



BenchMarks™

Critical Factors in Immunoassay Optimization

James W. Karaszkiwicz, Ph.D., Manager, Technical Service and Applications Development

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 without the use of radioisotope tracers, which have been commonly used in assay development for decades. The assay format that most closely meets all of these criteria in many situations is the Enzyme-Linked Immunosorbent Assay (ELISA). Today, most ELISAs 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

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

Table of Contents
Critical Factors in Immunoassay
Optimization 1
ELISA Protocols 3
KPL Products in the Literature 4
Frequently Asked Questions 5

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. Most commonly, the immobilized molecule is noncovalently adsorbed to the solid phase. Over the years, a number of materials have been used, each with

Table 1. Types of Immunoassay Solid Phases (from Reference 4)

Material ^a	Binding Capacity	Type of Interaction
Nitrocellulose	High	Hydrophobic, Hydrophilic
PVDF ^b	High	Hydrophobic
Nylon	High	Hydrophobic
<i>Plates and Tubes</i>		
Polystyrene	Low	Hydrophobic
Polyvinyl	Low	Hydrophobic
Derivatized microtiter plates	Low	Covalent, Hydrophobic, Hydrophilic
<i>Beads^c</i>		
Polystyrene	Moderate	Hydrophobic
Derivatized polystyrene	High	Covalent, Hydrophobic, Hydrophilic
Microparticles ^c	High	Covalent and hydrophobic

^aMaterials in each group are listed generally in order of increasing hydrophobicity.

^bPolyvinylidene difluoride

^cBeads are particles > 1 µm diameter, microparticles are < 1 µm diameter

Continued on page 2

Continued from page 1

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.

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 appropriate 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-

Continued on page 4

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

	Type of Antibody Available	Type of Antigen Available	Assay Choices (in recommended order)
Detect/Quantify Antigen	Polyclonal Antibodies	Purified	<ul style="list-style-type: none"> Antigen capture (Ag competition) Antibody capture (Ag competition)
		Unpurified	<ul style="list-style-type: none"> Antibody capture (Ab excess) Others possible but only with an additional technique (immunoprecipitation, blotting, cell staining)
	Affinity Purified Polyclonal Antibodies	Purified	<ul style="list-style-type: none"> Sandwich ELISA Antigen capture (Ag competition) Antibody capture (Ag competition)
		Unpurified	<ul style="list-style-type: none"> Sandwich ELISA Antibody capture (Ab excess)
	One Monoclonal Antibody	Purified	<ul style="list-style-type: none"> Antigen capture (Ag competition) Antibody capture (Ag competition)
		Unpurified	<ul style="list-style-type: none"> Antibody capture (Ab excess)
Detect/Quantify Antibody	2 or more Monoclonal Antibodies	Purified	<ul style="list-style-type: none"> Sandwich ELISA Antigen capture (Ag competition) Antibody capture (Ag competition)
		Unpurified	<ul style="list-style-type: none"> Sandwich ELISA Antibody capture (Ab excess)
	Polyclonal Antibodies	Purified	<ul style="list-style-type: none"> Antibody capture (Ag excess)
		Unpurified	<ul style="list-style-type: none"> Additional technique required (immunoprecipitation, blotting, cell staining)
Detect/Quantify Antibody	Affinity Purified Polyclonal Antibodies	Purified	<ul style="list-style-type: none"> Antibody capture (Ag excess)
		Unpurified	<ul style="list-style-type: none"> Additional technique required (immunoprecipitation, blotting, cell staining)
	One Monoclonal Antibody	Purified	<ul style="list-style-type: none"> Antibody capture (Ag excess)
		Unpurified	<ul style="list-style-type: none"> Additional technique required (immunoprecipitation, blotting, cell staining)
Two or more Monoclonal Antibodies	Purified	<ul style="list-style-type: none"> Antibody capture (Ag excess) 	
	Unpurified	<ul style="list-style-type: none"> Additional technique required (immunoprecipitation, blotting, cell staining) 	

ELISA Protocols

The following 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 antigens 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% v/v).

All incubations should be performed at room temperature.

DIRECT 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 to 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 solution to each well.
3. Read plate with plate reader.

Recommended filters:

ABTS: 405-415 nm

TMB: unstopped 620-650 nm
stopped 450 nm

pNPP: 405-415 nm

BluePhos: 595-650 nm

INDIRECT 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.

React Primary Antibody

1. Add 100 µl diluted primary antibody 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 to 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 µl of the appropriate stop solution to each well.
3. Read plate with plate reader.

SANDWICH/CAPTURE ELISA

Apply Capture Antibody

1. Add 100 µl capture antibody 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 Sample Antigen

1. Add 100 µl diluted antigen to each well.
2. Incubate 1 hour to 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 to 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 residual liquid from plate.

React Substrate

1. Dispense 100 µl substrate into each well.
2. If desired, after sufficient color development, add 100 µl of the appropriate stop solution 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.

Continued from page 2.

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.

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., Immunochimistry 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., Immunochimistry of Solid-Phase Immunoassay*. Boca Raton, FL, CRC Press, 221-231.

KPL Products In the Literature

- Laurence, J. *et al.* (1996) Apoptotic Depletion of CD4+ T Cells in Idiopathic CD4+ T Lymphocytopenia. *J. Clin. Invest.* 97 (3): 672-680.
- Lilly, C. *et al.* (1997) Expression of Eotaxin by Human Lung Epithelial Cells: Induction by Cytokines and Inhibition by Glucocorticoids. *J. Clin. Invest.* 99 (7): 1767-1773.
- Pon, R. *et al.* (1997) N-Propionylated Group B Meningococcal Polysaccharide Mimics a Unique Bactericidal Capsular Epitope in Group B *Neisseria meningitidis*. *J. Exp. Med.* 185 (11): 1929-1938.
- Budhai, L.; Oh, K., Davidson, A. (1996) An In Vitro Assay for Detection of Glomerular Binding IgG Autoantibodies in Patients with Systemic Lupus Erythematosus. *J. Clin. Invest.* 98 (7): 1585-1593.
- Rincon, M. *et al.* (1997) Interleukin (IL)-6 Directs the Differentiation of IL-4-producing CD4+ T Cells. *J. Exp. Med.* 185 (3): 461-469.
- Hogan, S. *et al.* (1997) Aeroallergen-induced Eosinophilic Inflammation, Lung Damage, and Airways Hyperactivity in Mice Can Occur Independently of IL-4 and Allergen-specific Immunoglobulins. *J. Clin. Invest.* 99 (6): 1329-1339.
- Kay, M. *et al.* (1997) Transient Immunomodulation With Anti-CD40 Ligand Antibody and CTLA4Ig Enhances Persistence and Secondary Adenovirus-Mediated Gene Transfer Into Mouse Liver. *Proc. Natl. Acad. Sci.* 94: 4686-4691.
- Uyemura, K. *et al.* (1996) Cross-regulatory Roles of Interleukin (IL)-12 and IL-10 in Atherosclerosis. *J. Clin. Invest.* 97 (9): 2130-2138.
- Hamad, A. R.; Marrack, P.; Kappler, J. (1997) Transcytosis of Staphylococcal Superantigen Toxins. *J. Exp. Med.* 185 (8): 1447-1454.
- Liang, H. *et al.* (1996) Activation of Human B Cells by Phosphorothioate Oligodeoxynucleotides. *J. Clin. Invest.* 98 (5): 1119-1129.
- Nanki, T. *et al.* (1996) Genetic Control of T Cell Receptor β Gene Expression in Peripheral Lymphocytes of Normal and Rheumatoid Arthritis Monozygotic Twins. *J. Clin. Invest.* 98 (7): 1594-1601.
- Rassenti, L. and Kipps, T. (1997) Lack of Allelic Exclusion in B Cell Chronic Lymphocytic Leukemia. *J. Exp. Med.* 185 (8): 1435-1445.
- Roben, P. *et al.* (1996) Repertoire Cloning of Lupus Anti-DNA Autoantibodies. *J. Clin. Invest.* 98 (12): 2827-2837.
- Schutzer, S. *et al.* (1997) Simultaneous Expression of *Borrelia* OspA and OspC and IgM Response in Cerebrospinal Fluid in Early Neurologic Lyme Disease. *J. Clin. Invest.* 99(9): 2192-2202
- Tilton, R. *et al.* (1997) Vascular Dysfunction Induced by Elevated Glucose Levels in Rats is Mediated by Vascular Endothelial Growth Factor. *J. Clin. Invest.* 99 (9): 2192-2202.

If you have published a paper using KPL products, send us a reprint and we will send you a free KPL T-shirt.

Send reprints to the attention of KPL Technical Services.

KPL, Inc. • www.kpl.com • phone: 800-638-3167 or 301-948-7755 • fax: 301-948-0169



NO POSTAGE
NECESSARY
IF MAILED
IN THE
UNITED STATES

BUSINESS REPLY MAIL

FIRST CLASS MAIL PERMIT NO. 35 GAITHERSBURG, MD

Postage will be paid by addressee.

Marketing Department
Kirkegaard & Perry Laboratories, Inc.
2 Cessna Court
Gaithersburg, MD 20879-9963



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. 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 μ l/well. An equal volume of stop solution, also 100 μ l/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 (pNPP and BluePhos[®]) compare in terms of sensitivity?

Our studies have shown BluePhos to be more sensitive than pNPP. The difference in sensitivity between the two 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 μ g/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. Other 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.

Visit our website at www.KPL.com.

KPL, Inc. • www.kpl.com • phone: 800-638-3167 or 301-948-7755 • fax: 301-948-0169

Product / Applications

General	Microwell ELISA	Immunoblotting	Immunohistochemistry
Nucleic Acid Detection	In situ Hybridization	Anti-Bacteria Antibodies	Flow Cytometry & Immunofluorescence

Primary Field of Interest

Biochemistry	Microbiology	Pathology	Immunohistochemistry
Cell Biology	Immunology	Molecular Biology	

Please send me the current catalog.

I am developing diagnostic products and need custom and bulk reagents and services.

NAME _____ TITLE _____ PHONE _____

INSTITUTION _____ DEPARTMENT _____

ADDRESS _____ BUILDING _____ ROOM# _____

CITY / STATE / ZIP _____

KPL does NOT distribute or exchange mail lists.