: one for coating, another for blocking, another for washing, and perhaps another for sample and antibody dilution. Buffers can be produced in house or sourced from a variety of commercial antibody and reagent suppliers. Basic ELISA buffer recipes can be found on our ELISA protocols page at **bio-rad-antibodies.com/elisa-protocols**.

Coating Buffers

Coating is the first step in any ELISA and is the process where a suitably diluted antigen or antibody is incubated until adsorbed to the surface of the well. Adsorption occurs passively as the result of hydrophobic interactions between the amino acids side chains on the antibody or antigen used for coating, and the plastic surface. It is dependent upon time, temperature, and the pH of the coating buffer, as well as the concentration of the coating agent.

Typical coating conditions involve adding 50-100 µl of coating buffer, containing antigen or antibody at a concentration of 1-10 µg/ml, and incubating overnight at 4°C or for 1-3 hours at 37°C. Alternative temperatures, times, buffers, and coating agent concentrations can be used and should be optimized for your experimental setup. During coating, it is important to maintain a moist environment in the well to minimize evaporation; plate sealers are generally used to achieve this in a repeatable and constant fashion.

It is best to test a range of concentrations of coating agent since higher concentrations of antibody/antigen may actually have a negative effect on coating, leading to oversaturation of the wells, which can inhibit antibody binding due to steric hindrance.

Conversely, when crude antigen or antibody preparations are used for coating, it is possible that the effective antigen/antibody concentration may be low and outcompeted by contaminating proteins, making the specific assay signal too low to be useful. In this case a sandwich assay is more suitable.

Coating buffers stabilize the antigen or antibody which is used to coat the ELISA multiwell plate, maximizing adsorption to the plate and optimizing interactions with the detection antibody. It is imperative that no other proteins are included in the coating buffer as these will compete with the antigen for binding to the plate.

The two most common coating buffers are bicarbonate buffer at pH 9.6 or PBS; basic buffer recipes can be found on our ELISA protocols page.

Blocking Buffers

Blocking is often necessary to prevent the nonspecific binding of detection antibodies to the multiwell plate surface itself. There are two main types of blocking agents, proteins and detergents. Proteins are classified as permanent blocking agents and hence added after the capture antibody has adsorbed to the well surface. Detergents only block temporarily,

meaning their blocking function disappears during washing steps. As there is no ideal universal blocking buffer, blocking is a compromise between achieving the desired sensitivity and reduced background. Starting with a buffer, that contains an unrelated protein or a protein derivative that does not react with any of the antibodies being used in the detection step, is a recommended starting point for finding an effective blocking buffer.

It is important to note that if a protein is used in the blocking reagent, that detergent should not be included before or during the blocking step as this will prevent effective blocking by the protein.

When a plate is fully blocked, assay sensitivity will be enhanced since additional nonspecific signal will be reduced. The most basic blocking buffer contains 1% BSA or milk proteins dissolved in PBS. Usually 150 µl of blocking buffer is added to the well to incubate for one hour at 37°C in order to fully block the plate.

Washing Buffers

Since the ELISA uses surface binding for separation, wash steps are repeated between each step to remove unbound materials. The wash steps are a critical part of the process and entail filling the wells entirely with buffer, usually PBS, with a small concentration of a nonionic detergent such as Tween-20.

Washing is typically repeated 3-5 times between each step in the ELISA to thoroughly remove unbound material. Usually the wash solution is only briefly retained on the plate. Excess wash solution must be removed in the final wash step to prevent the dilution of the reagents added in the subsequent stage. This is accomplished most simply by tapping the washed plate upside down on an absorbent paper to remove excess liquid or by careful aspiration. However, plate washers can provide a quicker and more effective method for performing these steps. It is crucial not to let the plate dry out.

Antibodies

The antibodies used in ELISA assays can be monoclonal, polyclonal, or a combination of both. Each antibody type offers distinct advantages in the development of ELISAs, so it is important to appreciate the differences between them and how these can be used to obtain an advantage during ELISA development.

The interaction between antibodies and their antigens is described in three ways: specificity, affinity, and avidity. During ELISA development, these factors influence the amount of optimization of, e.g. antibody concentration and buffers, required (see ELISA Optimization Section).

Specificity is an indication of whether an antibody binds solely to a unique epitope from a single antigen in a single species, or whether it binds to similar epitopes present on several molecules from a few different species. Cross-reactivity is the opposite of specificity.

Affinity describes the strength of binding of an antibody to a single epitope. Since binding is reversible, affinity determines how much antigen is bound by an antibody, how quickly

binding occurs, and for how long the binding lasts. High affinity antibodies are the best choice for all types of immunoassay because they rapidly produce the greatest number of stable immune complexes and therefore provide the most sensitive detection.

Avidity is a more complex term that accounts for the total stability of the antibody-antigen interaction. It is based upon affinity, but is also influenced by the valency of the antibody, or total number of antigen binding sites. Thus, avidity varies with isotype and whether the antibody is intact or fragmented. There is also a contribution made by the spatial arrangement of the whole complex.

Monoclonal Antibodies

Monoclonal antibodies are homogeneous by definition, with specificity for a single epitope or small region of a protein. As a result, they are less likely to interact with closely-related proteins and are not generally expected to trigger nonspecific signals in a given immunoassay.

Monoclonal antibodies can be used for all antibody-containing steps in all types of ELISAs. They are commonly used in sets as matched pairs in sandwich ELISAs, but can be used for capture or detection, in conjunction with a polyclonal antibody to enhance signal or to provide a greater chance of capturing antigen from a complex solution.

Polyclonal Antibodies

Polyclonal antibodies are complex antibody pools which represent a collection of specificities to various epitopes found in a single antigen. Some epitopes predominate or there may be wide representation of the epitopes available in any given antigen. Polyclonals can vary significantly from batch-to-batch, and must be tested and validated thoroughly.

As a result of their heterogeneity and the wide representation of epitopes present, polyclonal antibodies can be powerful tools for the thorough detection of an antigen, often yielding higher signal levels. It is also rare that they will fail to bind due to a single blocked antibody binding site, antigen configuration change, or misfolding. However, polyclonal antibodies are also more likely to share one or more epitopes with closely-related proteins, resulting in higher nonspecific signal. One solution to reduce this problem is to use affinity purified or cross-absorbed polyclonal antibodies.

Sometimes the detection method for an ELISA is switched from direct to indirect detection, and thus from a monoclonal to a polyclonal, in order to increase assay sensitivity due to higher levels of polyclonal antibody binding to the target antigen.

Polyclonal antibodies bring an additional aspect to ELISAs. They can be used as capture and detection antibodies. Antibodies from the same polyclonal batch can both capture the analyte and subsequently also detect it, in a biotin conjugated format.

Matched Pairs

Matched pairs are the basis of many sandwich ELISAs, either in kits or for in house assay set up. The name refers to sets of antibodies which are known to be capable of detecting different epitopes on the same protein antigen, so they can be used together for the capture and detection of a single antigen in a sandwich ELISA or related immunoassay. Matched pairs can consist of two monoclonal antibodies, two polyclonal antibodies, or a combination of both. If a monoclonal and polyclonal antibody are used together, the monoclonal antibody should be used as the coating antibody. If it is used as the detection antibody, its epitope may be masked by a component clone of the polyclonal antibody, resulting in reduced or lost signal.

Sample Handling and Preparation

A wide variety of samples can be tested in an ELISA and the choice of assay conditions will depend upon the complexity of the sample and the expected amount of antigen present.

Samples are usually considered to be homogeneous or heterogeneous, depending on their complexity. This is essentially equivalent to a purified antigen vs. a crude unpurified mixture. In the simplest case, ELISA samples are diluted in PBS, wash buffer, or other specialty buffers, and applied in a final volume of 100 μl. Blood presents special challenges due to the proteins present that can disrupt the assay results. Since sera can contain antibodies, there can also be unexpected cross-reactivity. Hence, special treatments and buffers are sometimes needed for the dilution of blood samples in order to obtain optimal results.

It is possible to use the samples to coat the wells themselves, as in a direct ELISA, or to capture and quantitate the antigen samples using a sandwich assay if a matched pair is available. A complex, heterogeneous protein mixture would be less suitable for coating a plate for direct ELISA detection unless the protein of interest is over-expressed and thus the majority of protein present in the sample.

It is important to test all samples in duplicate or triplicate in conjunction with a known standard to ensure the accuracy of results and for quantitation. If possible, it is better to test several dilutions of a sample to make sure the final results fall within the linear portion of the standard curve. This is because highly concentrated samples can underestimate concentration, while highly diluted samples can overestimate it. This will avoid the Hook effect, observed when very high levels of antigen are present in the sample, leading to reduced specific binding of the antigen, that is insufficient to match analyte levels and showing lower signal intensity than expected.

Antibody Labeling

It is generally advantageous to standardize the detection antibodies and source them from a commercial supplier, for consistency and convenience. In certain cases, e.g. direct ELISA it may be impossible to obtain a labeled detection antibody; in these cases the chosen antibody needs to be labeled.

If the antibody is purified and in 10-50 mM amine-free buffer (e.g. HEPES, MES, MOPS and phosphate) at a pH range of 6.5-8.5, it can be quickly and conveniently labeled with Horseradish Peroxidase (HRP) using Bio-Rad's LYNX Rapid HRP Antibody Conjugation Kit (#LNK001P) or to alkaline phosphatase with the LYNX Rapid Alkaline Phosphatase Antibody Conjugation Kit (#LNK0011AP).

Substrate

The final step in an ELISA is the enzyme catalyzed reaction to obtain a colored end product that can be read in a spectrophotometer as absorbance values, representing the analyte concentration. Bio-Rad supplies a range of substrates for HRP and AP enzyme based detection systems.

Visit **bio-rad.com/en-us/product/elisa-enzyme-antibody-conjugates** to browse Bio-Rad's selection of highly specific, affinity-purified HRP- and AP- conjugated antibodies, optimized for microtitration enzyme immunoassays.

TMB Core+

TMB Core+ (#BUF062) is a high-performance TMB (3,3´,5,5´- tetramethylbenzidine) solution, recommended for use in ELISAs as a substrate for HRP. TMB Core+ contains TMB, substrate buffer and hydrogen peroxide and has been optimized for increased sensitivity, minimal background and rapid development. It produces a deep blue color read at 655 nm. The reaction may be stopped with sulfuric acid, resulting in a yellow color read at 450 nm.

TMB Sensitive

TMB Sensitive (#BUF066) offers greater sensitivity than other TMB reagents offered by Bio-Rad.

TMB CORE

TMB CORE (#BUF056) is a high performance substrate for HRP that contains TMB, substrate buffer and hydrogen peroxide in a safe, ready-to-use solution.

TMB SIGNAL+

TMB SIGNAL+ (#BUF054) has been optimized to enable increased sensitivity and enhanced stability. It also provides minimal background and rapid development times.

pNPP

pNPP (#BUF044) is a high-performance p-NitroPhenyl Phosphate (pNPP) solution formulated to increase pNPP activity and stability. It is ready-to-use and recommended for ELISAs as a substrate for AP for kinetic and endpoint tests.

7 ELISA Optimization

Consistency between Wells

One of the most important aspects of any assay is consistency and standardization of conditions, as this will affect the reproducibility and accuracy of your results. In the initial stages of assay development, it is important to test a range of parameters, usually by completing a checkerboard dilution series to test various conditions in systematic manner. In addition, buffers, temperature, and humidity must be kept constant between and within experiments in order to produce standardized results.

In a typical ELISA, multiwell plates, multichannel pipets, and plate washers provide for more consistent and faster results, as well as higher throughput. It is very important to make sure that all pipettors used in ELISAs are properly calibrated on a regular basis, to prevent significant variation in the results. Furthermore, it is good practice to observe the level of the liquid in the pipet tip and the wells while following the procedure, to make sure no sample is far out of line with the others. This is particularly important when multichannel pipets are used, as sometimes the tips in the end rows do not always attach fully to the pipettes.

Use Bio-Rad's ImmunoWash Microplate Washer for accurate and reproducible plate washing. Create custom wash routines and control needle position. FDA-approved. Visit **bio-rad.com/en-us/product/model-1575-immunowash-microplate-washer**, for more information.

Suitability and Concentration of Antibodies

Using the assumption that a matched pair of antibodies is available to the analyte and that the aim is to establish a sandwich ELISA, the initial task is to determine working concentrations for the antibodies.

Prepare capture antibody dilutions in coating buffer at 0.5, 1, 2, and 5 µg/ml. Then follow standard procedure for a sandwich ELISA which can be found on our ELISA protocols page (**bio-rad-antibodies.com/elisa-protocol**), distributing the capture antibody as shown in Figure 7.

At the stage where sample addition would occur, add a high and low concentration of your analyte that reflects the expected working range. Finally include a blank, again following the layout in Figure 7 and standard sandwich ELISA protocol until the detection stage.

At the detection stage, prepare detection antibody dilutions at 1:200, 1:1,000, 1:5,000, and 1:25,000 in buffer, adding it as detailed in Figure 7. Including a high and low concentration of the analyte helps to determine the dynamic range. The low concentration analyte indicates the sensitivity of the assay. The blank will indicate nonspecific binding.

Detection Antibody	Capture Antibody A											
	5 µg/ml			2 µg/ml			1 µg/ml			0.5 µg/ml		
1:200	H	L	0	H	L	0	H	L	0	H	L	0
	H	L	0	H	L	0	H	L	0	H	L	0
1:1,000	H	L	0	H	L	0	H	L	0	H	L	0
	H	L	0	H	L	0	H	L	0	H	L	0
1:5,000	H	L	0	H	L	0	H	L	0	H	L	0
	H	L	0	H	L	0	H	L	0	H	L	0
1:25,000	H	L	0	H	L	0	H	L	0	H	L	0
	H	L	0	H	L	0	H	L	0	H	L	0

Fig. 7. Antibody concentration optimization plate layout.

Find the set with the maximum signal-to-noise ratio/largest difference between low and high analyte concentrations; these are the antibody concentrations for further optimization.

Should the blank sample show excessive readings, above 0.2 absorbance units, these key components need to be checked for optimization: ELISA plate type and the blocking and washing buffers. If the background readings are appropriate but the sensitivity is not high enough further experimentation with matrix conditions, buffers, and incubation timings should be carried out. However, if no improvement is possible different antibody combinations need to be generated.
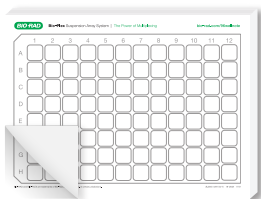
Bio-Plex assay preparation is made easy, when using Bio-Rad 96-well plate template Post-It Pad.

Request your free copy at **bio-rad.com/GetPlateTemplate**

Matrix Effects

Matrix effects occur most often in plasma and serum samples where a series of components can cause interference. These can be cross-reactive or nonspecific interactions to substances in the matrix or breakdown products that develop during the sample handling process. It is possible to reduce matrix effects by dilution of the sample; this should be verified by analyte recovery from spiked samples. Specialized buffers for sample dilution, coating, blocking, and washing can ameliorate matrix effects and provide constant performance.

Coating Buffers

Specialist coating buffers are available which have been optimized for ELISA, such as #BUF030 which has been developed to stabilize the adsorbed protein, preserving the antigenic regions and allowing greater binding reactivity in order to enhance the specific signal.

AbGuard Plate Stabilizer (#BUF063) simultaneously stabilizes and preserves microwell plates coated with proteins or other biomolecules and blocks any free binding sites. It preserves the biological activity of bound molecules and prevents degradation, denaturation and leaching. It also blocks free binding sites without creating any interference.

Blocking Buffers

A wide range of sophisticated commercial blocking buffers are available, some of which are BSA-based (#BUF032), but also contain preservatives to create a stable long-term environment. Another option is to use a high performance commercial blocking buffer, such as ELISA Ultrablock (#BUF033), which is an optimized formulation that includes small protein fragments which thoroughly block less accessible surfaces of the multiwell dish. Ultrablock is recommended for use on mammalian samples, particularly, human, pig, and cow, as there is less chance of cross-reactivity. When maximal blocking strength is required, or for most sandwich ELISAs, preparations such as ELISA SynBlock (#BUF034), an inert, synthetic blocking formula that reduces nonspecific interference.

Washing Buffers

For greatest consistency, specialized plate washers are used to add and remove the wash liquid. Specialized wash buffers are also available, such as #BUF031, which contains an optimized formulation of pH stabilizers, salts, and detergents that reduce background noise and enhance specific signal.

Sample Buffers

One type of specialty sample buffer used for sample dilutions is HISPEC assay diluent, #BUF049. This buffer is used to dilute a range of sample types or for the dilution of detection antibodies. It reduces cross-reactivity, nonspecific binding, and matrix effects.

#BUF037 is a specialized buffer that is recommended for use in sandwich ELISA assays with samples containing plasma, serum, or cell culture supernatant. This buffer contains goat serum proteins which reduce the difference between the sample matrix and the diluent used to generate the standard curve. It also contains a proprietary chelating agent that blocks interference from complement and thrombin in plasma and serum. It is easy-to-use and applied by pipetting 50-100 µl directly onto the plate before adding the sample. Since this buffer contains proteins derived from goat, it is not recommended for ELISAs that use anti-goat secondary antibodies for detection, as this will result in nonspecific signal.

Specialist Buffers

Some specialist buffers are multifunctional, allowing for simultaneous coating, stabilization, and blocking. One such example is AbGuard Plate Stabilizer (#BUF063), which preserves the biological activity of adsorbed molecules and prevents degradation, denaturation, and leaching of the coating material. As a result, this buffer can shorten the length of time required for the assay and will increase efficiency by extending the life of coated plates.

Another option is Block Ace (#BUF029), a buffer that can be used for blocking, sample dilution, and washing for ELISAs and western blots. It also improves ELISA results by reducing background and producing sharper standard curves.

8 Protocols

Direct ELISA with Streptavidin-Biotin Detection

Reagents

1. Coating buffer

   Na_2CO_2, 1.5 g

   $NaHCO_3$, 2.93 g

   Distilled water, 1 liter, pH to 9.6

   For an alternative coating buffer use ELISA coating buffer (#BUF030)

2. Blocking buffer

   Phosphate buffered saline (PBS) (#BUF036A) containing 1% w/v BSA

   For an alternative blocking buffer, use either ELISA BSA Block (#BUF032), ELISA Ultrablock (#BUF033), or ELISA SynBlock (#BUF034)

3. Wash buffer

   PBS containing 0.05% v/v Tween-20

   For an alternative wash buffer use ELISA wash buffer (#BUF031)

Recommended Substrates and Stop Solutions

TMB Core+ (#BUF062), for use with HRP-conjugated antibodies. Stop with 2M H_2SO_4.

pNPP (#BUF044), for use with alkaline phosphatase-conjugated antibodies. Stop with 1M NaOH.

Method

1. Coat microtiter plate wells with 100 µl of the appropriate coating antigen/analyte, at a concentration of between 1-10 µg/ml in coating buffer. Cover the plate and incubate overnight at 4°C. Wash the plate 3 times in wash buffer.

2. Add 150 µl of blocking solution to each well. Incubate for 60 minutes at 37°C. Wash 4 times in wash buffer.

3. Add 100 µl of biotin-conjugated detection antibody (appropriately diluted in wash buffer) to each well. Incubate for 1 hour at 37°C. Wash 3 times in wash buffer.

4. Add 100 µl of enzyme-conjugated streptavidin (appropriately diluted in wash buffer) to each well. Incubate for 60 minutes at 37°C. Wash 3 times in wash buffer.

5. Add 100 µl of the appropriate substrate solution to each well. Incubate at room temperature (and in the dark if required) for 30 minutes, or until desired color change is attained.

6. Read absorbance values immediately at the appropriate wavelength or add 50 µl of "stop solution". Gently tap plate to ensure thorough mixing. Measure absorbance within 30 minutes.

Indirect ELISA

Reagents

1. Coating buffer

   Na_2CO_2, 1.5 g

   $NaHCO_3$, 2.93 g

   Distilled water, 1 liter, pH to 9.6

   For an alternative coating buffer, use ELISA coating buffer (#BUF030)

2. Blocking buffer

   Phosphate buffered saline (PBS) containing 1% w/v BSA

   For an alternative blocking buffer, use either ELISA BSA Block (#BUF032), ELISA Ultrablock (#BUF033), or ELISA SynBlock (#BUF034)

3. Wash buffer

   PBS containing 0.05% v/v Tween-20

   For an alternative wash buffer use ELISA wash buffer (#BUF031)

Recommended Substrates and Stop Solutions

TMB Core+ (#BUF062), for use with HRP-conjugated antibodies. Stop with 2M H_2SO_4.

pNPP (#BUF044), for use with alkaline phosphatase-conjugated antibodies. Stop with 1M NaOH.

Method

1. Coat microtiter plate wells with 100 µl of the antigen/analyte solution, at a concentration of between 1-10 µg/ml in coating buffer. Cover the plate and incubate overnight at 4°C. Wash the plate 3 times in wash buffer.

2. Add 150 µl of blocking solution to each well. Incubate for 1 hour at 37°C. Wash 4 times in wash buffer.

3. Add 100 µl of unconjugated detection antibody (appropriately diluted in wash buffer) to each well. Incubate for 1 hour at 37°C. Wash 3 times in wash buffer.

4. Add 100 µl enzyme-conjugated secondary antibody (appropriately diluted in wash buffer) to each well. Incubate for 1 hour at 37°C. Wash 3 times in wash buffer.

5. Add 100 µl of the appropriate substrate solution to each well. Incubate at room temperature (and in the dark if required) for 30 minutes, or until desired color change is attained.

6. Read absorbance values immediately at the appropriate wavelength or add 50 µl of "stop solution". Gently tap plate to ensure thorough mixing. Measure absorbance within 30 minutes.

Sandwich ELISA with Direct Detection

Reagents

1. Coating buffer

   Na_2CO_2, 1.5 g
   $NaHCO_3$, 2.93 g
   Distilled water, 1 liter, pH to 9.6
   For an alternative coating buffer use ELISA coating buffer (#BUF030)

2. Blocking buffer

   Phosphate buffered saline (PBS) containing 1% w/v BSA
   For an alternative blocking buffer, use either ELISA BSA Block (#BUF032), ELISA Ultrablock (#BUF033), or ELISA SynBlock (#BUF034)

3. Wash buffer

   PBS containing 0.05% v/v Tween-20
   For an alternative wash buffer use ELISA wash buffer (#BUF031)

Recommended Substrates and Stop Solutions

TMB Core+ (#BUF062), for use with HRP-conjugated antibodies. Stop with 2M H_2SO_4.

pNPP (#BUF044), for use with alkaline phosphatase-conjugated antibodies. Stop with 1M NaOH.

Method

1. Coat microtiter plate wells with 100 μl of the appropriate coating antibody, at a concentration between 1-10 μg/ml in coating buffer. Cover the plate and incubate overnight at 4°C. Wash the plate 3 times in wash buffer.

2. Add 150 μl of blocking solution to each well. Incubate for 1 hour at 37°C. Wash 4 times in wash buffer.

3. Dilute samples and standards in wash buffer and add 100 μl of suitably diluted samples and standards to the relevant wells. Samples or standards should preferably be run in triplicate. Incubate for 90 minutes at 37°C. Wash 3 times in wash buffer.

4. Add 100 μl of appropriately diluted enzyme-conjugated detection antibody to each well. Incubate for 1 hour at 37°C. Wash 3 times in wash buffer.

5. Add 100 μl of appropriate substrate solution to each well. Incubate at room temperature (and in the dark if required) for 30 minutes, or until desired color change is attained.

6. Read absorbance values immediately at the appropriate wavelength or add 50 μl of "stop solution". Gently tap plate to ensure thorough mixing. Measure absorbance within 30 minutes.

Sandwich ELISA with Streptavidin-Biotin Detection

Reagents

1. Coating buffer

   Na_2CO_2, 1.5 g
   $NaHCO_3$, 2.93 g
   Distilled water, 1 liter, pH to 9.6
   For an alternative coating buffer use ELISA coating buffer (#BUF030)

2. Blocking buffer

   Phosphate buffered saline (PBS) containing 1% w/v BSA
   For an alternative blocking buffer, use either ELISA BSA Block (#BUF032), ELISA Ultrablock (#BUF033), or ELISA SynBlock (#BUF034)

3. Wash buffer

PBS containing 0.05% v/v Tween-20

For an alternative wash buffer use ELISA wash buffer (#BUF031)

Recommended Substrates and Stop Solutions

TMB Core+ (#BUF062), for use with HRP-conjugated antibodies. Stop with 2M H_2SO_4.

pNPP (#BUF044), for use with alkaline phosphatase-conjugated antibodies. Stop with 1M NaOH.

Method

1. Coat microtiter plate wells with 100 µl of the appropriate coating antibody, at a concentration between 1-10 µg/ml in coating buffer. Cover the plate and incubate overnight at 4°C. Wash the plate 3 times in wash buffer.

2. Add 150 µl of blocking solution to each well. Incubate for 1 hour at 37°C. Wash 4 times in wash buffer.

3. Add 100 µl of suitably diluted samples to the relevant wells. Ensure that appropriately diluted standards are included (dilute samples and standards in wash buffer). Samples or standards should preferably be run in triplicate. Incubate for 90 minutes at 37°C or overnight at 4°C. Wash 3 times in wash buffer.

4. Add 100 µl of biotin-conjugated detection antibody (appropriately diluted in wash buffer) to each well. Incubate for 1 hour at 37°C. Wash 3 times in wash buffer.

5. Add 100 µl of enzyme-conjugated streptavidin (appropriately diluted in wash buffer) to each well. Incubate for 1 hour at 37°C. Wash 3 times in wash buffer.

6. Add 100 µl of the appropriate substrate solution to each well. Incubate at room temperature (and in the dark if required) for 30 minutes, or until desired color change is attained.

7. Read absorbance values immediately at the appropriate wavelength or add 50 µl of "stop solution". Gently tap plate to ensure thorough mixing. Measure absorbance within 30 minutes.

Competitive ELISA

Reagents

1. Coating buffer

Na_2CO_2, 1.5 g
$NaHCO_3$, 2.93 g

Distilled water, 1 liter, pH to 9.6

For an alternative coating buffer use ELISA coating buffer (#BUF030)

2. Blocking buffer

Phosphate buffered saline (PBS) containing 1% w/v BSA

For an alternative blocking buffer, use either ELISA BSA Block (#BUF032), ELISA Ultrablock (#BUF033), or ELISA SynBlock (#BUF034)

3. Wash buffer

PBS containing 0.05% v/v Tween-20

For an alternative wash buffer use ELISA wash buffer (#BUF031)

Recommended Substrates and Stop Solutions

TMB Core+ (#BUF062), for use with HRP-conjugated antibodies. Stop with 2M H_2SO_4.

pNPP (#BUF044), for use with alkaline phosphatase-conjugated antibodies. Stop with 1M NaOH

Method

1. Coat microtiter plate wells with 100 µl of the antigen solution, at a concentration of between 1-10 µg/ml in coating buffer. Cover the plate and incubate overnight at 4°C. Wash the plate 3 times in wash buffer.

2. Add 150 µl of blocking solution to each well. Incubate for 1 hour at 37°C. Wash 4 times in wash buffer.

3. Prepare the antigen antibody mixture by adding 50 µl of antigen to 50 µl of antibody for each well in the assay (use a range of antigen concentrations appropriately diluted in wash buffer). Incubate for 1 hour at 37°C.

4. Add 100 µl of the mixture to each well. Incubate for 1 hour at 37°C. Wash 3 times in wash buffer.

5. Add 100 µl enzyme-conjugated secondary antibody (appropriately diluted in wash buffer) to each well. Incubate for 1 hour at 37°C. Wash 3 times in wash buffer.

6. Add 100 µl of the appropriate substrate solution to each well. Incubate at room temperature (and in the dark if required) for 30 minutes, or until desired color change is attained.

7. Read absorbance values immediately at the appropriate wavelength or add 50 µl of "stop solution". Gently tap plate to ensure thorough mixing. Measure absorbance within 30 minutes.

9 ELISA Troubleshooting

Problem	Possible Causes	Action/Solution
No signal	Assay set up incorrectly or used incorrect reagents	Review protocol. Repeat assay using a positive control
	Incorrect secondary antibody used	Retrace steps. Repeat assay using the correct secondary antibody
	Not enough antibody used	Increase concentration of the primary and/or secondary antibody
	Detection reagent too old or contaminated	Use fresh detection reagents
	Antigen not coated properly	Try longer coating times, different coating buffers, or avidin plates with biotinylated antigen
	Plate reader has the wrong settings	Check plate reader for wavelength, filters, gain etc
	Antibody stored at 4°C for several weeks or subjected to repeated freeze/thaw cycles	Use a fresh aliquot of antibody that has been stored at -20°C or below
Weak signal	Insufficient amount of antigen was coated to microtiter plate	Use more antigen for coating or very coating buffer
	Not enough antibody used	Increase concentration of the primary and/or secondary antibody. Optimize antibody concentrations for your assay
	Detection reagent too old, contaminated, or used at the wrong pH	Use fresh detection reagents at the correct pH
	Detection reagent too dilute	Use a higher concentration of detection reagent
	Plate reader has the wrong settings	Check plate reader for wavelength, filters, gain etc
	Incubation temperature too low	Optimize the incubation temperature for your assay. Reagents should be at room temperature before beginning the assay

High background signal	Too much antibody used	Reduce the concentration of the primary and/or secondary antibody. Optimize antibody concentrations for your assay
	Nonspecific antibody binding	Use a suitable blocking buffer or use an affinity-purified antibody
	Too much detection reagent used	Repeat assay with a higher dilution of detection reagent
	Too few washing cycles	Increase the number of washing cycles
	Contaminated blocking agent	Use fresh blocking agent
	Wrong concentration of blocking agent	Check the concentration of blocking agent in the recommended protocol
	Presence of blocking buffer interferes with antibody binding	Wash off blocking buffer before adding antibody
	Reaction not stopped	Use stop solution to prevent overdevelopment
	Plate left too long before reading	Start taking measurements shortly after the addition of detection reagent
	Wrong settings on the plate reader	Check settings and adjust as needed
	Insufficient amount of Tween in the buffers	Optimize the concentration of Tween in the blocking and wash buffers for your experiment using 0.05% Tween as a starting point
	Incubation with substrate carried out in the light	Perform substrate incubation in the dark
	Incubation temperature too high	Optimize the incubation temperature for your assay
	Plates stacked during incubations leading to uneven temperature distribution	Avoid stacking plates
	Pipetting errors	Calibrate pipets so that they dispense the correct volumes
	Reagents were not mixed properly	Before pipetting solutions into wells, make sure all reagents and samples have been thoroughly mixed
	Inconsistent washing of wells	Take precautions to reduce variability of washes, ensuring all liquid is removed from each well of the plate after each wash step. Consider using a plate washer to make wash steps more efficient

	Uneven evaporation of solution from wells during incubation	Always incubate with a lid on the plate
	Interference caused by dirt on the bottom of the plate	Clean the plate carefully and reread
Slow color development	Incubation temperature is wrong	Ensure plates and reagents are kept at room temperature
	Contaminated solutions	Make fresh solutions
	Detection reagent too old, contaminated or used at the wrong pH	Use fresh detection reagents at the correct pH

Technical Support

For further help and expert advice, contact our scientist staffed technical support department.

bio-rad-antibodies.com/contact

10 **Glossary**

Adsorption - the passive attachment of a liquid to a solid surface creating a thin film.

Antigen - substance, protein, chemical compound, or virus that is able to elicit an immune response against which antibodies are raised.

AP (Alkaline Phosphatase) - phosphatase enzyme that removes a phosphate group from a substrate. In ELISAs the substrate is p-NitroPhenyl Phosphate (pNPP).

Assay diluent (see also buffer) - buffer solution in which the sample to be analyzed is diluted in.

Assay sensitivity - a measure of the ability of the ELISA to distinguish between small changes in concentration.

Background - the signal readout attributable to all reagents excluding the analyte. Should be low.

Blocking - application of reagents, generally buffers, to lower background by binding to the potential nonspecific binding sites of antibodies and enzyme conjugates.

Buffer - solutions containing compounds, generally proteins, to reduce the nonspecific binding of antibodies; used in blocking to reduce background.

Cross-reactivity - an antibody binding to a target that is very similar to but not the intended target analyte, i.e. a closely related molecule with structural similarities to the target antigen.

Detection limit - the smallest quantity of analyte that can be reliably measured by the ELISA assay; it is often set to 2 standard deviations (2 SD) above background level.

Dilution - addition of a buffer to a protein solution to make it less concentrated, used in optimizing antibody concentration but also applied to samples to obtain readings within the dynamic range of the assay.

Dynamic range - range in the ELISA over which the absorbance reading increases in a linear mode and the analyte can be reliably measured.

Edge effect - the result of inconsistencies in the production of ELISA multiwell plates or when assay conditions, such as stacking plates, cause the outer wells to behave differently. As a result, unexpected values can appear in the outer wells which may be out of line with neighboring well. This can best be controlled for by using duplicates or triplicates for all samples, and noting any large variations in the results for a given sample.

Heterophilic interference - arises from antibodies found in the sample analyzed by the ELISA; it is binding by these antibodies to the detection antibody used in the assay. The best known example of heterophilic interference is HAMA (human anti-mouse antibody) found in some patients where it interferes with the accurate analyte determination, showing false positive readings.

HRP (Horseradish Peroxidase) - an enzyme that breaks down hydrogen peroxide to water – a peroxidase. Chromogenic substrates such as TMB serve as indicators of that enzyme activity.

Hook effect - caused by very high levels of antigen in the sample. As a result specific binding of the antigen by the antibody is insufficient to match analyte levels and signal is lower than expected. The best way to avoid this issue is to test several dilutions of each sample.

Immunoassay - any type of assay that uses antibodies to measure the concentration of an analyte in a sample.

Interference - effects on the immunoassay that interfere with the accurate measurement of the analyte (e.g. matrix effect, heterophilic interference).

Matrix effect - the effect compounds in the sample have on the measurement of the analyte.

pNPP (para-Nitrophenylphosphate) - colorimetric substrate for alkaline phosphatase, it precipitates as a yellow substance.

Protein stabilizers - reagents that promote maintenance of the native structure of proteins during adsorbtion to the assay surface.

Substrate - compound such as pNPP and TMB that is used to measure the analyte in an immunoassay.

TMB - a colorimetric substrate for horseradish peroxidase, turning blue upon completion of the enzymatic reaction.

Think Antibodies. Think Bio-Rad.
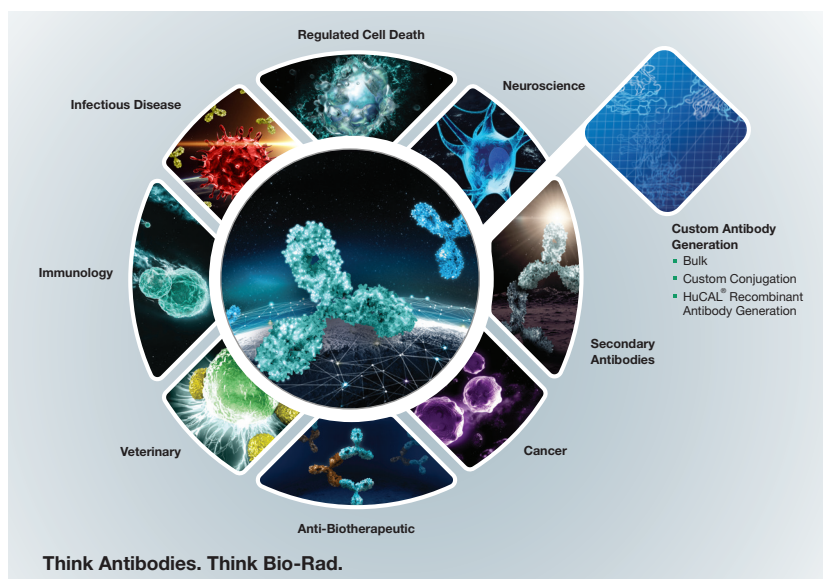
Sign Up to Receive Our Emailers

We are constantly expanding our range of antibodies and reagents, resources, and online tools to help you choose the right antibodies, design experiments with confidence so you can achieve more.

Don't miss out and sign up to receive our emailers with updates on:

- Free resources for your lab including application guides, posters, mini-reviews, protocols, and practical tips
- Online tools
- New product launches
- Webinar invitations
- Special offers
- And more…
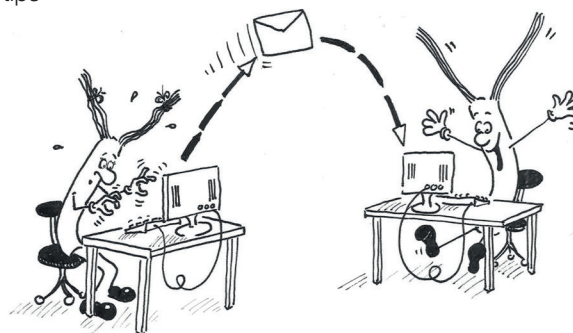
bio-rad-antibodies.com/signup

Visit bio-rad-antibodies.com for more information.

BIO-RAD

**Bio-Rad
Laboratories, Inc.**

*Life Science
Group*

Website bio-rad-antibodies.com