APPLICATION NOTE

Critical Factors in Immunoassay Optimization

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Since the mid-1970's, there has been an increasing demand for sensitive, relatively simple assays for use in basic research and clinical diagnosis. Over the same period, many practical considerations have led to the need to adapt existing assays and to develop novel ones. The assay format that most closely meets all of these criteria in many situations is the Enzyme-Linked Immunosorbent Assay (ELISA). Today, most ELISA's follow one of three strategies: Indirect ELISA, typically used to screen for antibodies; Sandwich (or Antigen Capture) ELISA, to assay the amount of target antigen which is present; or Competitive ELISA, to define antigenic specificity or to increase the specificity of an assay when samples contain cross-reacting species. Choosing the best format depends on the intended application of the assay; the type of samples to be analyzed; the availability of reagents; and whether the assay is intended for a single analysis in one lab, or is intended to be used in many laboratories by many different workers (1). Some guidelines for determining the best assay format for specific circumstances are found in Table 2. Common to all of these formats are several parameters that are critical to assay performance but which are often not fully considered during assay optimization, and which apply to membrane-based as well as microwell-based assays.

Antibodies

Table 1. Types of Immunoassay Solid Phases

Material[a]	Binding Capacity	Type of Interaction
Nitrocellulose	High	Hydrophobic, Hydrophilic
PVDF[b]	High	Hydrophobic
Nylon	High	Hydrophobic
plates and tubes		
Polystyrene	Low	Hydrophobic
Polyvinyl	Low	Hydrophobic
Derivatized microtiter plates	Low	Covalent, Hydrophobic, Hydrophilic
Beads		
Polystyrene	Moderate	Hydrophobic
Derivatized polystyrene	High	Covalent, Hydrophobic, Hydrophilic
Microparticles	High	Covalent and Hydrophobic

[a]Materials in each group are listed generally in order of increasing hydrophobicity.
[b]Polyvinylidene difluoride
[c]Beads are particles > 1 µm diameter, microparticles are < 1 µm diameter

The choice of antibodies is obviously of prime importance. A general rule of thumb is as follows. Monoclonal antibodies (MAb), because they recognize a single epitope, provide high specificity at the expense of sensitivity, since only one antibody molecule can bind to the antigen. Polyclonal antibodies (PAb) provide higher sensitivity due to the possibility of multiple antibodies binding to a single antigen molecule, but have a higher risk of cross-reactivity since the epitope is less precisely defined. When available, a MAb is often chosen as the primary antibody to establish the highest level of specificity in an assay,
and a PAb chosen as the secondary antibody, to amplify the signal via multiple binding events. There is no empirically correct choice. All candidate antibodies must be tested together with the intended sample type in order to optimize performance of the assay as a whole.

Sample

Sample type itself has a tremendous impact on assay performance and on the choice of assay components. Crude, complex mixtures (e.g., serum, cell or tissue extracts) are often the samples being assayed. These present problems of both sensitivity and specificity, since the analyte may be present at very low concentration, and may be similar to other molecules present, resulting in cross-reactivity. Processing samples to enrich the target molecule or to remove interfering molecules may be possible, but poses the risk that the sample may be compromised or that the processing method may reduce robustness and add complexity to the assay.

Solid Phase

The principal advantage of the ELISA lies in the ability of the user to carry out multiple assay steps in a single phase without the need to separate components from reaction products prior to the determination of the assay result. The assay is carried out on a solid-phase medium (a membrane, well, or bead) in which the reactants have been immobilized, covalently
or otherwise. See Table 1 for the types of immunoassay solid phases. Most commonly, the immobilized molecule is noncovalently adsorbed to the solid phase. Over the years, a number of materials have been used, each with properties which present advantages and disadvantages for specific purposes, and which must be considered. All of these materials are amphipathic (possessing both hydrophobic and hydrophilic properties),

allowing adsorption of a ligand to the surface while still permitting a degree of wetting in an aqueous environment. Some of these materials are described in Table 1. In general, the more hydrophobic the surface of the solid phase, the greater the binding capacity, but the more disruptive it is to the native structure of a macromolecular antigen (4).

It is important to remember that proteins are flexible molecules; adsorption to a nonfluid surface will likely result in changes to the conformation of the molecule, with potential loss of critical epitopes. This effect is greater when protein is adsorbed at low concentration. Adsorption at high concentration, however, increases the likelihood of aggregation and binding both to the solid phase and the protein already bound, again with possible loss or masking of critical epitopes. A number of manufacturers produce materials which are modified in order to increase binding capacity while decreasing damage to the native protein conformation. This is typically accomplished by placing polar or charged groups on the plastic, facilitating hydrophilic interactions. While this is effective, it often results in a need for more extensive blocking procedures in order to minimize background (4). Similarly, covalent attachment of the ligand to the solid phase may mask or remove surface functional group(s) required for recognition and is strongly dependent upon the conditions of the reaction. Detailed discussions of all these factors are presented in References 4 and 5.

Table 2. Guidelines for Choosing an Assay Format (From Reference 2)

	Type of Antibody Available	Type of Antigen Available	Assay Choices (in recommended order)
Detect/Quantify Antigen	Polyclonal Antibodies	Purified	Antigen capture (Ag competition) Antibody capture (Ag competition)
		Unpurified	Antibody capture (Ab excess) Others possible but only with an additional technique (immuno-precipitation, blotting, cell statiing
	Affinity Purified Polyclonal Antibodies	Purified	Sandwich ELISA Antigen capture (Ag competition) Antibody capture (Ag competition)
		Unpurified	Sandwich ELISA Antibody capture (Ab excess)
	One Monoclonal Antibody	Purified	Antigen capture (Ag competition) Antibody capture (Ag competition)
		Unpurified	Antibody capture (Ab excess)
	Two or more Monoclonal Antibodies	Purified	Sandwich ELISA Antigen capture (Ag competition) Antibody capture (Ag competition)
		Unpurified	Sandwich ELISA Antibody capture (Ab excess)
Detect/Quantify Antibody	Polyclonal Antibodies	Purified	Antibody capture (Ag excess)
		Unpurified	Additional technique required (immunoprecipitation, blotting, cell staining)
	Affinity Purified Polyclonal Antibodies	Purified	Antibody capture (Ag excess)
		Unpurified	Additional technique required (immunoprecipitation, blotting, cell staining)
	One Monoclonal Antibody	Purified	Antibody capture (Ag excess)
		Unpurified	Additional technique required (immunoprecipitation, blotting, cell staining)
	Two or more Monoclonal Antibodies	Purified	Antibody capture (Ag excess)
		Unpurified	Additional technique required (immunoprecipitation, blotting, cell staining)

Fluid Phase

Additional consideration must be given to the fluid phase in each step in the assay. It is extremely important to recognize that antibodies, like all other proteins, possess properties (hydrophobicity, pI) which are the net effect of their primary structure. Because antibodies are formed as the result of gene rearrangements, each antibody differs somewhat in its amino acid composition. This means that the physical properties of one antibody are not identical to those of another. A purified polyclonal antibody is actually a heterogeneous pool of molecules with similar functional activity. Conditions which are optimal for adsorption or for antigen binding of one antibody molecule may not be the same for another. Standard conditions may be a good starting point for optimization, but they should not be considered optimal in all circumstances. The best buffer composition, pH, and ionic strength must be determined for an individual assay, as these factors all influence the ultimate specificity and sensitivity of the assay (4).

Substrate

A final consideration in ELISA development is the choice of substrate. It is important to remember that in an ELISA, optimization does not end with plate coating and antibody binding. These events are essentially the means for the specific localization of an enzyme. Once the binding conditions are determined, an ELISA is essentially like any other enzyme assay. As such, it is necessary to identify the appropriate substrate, and to determine the concentration, temperature and incubation time which provides a linear response over the duration of the assay. When choosing a substrate, it is important to note that the sensitivity of the assay is not the same as that of the enzyme substrate. Substrate sensitivity refers to the signal intensity produced by a unit of enzyme activity. Assay sensitivity refers to the minimum detectable amount of antigen. Fortunately, the use of commercially available substrates prepared in appropriate buffers and at apppropriate concentrations permits the user to focus on optimizing the earlier stages of the assay, and limits substrate concerns to reaction temperature and time, since the other values are fixed. These parameters should be determined under three conditions: definitive negative controls, unequivocal positive controls, and weakly positive controls. This range of samples will enable determination of the sensitivity of the assay, its linear range at both high and low analyte concentration, and the expected level of non- specific signal (background). Possibly the most important lesson is that optimization of all of the assay parameters should be done in the same system in which they will be used: samples, components, substrates, and all other factors should be representative of the intended application, otherwise the data generated is not predictive of the intended use of the assay (4). ELISA optimization is complex and laborious. However, the outcome of appropriate assay optimization and validation is a robust, reproducible assay which delivers results in which one can have great confidence and which is simpler to troubleshoot.

ELISA Protocols

On the next page, there are general protocols for 3 common ELISA formats. In each case, the precise conditions should be optimized for a particular assay.

Solution Preparation

Coating Solution: Antigen or antibody are diluted in coating solution to immobilize them to the microplate. Commonly used coating solutions are: 50 mM carbonate, pH 9.6; 20 mM Tris-HCl, pH 8.5; and 10 mM PBS, pH 7.2. A protein concentration of 1-10 µg/ml is usually sufficient.

Blocking Solution: Commonly used blocking agents are: BSA, nonfat dry milk, casein, gelatin, etc. Different assay systems may require different blocking agents.

Primary/Secondary Antibody Solution: Primary/secondary antibody should be diluted in 1X blocking solution to help prevent non-specific binding. A concentration of 0.1-1.0 µg/ml is usually sufficient.

Antigen Solution (Capture ELISA ONLY): Sample antigen should be diluted in 1X blocking solution to help prevent non-specific binding. A concentration of 0.1-1.0 µg/ml is usually sufficient.

Wash Solution: Typically 0.1 M Phosphate-buffered saline or Tris-buffered saline (pH 7.4) with a detergent such as Tween 20 (0.02%-0.05% v/v).

All incubations should be performed at room temperature.

Direct ELISA	Indirect ELISA	Sandwich/Capture ELISA
Apply Antigen 1. Add 100 µl antigen diluted in coating solution to appropriate wells. 2. Incubate 1 hour. 3. Empty plate and tap out residual liquid. **Block Plate** 1. Add 300 µl blocking solution to each well. 2. Incubate 15 minutes, empty plate and tap out residual liquid. **Add Secondary Antibody Solution** 1. Add 100 µl secondary antibody solution to each well. 2. Incubate 1 hour. 3. Empty plate, tap out residual liquid. **Wash Plate** 1. Fill each well with wash solution. 2. Empty plate, tap out residual liquid. 3. Repeat 3 - 5 times. 4. Give final 5 minute soak with wash solution; tap out residual liquid. **React Substrate** 1. Dispense 100 µl substrate into each well. 2. If desired, after sufficient color development add 100 µl of the appropriate stop soluton to each well. 3. Read plate with plate reader. **Recommended filters:** **ABTS: 405-415 nm** **TMB-based substrates:** **unstopped 620-650 nm** **stopped 450 nm** ***p*NPP: 405-415 nm** **BluePhos: 595-650 nm** **FirePhos: 460-505 nm**	**Apply Antigen** 1. Add 100 µl antigen diluted in coating solution to appropriate wells. 2. Incubate 1 hour. 3. Empty plate and tap out residual liquid. **Block Plate** 1. Add 300 µl blocking solution to each well. 2. Incubate 15 minutes, empty plate and tap out residual liquid. **React Primary Antibody** 1. Add 100 µl secondary antibody solution to each well. 2. Incubate 1 hour. 3. Empty plate, tap out residual liquid. **Wash Plate** 1. Fill each well with wash solution. 2. Empty plate, tap out residual liquid. 3. Repeat 3 - 5 times. **Add Secondary Antibody Solution** 1. Add 100 µl diluted secondary antibody to each well. 2. Incubate 1 hour at room temperature. 3. Empty plate, tap out residual liquid and wash as above. 4. Give final 5 minute soak with wash solution; tap out residual liquid. **React Substrate** 1. Dispense 100 µl substrate into each well. 2. If desired, after sufficient color development add 100 ml of the appropriate stop soluton to each well. 3. Read plate with plate reader.	**Apply Capture Antibody** 1. Add 100 µl antigen diluted in coating solution to appropriate wells. 2. Incubate 1 hour. 3. Empty plate and tap out residual liquid. **Block Plate** 1. Add 300 ml blocking solution to each well. 2. Incubate 15 minutes, empty plate and tap out residual liquid. **React Sample Antigen** 1. Add 100 µl secondary antibody solution to each well. 2. Incubate 1 hour or overnight. 3. Empty plate, tap out residual liquid. **Wash Plate** 1. Fill each well with wash solution. 2. Empty plate, tap out residual liquid. 3. Repeat 3 - 5 times. **Add Secondary Antibody Solution** 1. Add 100 µl diluted secondary antibody to each well. 2. Incubate 1 hour at room temperature. 3. Empty plate, tap out residual liquid and wash as above. 4. Give final 5 minute soak with wash solution; tap out residual liquid. **React Substrate** 1. Dispense 100 µl substrate into each well. 2. If desired, after sufficient color development add 100 ml of the appropriate stop soluton to each well. 3. Read plate with plate reader.

1. Perlmann, H. and Perlmann, P. (1994). Enzyme-Linked Immunosorbent Assay. In: Cell Biology: A Laboratory Handbook. San Diego, CA, Academic Press, Inc., 322-328.
2. Crowther, J.R. (1995). Methods in Molecular Biology, Vol. 42 ELISA: Theory and Practice. Humana Press, Totowa, NJ.
3. Harlow, E. and Lane, D. (1988). Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 553-612.

Frequently Asked Questions

What is the difference between the 1 and 2 Component ABTS and TMB formulations?

Our studies have shown that the 1 and 2 Component formulations of both ABTS and TMB are equivalent in performance. Both substrates were originally developed as 2 component systems. After further research, we were able to stabilize them as 1 component substrates. Although the 1 component formulation is more convenient, many customers still prefer the 2 Component formulation for longer shelf life, so we continue to offer both.

How do the peroxidase microwell substrates (ABTS, TMB and OPD) compare in terms of sensitivity?

Based on an in-house comparison, TMB proved to be the most sensitive, OPD was less sensitive than TMB but more sensitive than ABTS, and ABTS was the least sensitive. SureBlue™ and SureBlue Reserve™ are TMB based with SureBlue Reserve the substrate of choice when highest sensitivity is desired. In many assays where the high sensitivity of TMB or OPD is not needed, ABTS works very well to give good results with low background.

What volume of substrate should be added to each well of an ELISA plate? What volume of stop solution?

The recommended volume of substrate is 100 µl/well. An equal volume of stop solution, also 100 µl/well, should be added.

Can a microwell substrate be diluted to reduce the intensity of the signal?

Dilution of the substrate is not recommended. The reagents have been optimized in terms of pH and buffer concentration. Dilution may change the sensitivity and stability of the substrate. To reduce the intensity of the reaction, dilution of the enzyme-labeled conjugate is recommended.

How do the phosphatase microwell substrates (*p*NPP and BluePhos® and FirePhos™) compare in terms of sensitivity?

Our studies have shown BluePhos® and FirePhos™to be more sensitive than *p*NPP. The difference in sensitivity among these substrates will vary depending upon the vendor of the pNPP and the conditions of the assay.

What are the contents of the Coating Solution Concentrate? How should it be used?

The Coating Solution Concentrate contains 0.1 M PBS. The solution should be diluted 1:10 with reagent quality water and then used to dilute antigen or coating antibody for binding to an ELISA plate.

What is the recommended dilution of an HRP conjugate in a microwell ELISA? An AP conjugate?

For both HRP and AP conjugates, usually a concentration in the range of 0.1-1.0 µg/ml is sufficient for use as a secondary antibody in ELISA.

What are some recommendations for reducing background in an ELISA?

A common cause of high background in ELISA is an overly concentrated conjugate. Often the conjugate concentration can be reduced significantly to lower the background and still maintain a strong positive signal. Others causes of background are insufficient blocking or washing steps. To decrease background, try increasing the amount of protein in the blocking solution, or block for a longer period of time. For effective washing, we recommend adding a detergent (such as Tween 20) to the wash solution and washing 3 times for 5 - 10 minutes.

How does the chemiluminescent substrate, LumiGLO, compare in terms of sensitivity to the chromogenic substrates in a microwell ELISA?

Our studies show that LumiGLO® Chemiluminescent Peroxidase Substrate is approximately 10 times more sensitive than TMB.

References

1. Crowther, J.R. (1995). Methods in Molecular Biology, Vol. 42, ELISA: Theory and Practice. Humana Press, Totowa, NJ.

2. Harlow, E. and Lane, D. (1988). Antibodies: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 553-612.

3. Perlmann, H. and Perlmann, P. (1994). Enzyme-Linked Immunosorbent Assay. In: Cell Biology: A Laboratory Handbook. San Diego, CA, Academic Press, Inc., 322-328.

4. Butler, J.E. (1991). Perspectives, configurations, and principles. In: Butler, J.E., ed., Immunochemistry of Solid-Phase Immunoassay. Boca Raton, FL, CRC Press, 3-26.

5. Butler, J.E., Joshi, K.S., and Brown, W.R. (1991). The application of traditional immunochemical methods to evaluate the performance of capture antibodies immobilized on microtiter wells. In: Butler, J.E., ed., Immunochemistry of Solid-Phase Immunoassay. Boca Raton, FL, CRC Press, 221-231.

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