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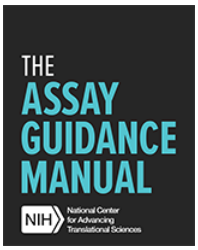

Immunoassay Methods

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Abstract

Immunoassays are used to quantify molecules of biological interest based on the specificity and selectivity of antibody reagents generated. In HTS and lead optimization projects, assays are designed to detect molecules that are produced intracellularly or secreted in response to compounds screened. This chapter describes the basics of designing and implementing robust, automation friendly immunoassays for HTS, modes of immunoassay formats (competitive and sandwich), instrumentation, reagent selection, experimental design and detailed data analysis concepts. The importance of an appropriate curve-fitting model for calibration curves used for quantification is also addressed in detail. This is an excellent primer for beginners as well as for experienced investigators.

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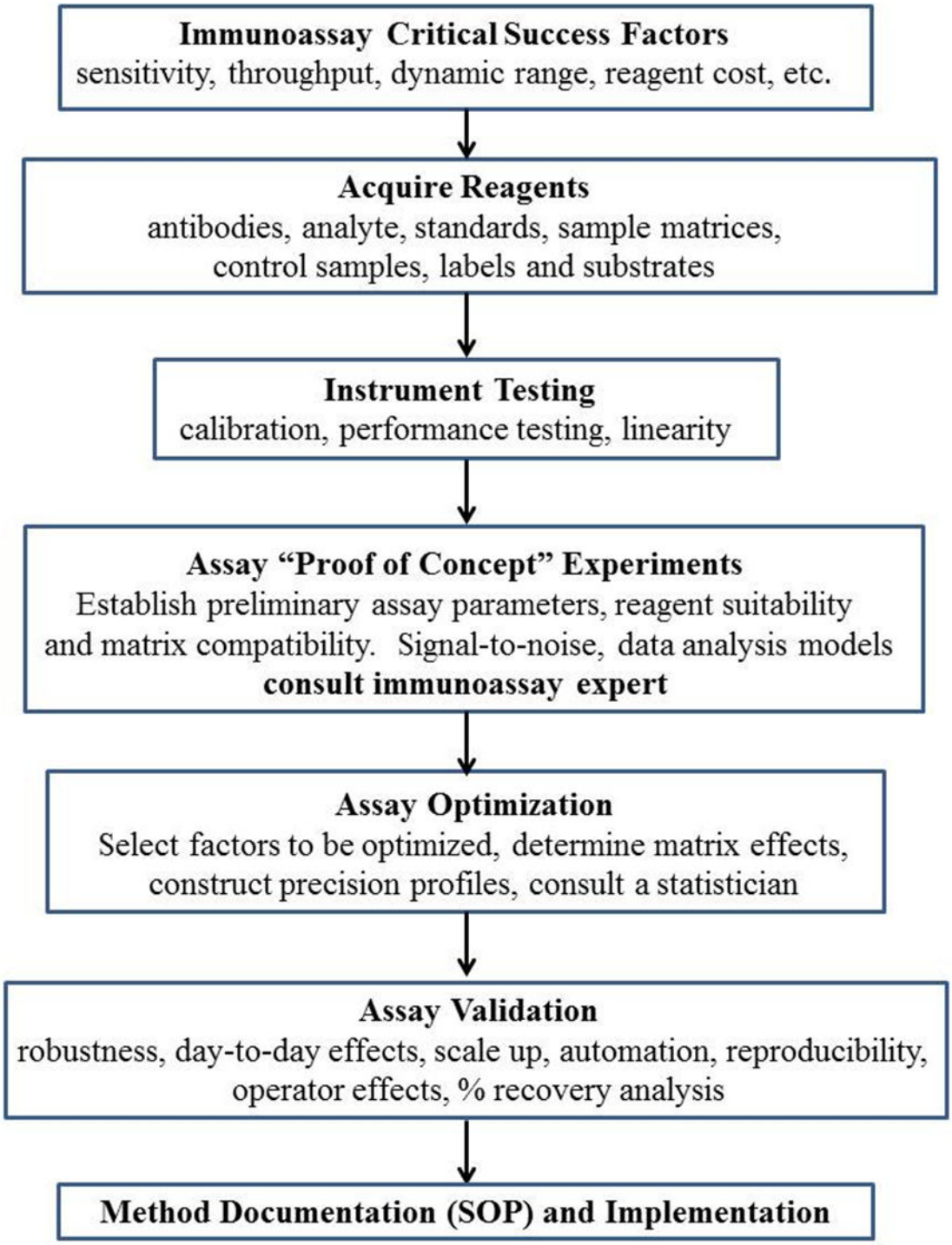
Immunoassay Development, Optimization and Validation Flow Chart



Introduction

The intent of this document is to provide general guidelines to aid in the development, optimization and validation of an immunoassay. Following these guidelines will increase the likelihood of success in developing a robust immunoassay that will measure consistent values for unknown samples.

Immunoassays are used when an unknown concentration of an analyte within a sample needs to be quantified. To obtain the most accurate determination of the unknown concentration, an immunoassay must be developed based not only on the usual assay development criteria (standard deviation or optimal signal window) but also on how well the immunoassay can predict the value of an unknown sample. First, one needs to establish the assay critical success factors. Then the immunoassay needs to be developed, which establishes proof of concept. During the optimization phase, the quantifiable range of the immunoassay method is determined by calculating a precision profile in the matrix in which the experimental samples will be measured. A spiked recovery is then performed by spiking the analyte into the matrix and determining the percent recovery of the analyte in the matrix. If the precision profile is within the desired working range, then assaying spiked recovery samples over several days completes the validation of the immunoassay. If the precision profile limits are not within the desired working range, further optimization of the immunoassay is required prior to validation.

Basic Steps for Developing and Running an Immunoassay

1. Establish assay critical success factors (i.e. sensitivity required).
2. Ensure appropriate antibody and antigen reagents are available.
3. Adsorb antigen or capture antibody to a solid surface.
4. Wash off unbound reagents.
5. Block nonspecific binding sites to reduce background.
6. Incubate the secondary antibody with the sample.
7. Wash off unbound reagents.
8. Incubate secondary antibody-conjugate with sample.
9. Wash off unbound reagents.
10. Incubate substrate to generate signal.
11. Calibration curve fitting, data analysis and quantitation by non-linear regression.

Basic Steps in Using Immunoassays for High Throughput Screening (HTS)

Immunoassays are used in screening to quantify the production or inhibition of antigens/haptens related to a disease target. These antigens or haptens are characteristic of the disease process and mediated by the target, such as cytokines or growth factors. Hence the screening procedure will involve incubating compounds with the specified target, usually expressed in cells, and collecting the cell medium or lysates to quantify the activity of the compounds. Several examples of this approach for using immunoassay procedures have been described in the literature (1-5). The critical steps in setting up a screen are as follows:

1. Develop a validated immunoassay as described above.
2. Acquire antibody, antigen/calibrator, label and buffer reagents in quantities needed for HTS.
3. Establish liquid handling and automation procedures for screening and immunoassay methods.
4. Establish stability of the capture antibody or antigen bound to a plate. Determine compound collections to be tested.
5. Develop and validate a method for incubation of compounds with a relevant target in the screening mode.
6. Develop a sample collection procedure from screening experiments.
7. Develop data analysis procedures to use immunoassay data to derive compound potency such as IC_{50} or EC_{50}.

Immunoassay Parameters

It is important to define the relevant immunoassay parameters before one begins the development, optimization and validation of an immunoassay:

1. Analyte (hapten or antigen) to be measured.
2. Sample matrices in which measurements will be made (serum, plasma, cell lysates, culture media, etc.).
3. Source of antibody, analyte standards and detection reagents (labeled antibody, enzyme substrates, etc.). Availability of these reagents is a critical requirement.
4. Detection mode (colorimetric, fluorescence or chemiluminescence) and appropriate plate readers.
5. Type of immunoassay to develop: Sandwich, competitive or antigen-down formats.
6. Expected analyte concentration ranges to be measured: pg/ml, ng/ml or µg/ml in the sample matrix of choice. This would determine the detection limits and the measurable range that should be achieved in a validated assay.
7. Data analysis models and format for reporting results.
8. Validation and optimization criteria using statistical experimental design tools.
9. Recovery, accuracy and precision expected at the limits of quantification and the measurable range.
10. Sample throughput, frequency of use, automation and the number of laboratories that would run the assay.
11. Control samples that would be used for optimization, validation and quality control runs.

Reagents

Reagents are a critical piece of any assay development process. This refers to all of the reagents that will be used in the assay. There are certain items that need to be considered when obtaining reagents:

1. Quality of standards and antibodies.
2. Quantity of standards and antibodies.
3. Purity of standards and antibodies (when possible antibodies are affinity purified).
4. Selectivity and specificity of antibodies.

Example Plate Types

Greiner high binding plates, Costar EIA/RIA high-low binding plates, Immunotech, Falcon, Nunc

Note: Other plate types can also be used based on the experience of the investigator and appropriate quality control to demonstrate acceptability.

Coating Buffers

50 mM sodium bicarbonate, pH 9.6

0.2 M sodium bicarbonate, pH 9.4

PBS - 50 mM Phosphate, pH 8.0, 0.15 M NaCl

Carbonate-bicarbonate

Phosphate Buffer: 1.7 mM NaH_2PO_4, 98 mM $Na_2HPO_4 \cdot 7H_2O$, 0.1% NaN_3, pH 8.5

TBS - 50 mM TRIS, pH 8.0, 0.15 M NaCl

Blocking Buffers

1% BSA or 10% host serum in TBS, or TBS with 0.05% Tween-20

Phosphate Buffer: 73 mM Sucrose, 1.7 mM NaH_2PO_4, 98 mM $Na_2HPO_4 \cdot 7H_2O$, 0.1% NaN_3, pH 8.5

1% HSA in PBS

Casein Buffer: Pierce Blocker cat# 37528

Protein Free Block: Pierce cat# 37573

Pierce has many blocking buffers that are available in their catalog.

Heterophilic Blocking Reagent (HBR): Scantibodies Laboratory, Inc., cat# 3KC533

Scantibodies has many other blocking reagents that are available in their catalog.

Wash Buffers

PBST, 0.05% Tween-20

TBST, 0.05% Tween-20

Antibody Diluents Buffers

1% BSA or 10% host serum in TBS, or TBS with 0.05% Tween-20

1% BSA or 10% host serum in PBS, or PBS with 0.05% Tween-20

50 mM HEPES, 0.1 M NaCl, 1% BSA, pH 7.4

Blocking buffer

Matrix Diluent

1. Serum or plasma from the sample species (this might contain the analyte to be measured which will interfere with the assay)
2. Serum or plasma from a species different from the sample (the analyte, if present, might not cross react with the antibody)
3. 0.1 M HEPES, 0.1 M NaCl, 1% BSA, 0.1% Tween-20
4. Tissue culture medium for samples
5. Cell lysates (these might contain SDS or other denaturing reagents that might interfere with the assay)

Enzymes and Substrates

1. Horseradish peroxidase (HRP) substrates:
    a. TMB: 3, 3', 5,5'-tetramethyl benzidine (colorimetric)
    b. OPD: o-phenylene diamine (colorimetric)
    c. ABTS: 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (colorimetric)
    d. Pierce Supersignal (chemiluminescent)
    e. Pierce QuantaBlu (chemifluorescent)
    f. Pierce QuantaRed (chemifluorescent)
    g. Pierce has other substrates that provide strong signal and sensitivity with HRP enzyme conjugates that are available in their catalog.
2. Alkaline phosphatase substrate:

a pNpp (p-Nitrophenyl Phosphate)

Stop Solutions

1. HRP/TMB: 2M H_2SO_4 solution (at a 1:1 volume with the HRP/TMB substrate/enzyme solution)
2. OPD: 3M H_2SO_4 solution, (at a 1:1 volume with the OPD substrate/enzyme solution)
3. ABTS: 1% SDS

Absorbance Readout

1. HRP TMB: 450 nm
2. OPD: 490 nm
3. ABTS: 405 nm

Fluorescent Readout (Emax/Amax)

1. QuantaBlu 420/325
2. QuantaRed 585/570
3. FITC 518/494

Luminescent Readout

1 An immunoassay technique where the antibody or the antigen is labeled with a molecule capable of emitting light during a chemical reaction. For detection, a luminescent plate reader is required (available from PerkinElmer).

Specific Antibodies

1. Sandwich Immunoassay: matched pair of antibodies, one for analyte capture on a solid surface and one for detection that binds to the antigen/hapten/analyte. Antibodies need to be affinity purified for optimal results.
2. Competitive Immunoassay: a single antibody specific for the hapten/analyte. For optimal results affinity purified reagents are preferred.

Standards or Antigen (Analyte)

1. The analyte to be measured is typically a recombinant form of the natural analyte or peptide.
2. Enough standard should be obtained for use in the development phase, validation phase and the continued support of the method to avoid changing lots and/or running out of standard.
3. Standard quality: Can vary from vendor to vendor and from lot to lot from a vendor.
4. Standard stability: Information on the stability of a standard can be obtained from the vendor and their recommendations should be followed in storing the standards.

Control Samples

1. Control samples are real samples where the antigen analyte level has been determined by another validated method. Samples are aliquoted, frozen and used as control samples in each experiment to track assay performance.
2. Spiked controls are created by adding a known concentration of the standard analyte into the matrix (for example: tissue culture, serum, plasma, or cell lysates). Spiked controls can be used to determine assay performance based on calculating the percent recovery.

Instrumentation

Instrument Linearity and Performance

The instrument used to read the output of the immunoassay should be tested initially for both linearity and performance. Instrument performance should be regularly calibrated according to manufacturer's specifications. The majority of plate readers employ UV-Vis Absorbance, fluorescence or chemiluminescence signals as the measured response, because the products of enzyme labels are chromophores, fluorophores or emit luminescent signals. Linearity in response to the specific enzyme product of an enzyme-linked immunoassay (ELISA) should be checked at the appropriate wavelengths and instrument settings.

Spectrophotometric/Colorimetric Plate Readers

Lamp sources and Photomultiplier Tubes (PMT) vary in quality and performance in many plate readers. The linear range of many plate readers is generally between 0-3 Absorbance Units (AU), but other instruments have a linear range up to 4.0 AU. A malfunctioning lamp source or photomultiplier tube can significantly affect the linear response range.

Fluorescence Plate Readers

These readers employ excitation and emission filter sets in addition to excitation lamp sources and PMTs. In addition to the lamps and PMTs, the filter sets also vary in quality, light throughput and bandwidth. Fluorescence signals are generally in Relative Fluorescence Units (RFU) and linearity should be verified with appropriate filter sets for the fluorophore employed according to instrument specifications.

Chemiluminescence Readers

These instruments have sensitive photomultipliers to detect light emitted from a chemical reaction. No Lamp sources are necessary. These readers usually have a much larger dynamic range, thus allowing for the increase in sensitivity. Signals or responses are measured in Relative Light Units (RLU) and can be significantly different depending on the instrument design.

Immunoassay Formats

An ELISA is one of several methods used in the laboratory to detect and quantify specific molecules. ELISAs rely on the inherent ability of an antibody to bind to the specific structure of a molecule. In order to optimize an ELISA and obtain the sensitivity and dynamic range required for the particular assay being developed, all the various components of the assay must be evaluated. The components will vary depending on the immunoassay format selected. The following is a description of the various types of ELISA formats as well as reagents that need to be optimized in order to obtain a robust assay.

Types of ELISA Formats

Three frequently used types of ELISA are: sandwich assays, competitive assays and antigen down assays. The format selected depends on the reagents that are available and the dynamic range required for the particular assay. Sandwich assays tend to be more sensitive and robust and therefore tend to be the most commonly used.

Sandwich Immunoassay (ELISA)

A sandwich immunoassay is a method using two antibodies, which bind to different sites on the antigen or ligand (Figure 1). The capture antibody, which is highly specific for the antigen, is attached to a solid surface. The antigen is then added, followed by addition of a second antibody referred to as the detection antibody. The

detection antibody binds the antigen at a different epitope than the capture antibody. As a result, the antigen is 'sandwiched' between the two antibodies. The antibody binding affinity for the antigen is usually the main determinant of immunoassay sensitivity. As the antigen concentration increases, the amount of detection antibody increases, leading to a higher measured response. The standard curve of a sandwich-binding assay has a positive slope. To quantify the extent of binding, different reporters can be used. These reporters (i.e. enzyme, fluorophore, or biotin) can be directly attached to the detection antibody or to a secondary antibody which binds the detection antibody (i.e. goat, anti-mouse IgG – HRP). In this latter case, the capture antibody and the detection antibody must be from different species (i.e. if the capture antibody is a rabbit antibody, the detection antibody would be from goat, chicken, etc., but not rabbit). If the detection antibody is directly labeled, then the capture and detection antibodies can be from the same species. Polyclonal antibodies often contain multiple epitopes and the same affinity purified polyclonal can be used as the capture and labeled detection antibody. The substrate for the enzyme is added to the reaction that forms a colorimetric readout as the detection signal. The signal generated is proportional to the amount of target antigen present in the sample.

The antibody linked reporter used to measure the binding event determines the detection mode. For an ELISA, where the detection is colorimetric, a spectrophotometric plate reader is used. Several types of reporters have been developed in order to increase sensitivity in an immunoassay. For example, chemiluminescent substrates have been developed which further amplify the signal and can be read on a luminescent plate reader. Also, a fluorescent readout where the enzyme step of the assay is replaced with a fluorophore tagged antibody is becoming quite popular. This readout is then measured using a fluorescent plate reader. When the detection antibody is labeled with biotin, you have the flexibility to use a number of different types of streptavidin conjugated reporters.

Competitive Binding Assay

A competitive binding assay is based upon the competition of labeled and unlabeled ligand for a limited number of antibody binding sites (Figure 2). Only one antibody is used in a competitive binding ELISA. Competitive binding assays are often used to measure small analytes. These assays are also used when a matched pair of antibodies to the analyte does not exist. A fixed amount of labeled ligand (tracer) and a variable amount of unlabeled ligand are incubated with the antibody. According to the law of mass action, the amount of bound labeled ligand is a function of the total concentration of labeled and unlabeled ligand. As the concentration of unlabeled ligand is increased, less labeled ligand can bind to the antibody and the measured response decreases. Thus the lower the signal, the more unlabeled analyte there is in the sample. The standard curve of a competitive binding assay has a negative slope. Alternatively, the antigen can be coated on the plate with the antibody and the sample in solution. Fewer antibodies will be available to bind the coated antigen as the amount of antigen in the sample increases. The antibody and labeled antigen concentrations are the important parameters that need to be optimized.

Antigen-Down Immunoassay or Immunometric Assay

An antigen-down immunoassay or immunometric assay involves binding the antigen to a solid surface instead of an antibody (Figure 3). This is done by coating the solid surface with the antigen, allowing for passive absorbance to the solid surface. Antigen-down immunoassays are used to bind antibodies found in a sample or in a competitive ELISA format (discussed above). When the sample is added (such as human serum), the antibodies (IgE for example) from the sample bind to the antigen coated on the plate. A species-specific antibody (anti-human IgE for example) labeled with HRP is added next. The signal is directly proportional to the amount of antibody present in the sample; the more antibodies there are in the sample, the higher the signal.

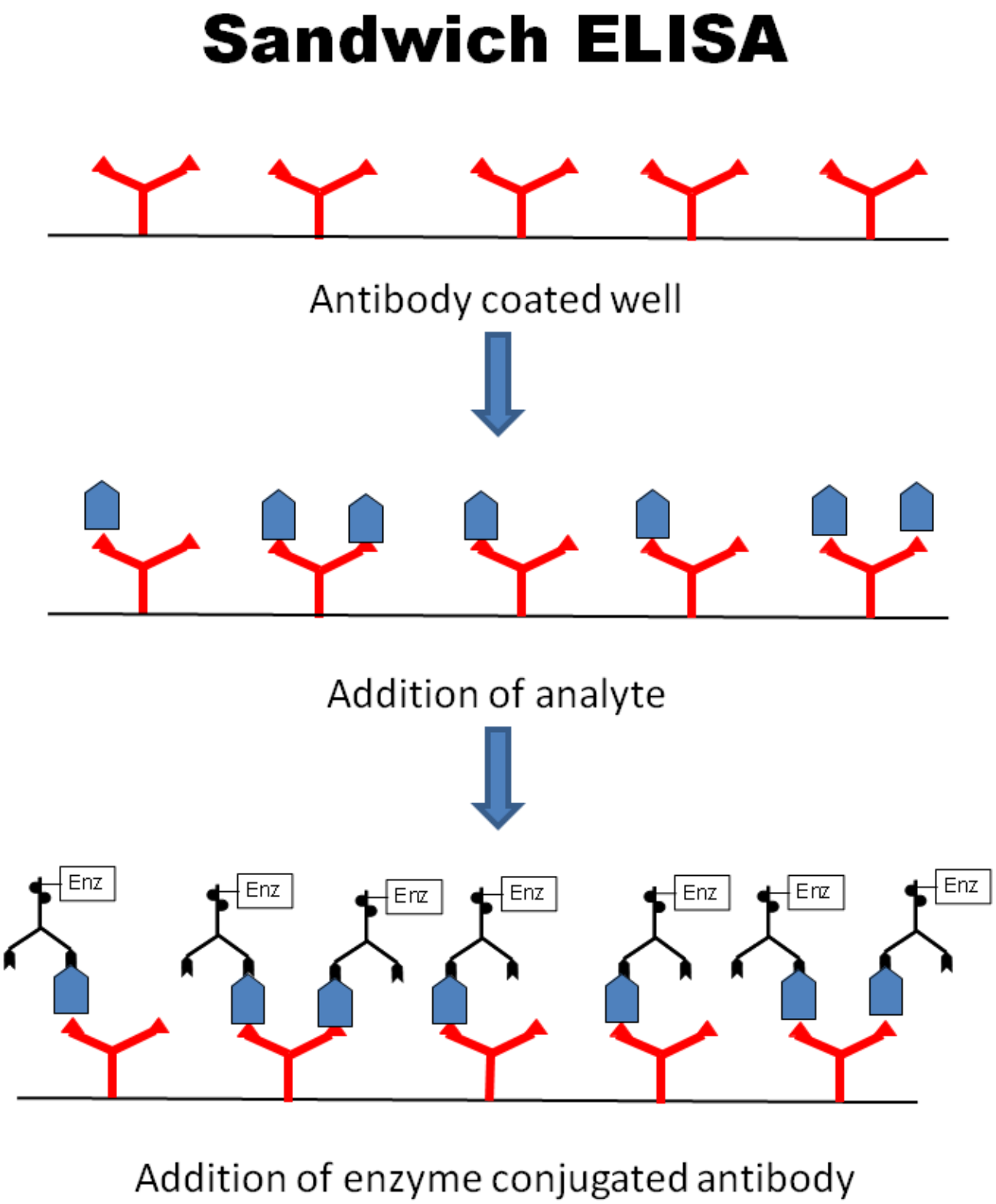


Figure 1: Diagram of a sandwich ELISA. The addition of the enzyme's substrate leads to color development. The amount of color (absorbance) is directly proportional to the analyte concentration.

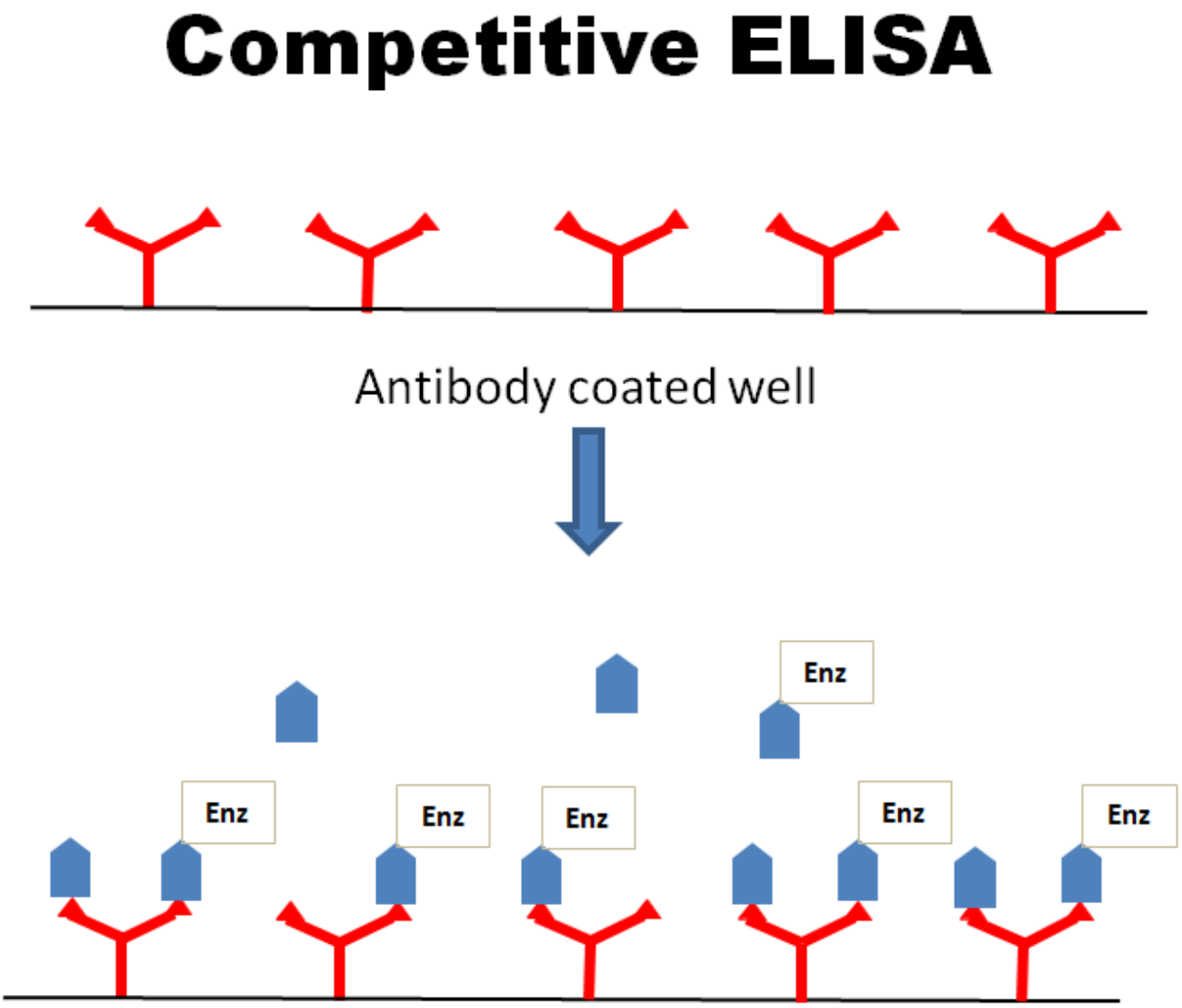


Figure 2: Diagram of a competitive binding assay. After addition of both the analyte and the enzyme-conjugated analyte, competition occurs between the two for binding to the antibody. The addition of the enzyme's substrate leads to color development. The amount of color (absorbance) is indirectly proportional to the analyte concentration

Single Antibody Two Plate ELISA Format

A single antibody ELISA format is considered when there is only one antibody available that recognizes the analyte (Figure 4). The assay is configured using the same antibody as both capture and detection. The antibody is biotinylated for use as the detection antibody. The method also utilizes two plates. The basic concept is to capture the antibody to an ELISA plate and allow the analyte of interest to bind to the capture antibody. Unbound material is removed by washing the plate and then adding an acid solution to elute the analyte from the capture antibody. The eluted analyte is then transferred to another ELISA plate containing the neutralization solution. The eluted analyte is then allowed to bind to the second ELISA plate. The unbound material is removed and the plate is blocked followed by a wash step. The detection antibody, which is biotinylated, is then added to the plate, followed by an incubation period. Another wash step is performed to remove excess detection antibody, followed by addition of a streptavidin reporter. The last wash step is performed to remove the excess

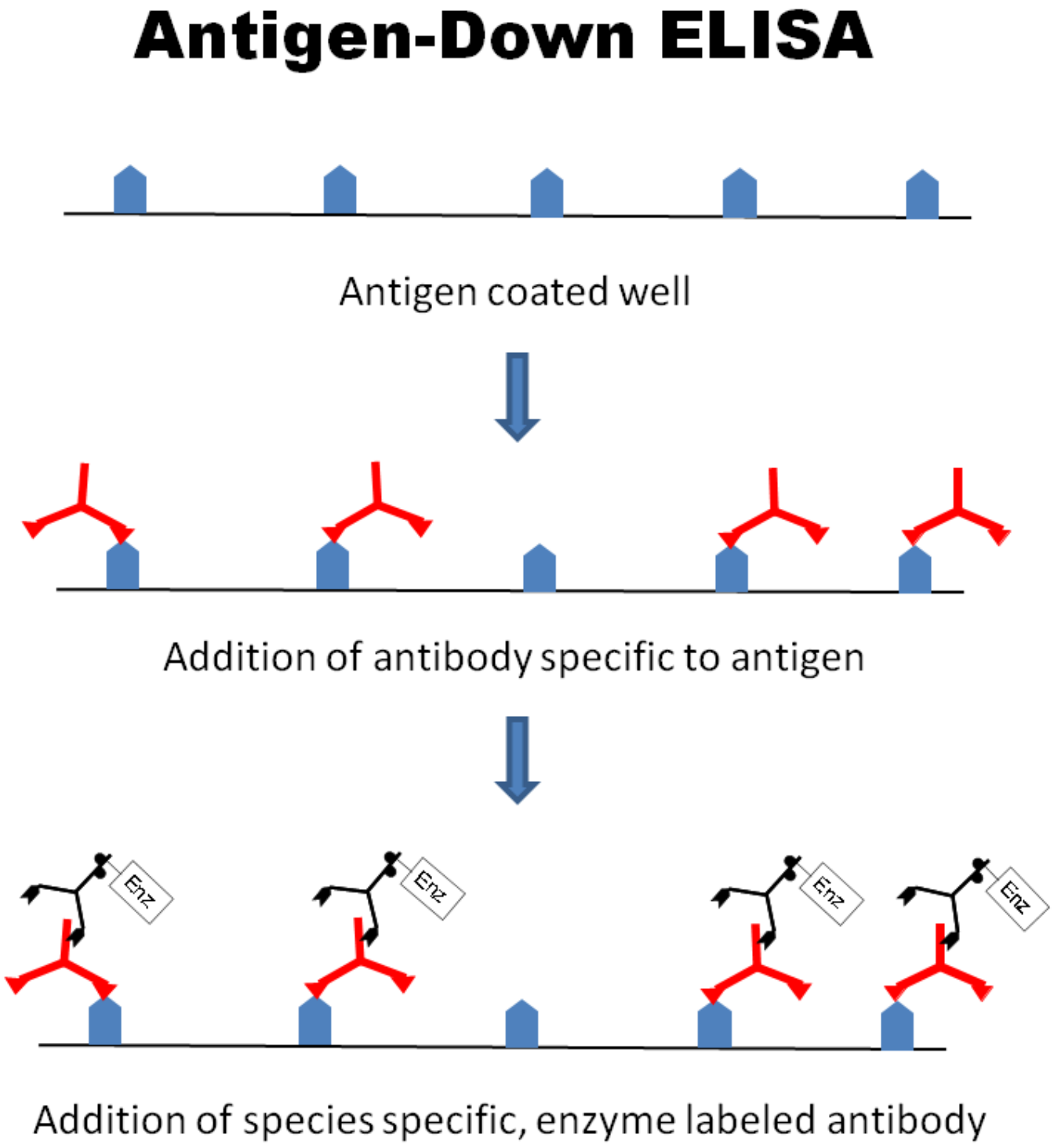


Figure 3: Diagram of an antigen-down ELISA. The addition of the enzyme's substrate leads to color development. The amount of color (absorbance) is directly proportional to the antigen specific antibody concentration.

reporter, followed by addition of the substrate. Below is a generic protocol that can be used to set up a Single Antibody Two Plate ELISA Method.

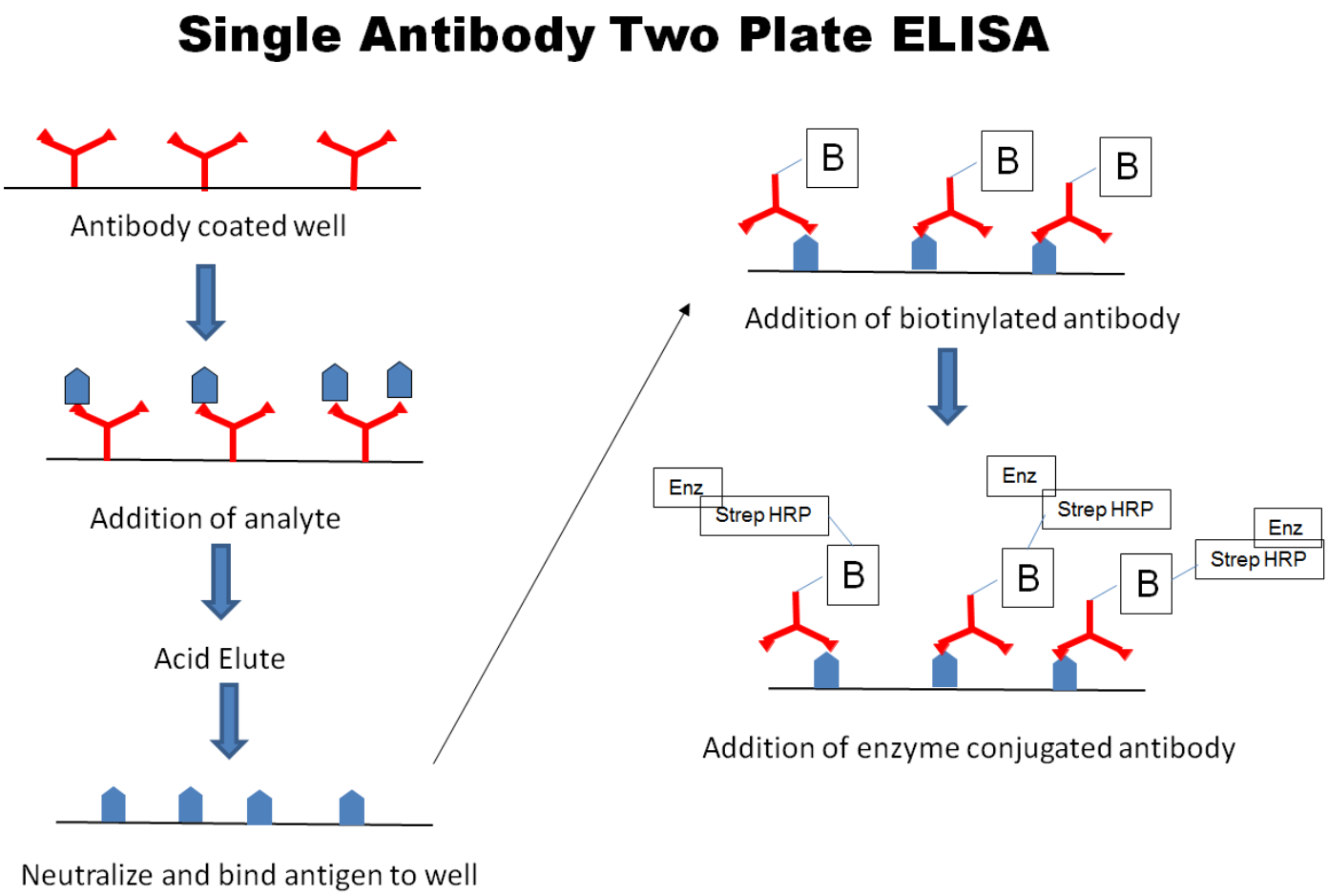


Figure 4: Diagram of a Single Antibody Two Plate ELISA. The addition of the enzyme's substrate leads to color development. The amount of color (absorbance) is directly proportional to the analyte concentration.

Reagents and basic concepts

Included in the list below are the plate type and buffers that are a good starting point for single antibody ELISA assays.

1. One antibody that recognizes the analyte.
2. The optimal capture and detection antibody concentrations need to be determined experimentally.
3. Nunc immunoassay plate
4. Coating buffer: TBS
5. Blocking buffer: 1% BSA, TBS, 0.1% Tween-20
6. Antibody diluent buffer: 1% BSA, PBS or TBS, or 0.1% Tween-20
7. Acid elution buffer: 200 mM Acetic Acid
8. Neutralizing buffer: 1M Tris pH 9.5
9. Wash buffer: TBS 0.1% Tween-20
10. TMB and HRP are used for enzyme/substrate readout.
11. Acid stop buffer
12. The conditions for the following need to be tested and optimized for optimal assay performance: pH, buffers, incubation times, concentrations of antibodies, volumes used, etc.

Basic Protocol for Single Antibody Two Plate ELISA Method Day1

1. Prepare the capture antibody in coating buffer

2. Add 100 µl of the capture antibody in coating buffer to Plate 1- Nunc 96-well microtiter plate
3. Incubate plate with gentle shaking for 1 hour at room temperature
4. Remove the unbound capture antibody from the microtiter plate by dumping the plate
5. Wash the plate 3 times with wash buffer
6. Prepare serial dilutions of the analyte in dilution buffer
7. Add 100 µl of the analyte to the plate
8. Incubate plate with gentle shaking for 1 hour at room temperature
9. Wash the plate 3 times with wash buffer
10. Add 65 µl of 200 mM Acetic Acid for 5 minutes at room temperature with gentle shaking
11. Prepare Plate 2 by adding 100 µl of 1 M Tris pH 9.5 to the microtiter plate
12. After 5 minutes of incubation transfer the 65 µl of acidified analyte from Plate 1 to Plate 2
13. Incubate overnight at 4°C with gentle shaking

Day2: Plate 2

1. Wash Plate 2, 3 times with wash buffer
2. Add 200 µl of blocking buffer to the plate
3. Incubate for one hour at room temperature with gentle shaking
4. Wash the plate 3 times with wash buffer
5. Add 100 µl of the biotinylated detection antibody (this is the same antibody that was used as the capture antibody)
6. Incubate for one hour at room temperature with gentle shaking
7. Wash the plate 3 times with wash buffer
8. Add Streptavidin HRP
9. Incubate for 20-30 minutes at room temperature with gentle shaking
10. Wash the plate 3 times with wash buffer
11. Add TMB substrate to the plate
12. Incubate according to manufacturer's suggestions with gentle shaking
13. Add Stop Solution, mix thoroughly
14. Read optical density at 450 nm

Multiplex Immunoassay Technologies

Biomarker research has expanded over the years, producing a need to quantitatively measure multiple analytes simultaneously from one sample. In the pre-clinical research area there is a need to measure endpoints from rodents and non-human primates to determine safety and efficacy of drug candidates. Typically the samples from these animal models are limited in volume and expensive to obtain, which produces a challenge in obtaining data if more than one analyte needs to be quantified. The same issues apply to clinical samples being assayed for Biomarker, Pharmacokinetic, and Pharmacodynamic studies. A single endpoint ELISA tends to use a larger volume of sample than a multiplexed assay.

Two of the widely used multiplex technologies that have been developed are the Luminex xMAP technology (LMX) and the Meso Scale Discovery (MSD). Both technologies have well validated immunoassays that cover a wide range of secreted and intracellular proteins. In most cases, numerous analytes can be measured with sample volumes of less than 50 µl.

Meso Scale Discovery is a multiplexed technology based on MULTI-ARRAY® technology. The technology utilizes a proprietary electrochemiluminescence detection system and an array of spots in a standard 96-well format. The electrochemiluminescence technology allows for an increase in dynamic range of the standard curve as well as an increase in sensitivity over normal ELISA readouts, such as HRP/TMB. The MSD technology utilizes a 96- or 384-well microtiter plate, allowing an immunoassay to be developed and optimized using the

same variables of antibody concentrations, buffers, and incubation times that are used in a standard ELISA. The plates are read using a Sector 6000 instrument yielding Relative Light Units that can be back calibrated off the standard curve to the analyte concentration that is being analyzed. Additional information on the MSD platform can be found at: http://www.mesoscale.com/.

Luminex xMAP is a bead based technology that allows capture antibodies to be coupled to color coded beads or microspheres that contain different emission spectra. A sandwich assay format is performed with the analyte added to the capture antibody bound beads, followed by the addition of a biotinylated antibody. The detection occurs by adding a streptavidin-conjugated flourochrome to the complex containing the sandwiched immunoassay. The fluorescent readout is detected using a Luminex xMAP which is a flow cytometry based instrument. The microspheres are classified based on their emission spectra and the amount of analyte detected is directly proportional to the fluorescent signal. Additional details on the LMP technology can be found at: http://www.luminexcorp.com/TechnologiesScience/xMAPTechnology/.

Studies have been performed by numerous laboratories directly comparing the MSD technology to the Luminex xMAP as well as to commercially available enzyme-linked immunosorbent assays (6-12). Validation data has been generated for spiked recovery in various matrixes, including other validation parameters for both the MSD and Luminex technologies. Overall the results from numerous studies show that while there usually is a quantitative difference between the technologies (most likely due to the use of different antibodies), the relative differences are comparable. Data from these numerous studies demonstrate that multiplexed technologies are suitable for screening for trends in cytokine profiles and other secreted proteins to support pre-clinical and clinical studies.

Important Parameters for Development of an Immunoassay

1. Capture antibody
2. Detection antibody
3. Plate type
4. Coating buffer
5. Blocking buffer/diluent buffer
6. Wash buffer
7. Coating antibody concentration
8. Coated antibody stability
9. Timing of each step in the immunoassay
10. Detection and/or secondary antibody concentration
11. Reporter concentration
12. Readout
13. Instrument linearity

Initial Concept and Method Development for a Sandwich Immunoassay

Initial Development Experiment

The goal is to develop a basic working method by determining the antibody which should be the capture antibody and which antibody should be the detection antibody. Determine the optimum antibody concentrations for both the capture and detection antibody. The optimal antibody for capture vs. detection can only be determined empirically. If multiple antibodies to the analyte exist, it is best to examine all possible pairs of the antibodies.

Experiment

Coat the ELISA plate with several dilutions of each antibody that will be used as part of the sandwich assay. Add the analyte to be measured at a high, low and zero concentration. Use each of the antibodies, at several concentrations, as a detection antibody. The results of this experiment will determine which antibody is best for the capture antibody and which is best for the detection antibody. Furthermore, the dilution needed for both antibodies will also be determined.

Reagents

Included in the list below are the plate type and buffers that will work for the majority of immunoassays. Use these conditions as a starting point.

1. Two antibodies that recognize different epitopes on the analyte.
2. The optimal antibody pair for the sandwich assay was determined empirically in the experiment above.
3. Greiner immunoassay plate
4. Coating buffer: PBS
5. Blocking buffer: 1% BSA, TBS, 0.1% Tween-20
6. Antibody diluent buffer: 1% BSA, PBS or TBS, or 0.1% Tween-20
7. Wash buffer: PBS or TBS 0.1% Tween-20
8. TMB and HRP are used for enzyme/substrate readout
9. Acid stop buffer

Protocol (see plate layouts in Table 1 and Table 2):

1. Dilute both antibodies in coating buffer at 0.5, 1, 2 and 5 µg/ml and add 100 µl of each concentration to 24 wells of the 96-well microtiter plate.
2. Incubate the plate containing the capture antibody overnight at 4°C and continue the experiment the next day. (Stability of the capture antibody bound to the plate can be determined in later experiments.)
3. Remove the unbound capture antibody solution from the microtiter plates by aspirating or dumping the plate.
4. Add 200 µl of blocking buffer to each well of the 96-well microtiter plate. Incubate the plate for one hour at room temperature.
5. Remove the blocking buffer from the plate by aspirating or dumping the plate.
6. Determine the desired working range of the analyte. This will give you the high and low concentrations to incubate with each capture antibody dilution. The zero analyte wells will give you the non-specific binding (NSB).
7. Add 100 µl of the analyte to each well in the microtiter plate and incubate for 2.5 hours at room temperature.
8. Wash the plates 3 times with wash buffer.
9. Dilute the detection antibody serially at 1:200, 1:1000, 1:5000 and 1:25000 in diluent.
10. Add 100 µl of detection antibody to each well of the microtiter plate and incubate for 1.5 hours at room temperature.
11. Wash the plates 3 times with wash buffer.
12. Dilute streptavidin-HRP (if detection antibodies are biotinylated) or appropriate secondary antibody (if capture and detection antibodies are from different species) according to manufacture instructions in antibody diluent and add 100 µl to each well in the microtiter plate and incubate for 1 hour at room temperature.
13. For HRP readout add TMB as a substrate to allow color development and incubate for 10-20 minutes at room temperature.
14. Add acid stop reagent to stop the enzyme reaction.

15. Read at 450 nm for TMB/HRP.

Table 1: Plate 1 layout for initial experiment. H=High, L=Low and 0=Zero. High analyte or ligand concentration in combination with the low ligand concentration will give an indication of the dynamic range. Low analyte or ligand concentration will give an indication of sensitivity. Zero ligand will give the non-specific binding and indicate if there are background issues.

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
1:1000	H	L	0	H	L	0	H	L	0	H	L	0
	H	L	0	H	L	0	H	L	0	H	L	0
1:5000	H	L	0	H	L	0	H	L	0	H	L	0
	H	L	0	H	L	0	H	L	0	H	L	0
1:25000	H	L	0	H	L	0	H	L	0	H	L	0
	H	L	0	H	L	0	H	L	0	H	L	0

Table 2: Plate 2 layout for the initial experiment. H=High, L=Low and 0=Zero. High analyte or ligand concentration in combination with the low ligand concentration will give an indication of the dynamic range. Low analyte or ligand concentration will give an indication of sensitivity. Zero ligand will give the non-specific binding and indicate if there are background issues.

Detection Antibody	Capture Antibody B											
	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
1:1000	H	L	0	H	L	0	H	L	0	H	L	0
	H	L	0	H	L	0	H	L	0	H	L	0
1:5000	H	L	0	H	L	0	H	L	0	H	L	0
	H	L	0	H	L	0	H	L	0	H	L	0
1:25000	H	L	0	H	L	0	H	L	0	H	L	0
	H	L	0	H	L	0	H	L	0	H	L	0

Results

Determine the absorbance units that yield the maximum signal to noise ratio or the greatest difference between the high and low analyte concentrations with the lowest variability. These are the conditions that will be selected for the antibody to be used as the capture antibody and the dilution of the antibodies to be used in the next experiment.

- If the background signal is unacceptably high (greater than 0.2 A.U.) then run additional experiments varying the plate type, blocking buffers, blocking buffers with a diluent agent like species specific IgG, antibody diluent buffers, wash buffers, and the reporter type.
- If the above general conditions have an acceptable NSB then it can be determined if the dynamic range and sensitivity are in the appropriate range. To improve the sensitivity of the assay, the buffers, timing of incubations and matrix conditions can be varied in the next experiment.

- Antibodies are the reagents that play a major role in the sensitivity and dynamic range of an immunoassay. This is due to the actual antibody affinity for the analyte. If after attempting to develop the assay the sensitivity is still not in the desired range, different antibody pairs will need to be evaluated.

Example 2

An ELISA was set up to measure the amounts of a protein where there is only one polyclonal antibody available. The polyclonal antibody was used as both the capture antibody and the detection antibody. In this example the detection antibody is biotinylated.

Reagents:

1. Affinity pure polyclonal antibody
2. Analyte protein
3. Biotinylated affinity pure polyclonal antibody

Protocol:

Follow the same basic protocol above using these parameters (see plate layout in Table 3).

1. Coat the affinity purified antibody at 3 levels: 2, 1 and 0.5 µg/ml.
2. Dilute the biotinylated antibody at 3 levels: 1:1000, 1:5000, and 1:25000.
3. Dilute the analyte protein in buffer to 50 ng/ml, 1 ng/ml, and zero.

Conclusions:

As seen in Table 4, the lowest NSB and best signal to noise ratio from low to high analyte concentration are the 0.5 µg/ml concentration for the capture antibody and the 1:25000 dilution of the biotinylated detection antibody.

Detection Antibody	Capture Antibody											
	2 µg/ml			1 µg/ml			0.5 µg/ml					
1:1000	H	L	0	H	L	0	H	L	0			
	H	L	0	H	L	0	H	L	0			
1:5000	H	L	0	H	L	0	H	L	0			
	H	L	0	H	L	0	H	L	0			
1:25000	H	L	0	H	L	0	H	L	0			
	H	L	0	H	L	0	H	L	0			

Table 3: Plate layout to determine the capture and detection antibody concentrations.

Table 4: Results from Example 2: to determine the capture and detection antibody concentrations. Values are averages of absorbance measurements at A450.

Capture Antibody		2 µg/ml			1 µg/ml			0.5 µg/ml		
Analyte protein ng/ml		50	1	0	50	1	0	50	1	0
Detection Antibody	1:1000	3.69	1.81	1.37	3.63	1.89	1.33	3.3	1.79	0.99
	1:5000	3.22	0.7	0.49	3.24	0.81	0.47	3.1	0.83	0.36
	1:25000	1.61	0.21	0.15	1.75	0.25	0.16	1.72	0.26	0.12

Second Development Experiment-Matrix Compatibility

The goal is to determine the matrix effect or sample type on the immunoassay method. The matrix is based on what the sample is found in, for instance tissue culture media, serum, plasma, cell lysate, buffers, etc. Serum matrix, due to its complexity, can have a significant effect on the method. In this example the samples are in rat serum so the matrix effect of rat serum needs to be determined.

Experiment

The samples that need to be measured in this assay will be in either mouse or rat serum. Use the conditions established in the first experiment for the concentration of the capture antibody and the detection antibody. Serially dilute the standard (analyte) to obtain a full standard curve in 3 different matrices (10% rat serum, 30% rat serum and the original buffer diluent used in the first experiment). This will determine the effect of the matrix used for the experimental samples.

Reagents:

1. Use all of the reagents and buffers listed in the first experiment (Example 2).
2. Matrix diluent: 10% rat serum in antibody diluent or 30% rat serum in antibody diluent.

Protocol:

Follow the standard protocol, changing only the matrix diluent to include rat serum.

1. Dilute the coating antibody in coating buffer at 0.5 µg/ml and add 100 µl to each well of the 96-well microtiter plate.
2. Incubate the plate containing the capture antibody overnight at 4°C and use the next day.
3. Stability of the capture antibody bound to the plate can be determined in later experiments.
4. Remove the capture antibody solution from the microtiter plates by aspirating or dumping the plate.
5. Add 200 µl of blocking buffer to each well of the 96-well microtiter plate.

6. Incubate the plate for one hour at room temperature.
7. Remove the blocking buffer from the plate by aspirating or dumping the plate.
8. Serially dilute the standard in antibody dilution buffer containing either 10% or 30% rat serum, or diluent alone.
9. Add 100 µl of the standard to each well in the microtiter plate and incubate for 2.5 hours at room temperature.
10. Wash the plates 3 times with wash buffer.
11. Dilute the detection antibody to 1:25000 in antibody diluent.
12. Add 100 µl of detection antibody diluent to each well of the microtiter plate and incubate for 1.5 hours at room temperature.
13. Wash the plates 3 times with wash buffer.
14. Dilute streptavidin-HRP according to manufacturer's instructions in antibody diluent and add 100 µl to each well in the microtiter plate and incubate for 1 hour at room temperature.
15. For HRP readout add TMB as substrate to allow color development and incubate for 10-20 minutes at room temperature.
16. Add acid stop reagent to stop the enzyme reaction.
17. Read at 450 nm for TMB/HRP.

Results:

Use the standard curve data and construct a precision profile. Check the background levels. See the next section for standard or calibration curve model fitting. Note that the standard curves under all three matrix diluent conditions give the dynamic range and sensitivity necessary for the intended use (Figure 5). For this particular assay, no further development is needed (based on the standard curve, low background and precision profile).

Precision Profile:

Generate the precision profile for the standard curve of the appropriate matrix for the experiment. The precision profile is a plot of coefficient of variation (CV) for the calibrated concentration levels of the replicates of each calibrator versus the nominal analyte concentration in the calibrator samples. The dynamic range of the calibration curve (quantification limits) are then defined by the concentrations where the precision profile intersects the 20% CV. The calculation of this CV has to take into consideration both the sampling variability and the lack of fit to the calibration curve, and is therefore not straightforward. A statistician should be consulted for this evaluation. An SAS program for this evaluation has been published (13).

Calibration Curve and Precision Profile for the Three Different Matrix Conditions

Calibration Curve Model Selection:

A significant source of variability in the calibration curves can come from the choice of the statistical model used for the calibration curve. It is therefore extremely important to choose an appropriate calibration curve model. For most immunoassays, the following models are commonly available from most instrument software.

Linear Model:

$$\text{Response} = a + b^{*}(\text{Concentration}) + \text{error}$$

where parameters, a and b are the intercept and slope respectively, and "response" refers to signal readout, such as optical density or fluorescence from an immunoassay. Often this linear model is fitted after log transformation of the response and concentration. This is sometimes referred as the "log-log linear model".

Quadratic Model:

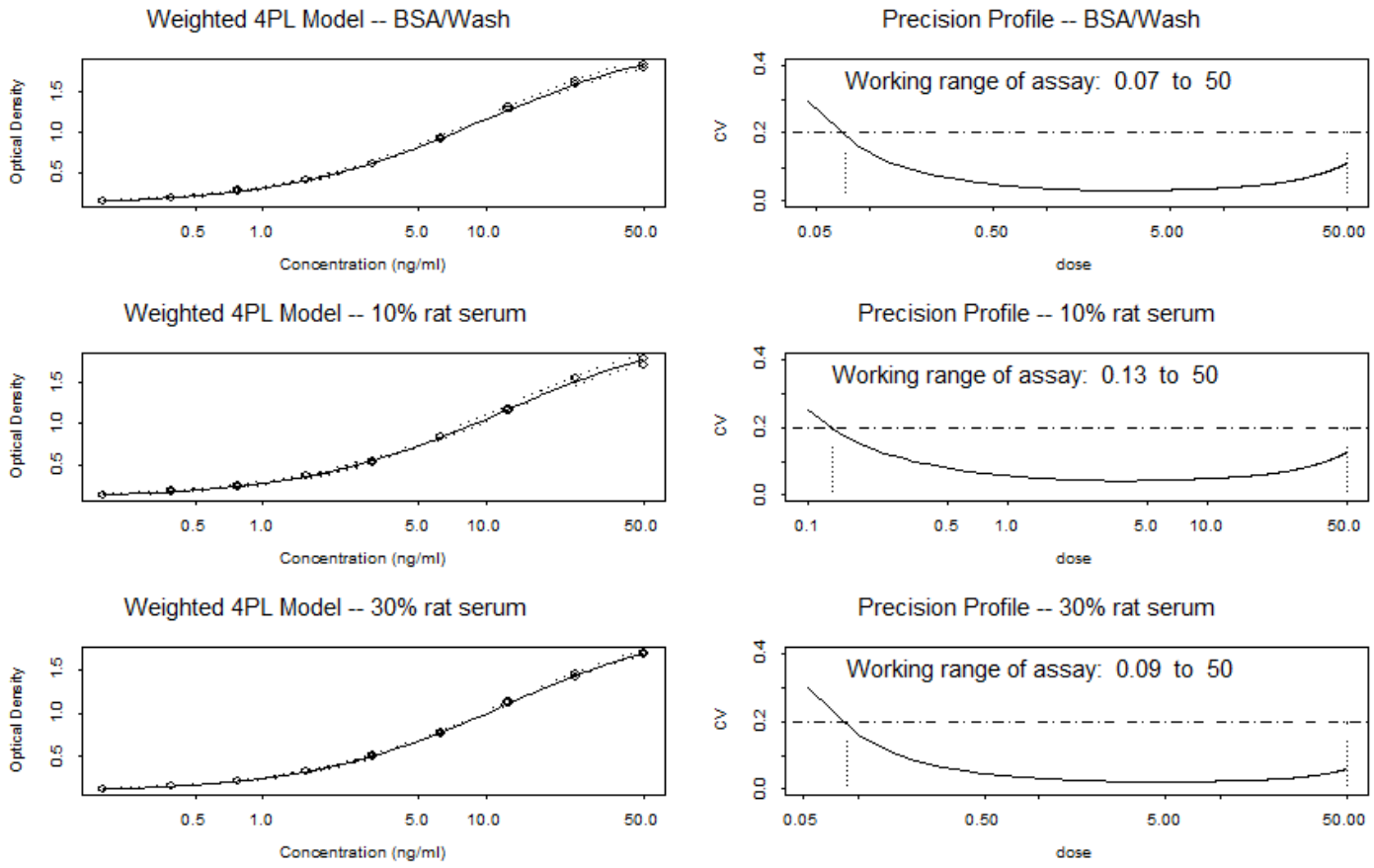


Figure 5: Calibration curve and precision profile for the three different matrix conditions using a Four Parameter Logistic (4PL) Model.

$$\text{Response} = a + b^{*}(\text{Concentration}) + c^{*}(\text{Concentration})^{2} + \text{error}$$

where a, b and c are the intercept, linear and quadratic term coefficients, respectively, of this quadratic model.

Four Parameter Logistic Model:

$$\text{Response} = \text{Top} + \frac{(\text{Bottom} - \text{Top})}{1 + \left(\frac{\text{concentration}}{EC_{50}}\right)^{\text{Slope}}}$$

The four parameters to be estimated are Top, Bottom, EC_{50} and Slope. Top refers to the top asymptote, Bottom refers to the bottom asymptote, and EC_{50} refers to the concentration at which the response is halfway between Top and Bottom.

Five Parameter Logistic Model:

$$\text{Response} = \text{Top} + \frac{(\text{Bottom} - \text{Top})}{\left[1 + \left(\frac{\text{concentration}}{EC_{50}}\right)^{\text{Slope}}\right]^{\text{Asymmetry}}}$$

Asymmetry is the fifth parameter in this model. It denotes the degree of asymmetry in the shape of the sigmoidal curve with respect to "EC_{50}". A value of 1 indicates perfect symmetry, which would then correspond to the four-parameter logistic model. However, note that the term referred to as "EC_{50}" in this model is not truly the EC_{50}. It is the EC_{50} when the asymmetry parameter equals 1. It will correspond to something very different such as EC_{20}, EC_{30}, EC_{80}, etc., depending on the value of the asymmetry parameter for a particular data set.

For most immunoassays, the four or five parameter logistic model is far better than the linear, quadratic or log-log linear models. These models are available in several software packages, and are easy to implement even in an Excel-based program. As illustrated in the plots shown in Figure 6, the quality of the model should be judged based on the dose-recovery scale instead of the lack-of-fit of the calibration curve (R^2). In this illustration, even though the R^2 of the log-log linear model is 0.99, when assessed in terms of the dose-recovery plot, this model turns out to be significantly inferior to the four parameter logistic model. Before the assay is ready for production, the best model for the calibration curve should be chosen based on the validation samples using dose-recovery plots.

Importance of Weighting in Calibration Curves

The default curve-fitting method available in most software packages assigns equal weight to all of the response values, which is appropriate only if the variability among the replicates is equal across the entire range of the response. However, for most immunoassays, the variability of assay signal among replicates of each calibrator increases proportionately with the response (signal) mean. Giving equal weight can lead to highly incorrect conclusions about the assay performance and will significantly affect the accuracy of results from the unknown samples. More specifically, lack of weighting leads to higher variability of results in the lower end of the assay range, thus greatly compromising the sensitivity of the assay. It is therefore extremely important to use a curve-fitting method/software that has appropriate weighting methods/options. This is illustrated in Figure 7 where we compare the total error results from the validation controls after fitting the calibration curves using log-log linear, four-parameter logistic and five-parameter logistic models. For this example, the performance of the validation samples is better overall when the five-parameter logistic model is used.

Third Development Experiment

The two-step experiment detailed above is a very simple example of how to develop a sandwich ELISA method. If the dynamic range and sensitivity of the assay does not meet the experimental needs then further experimental parameters should be tested using experimental design. With experimental design all of the factors involved in the ELISA including buffers, incubation time and plate type can be analyzed.

In a sandwich ELISA method the antibodies chosen are the major drivers of the assay parameters. If at this point in the method development, the precision profile of the standard curve does not encompass the desired dynamic range and sensitivity, instead of continuing with the development experiment, antibodies should be further characterized. Changing some of the variables such as the antibody concentrations can significantly improve the calibration curve and hence its precision profile.

The goal is to determine the optimal conditions for the variables in the immunoassay, including incubation steps, buffers, substrate, etc. Also, determine the optimal antibody concentrations and the stability of the capture antibody bound to the plate.

Experiment:

Dilute the standard in the matrix compatible to the sample (as determined in the second experiment). Vary the incubation times, dilution buffers and other variables in order to optimize the immunoassay. Analyze by using experimental design software and precision profiles.

Reagents:

1. Antibodies
2. Coating buffers
3. Blocking buffers
4. Wash buffers

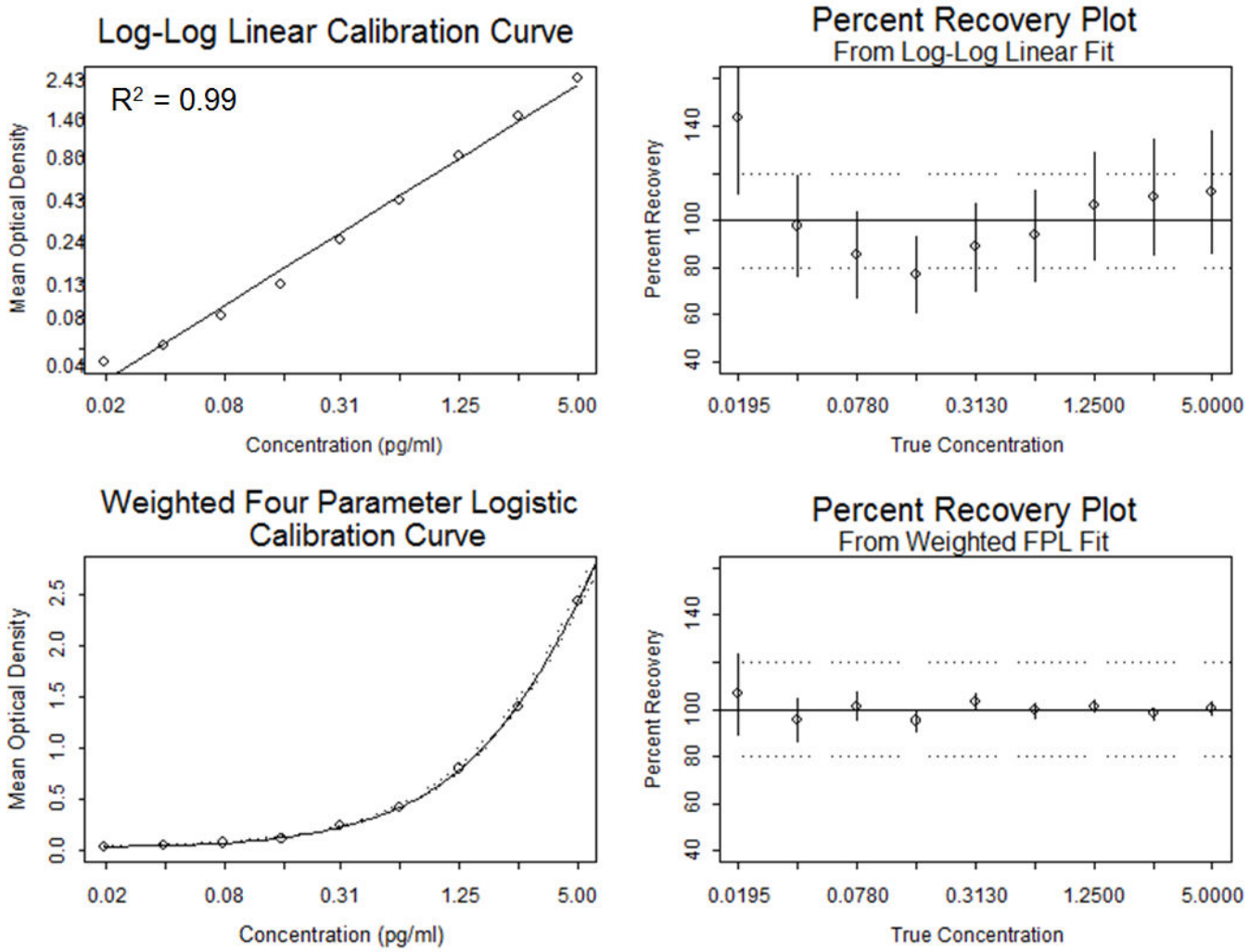


Figure 6: Example of log-log linear and weighted four parameter logistic calibration curves.

5. Antibody diluents
6. Substrate

Protocol:

1. Coat the microtiter plate with the capture antibody at the concentration determined in the initial experiment. Incubate overnight at 4°C.
2. Discard the capture antibody solution from the microtiter plate.
3. Block the plate for 1 hour at room temperature using various blocking reagents.
4. Store plates at 4°C, desiccated, for several periods of time 0-5 days.
5. Repeat steps 1-3 the day of the actual experiment.
6. Serially dilute, using an 8-point standard curve, the known standard in the appropriate matrix for the experiment. For the control also dilute the standard in the same buffer as was used in the initial experiment. Add 100 µl of standard to each well in the 96-well microtiter plate.
7. Incubate the diluted standard with the capture antibody for 1 hour and 3 hours at room temperature and overnight at 4°C. Each time point will have to be run in a separate plate.
8. Wash plates 3 times (if background or NSB is high, try different wash buffers).
9. Add 100 µl of diluted detection antibody. If background is high again different diluents can be tested.

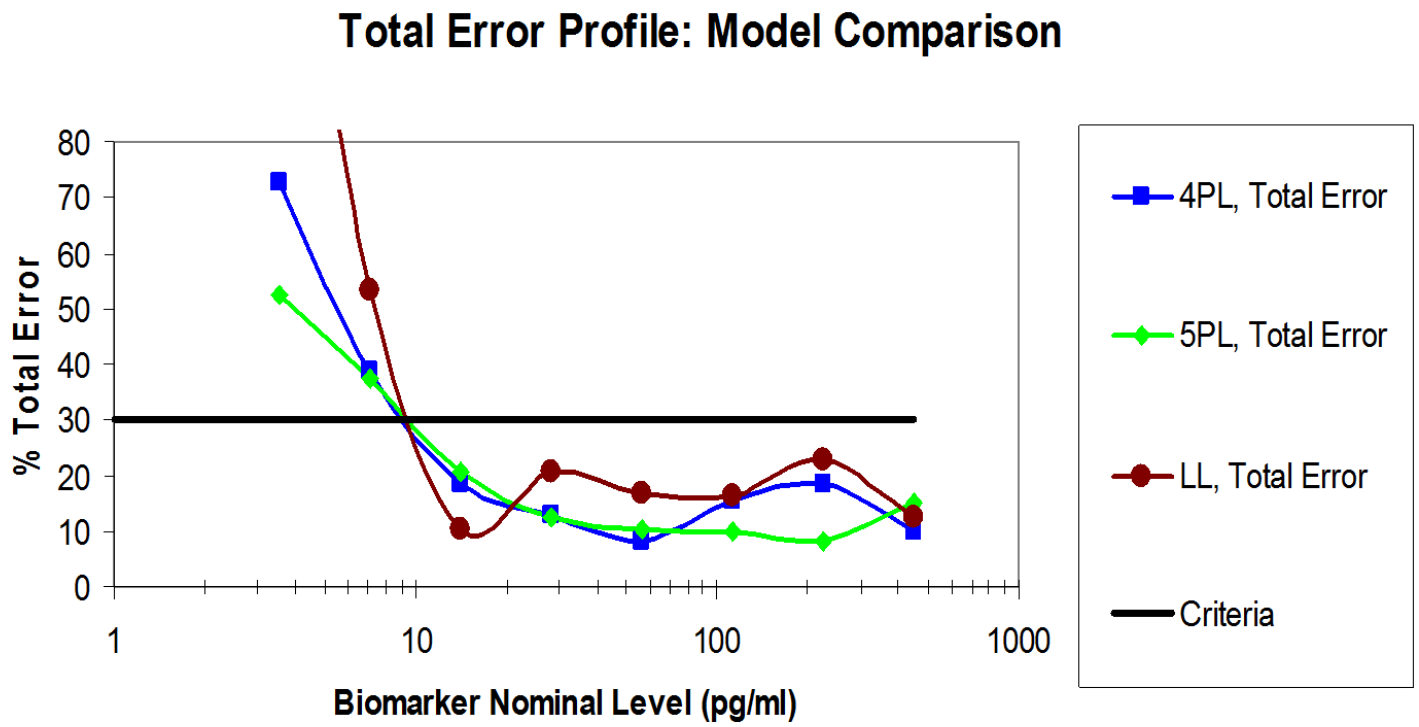


Figure 7: Validation samples are plotted with different calibration curve models. It is clear from the plot that the five-parameter logistic (5PL) model is better than the four-parameter logistic (4PL) and log-log linear (LL) models. For this particular assay, 5PL is the optimal choice for the in-study (production) phase.

10. Incubate the detection antibody for different time periods and again different plates will have to be used for each time condition.
11. Wash plates 3 times.
12. Add 100 µl of substrate to the wells containing the detection antibody conjugated to the enzyme and allow incubation according to the manufacturer's conditions.
13. Add 100 µl of stop buffer.
14. Read at 450 nm.

Data Analysis:

Compute the standard curves and their precision profiles for all the experimental design conditions. Derive the optimization endpoints using the precision profiles. Then analyze the optimization endpoints using software such as JMP (http://www.jmp.com) to determine the optimum levels of the assay factors. See next section for the details and illustration.

Experimental Designs for Increasing Calibration Precision

Step 1:

Identify all the factors/variables that potentially contribute to assay sensitivity and variability. Choose appropriate levels for all the factors (high and low values for quantitative factors, different categories for qualitative factors). Then use fractional-factorial experimental design in software such as JMP to derive appropriate experimental "trials" (combinations of levels of all the assay factors). Run 8-point calibration curves in duplicate for each trial. With each trial taking up two columns in a 96-well plate, 6 trials per plate can be tested. All trials should be randomly assigned to different pairs of columns in the 96-well plates. However,

certain factors such as incubation time and temperature are inter-plate factors. Therefore, levels of such factors will have to be tested in separate plates (see Table 5).

After the above experiment is run, the calibration curves should be fit for each trial using an appropriately weighted-nonlinear regression model. Then the precision profile for the calibration curve of each trial should be obtained along with the important optimization end-points such as working-range, lower quantitation limit and precision area (area of the region intersected by the precision profile with 20% CV). Now analyze these data to determine the optimal level of all qualitative factors and determine which subset of quantitative factors should be further investigated.

Table 5: Example plate layout to increase calibration precision.

	Trial 1		Trial 2		Trial 3		Trial 4		Trial 5		Trial 6	
	1	2	3	4	5	6	7	8	9	10	11	12
A	8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate	
B	8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate	
C	8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate	
D	8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate	
E	8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate	
F	8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate	
G	8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate	
H	8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate		8pt calibration curve; duplicate	

Step 2:

We now need to determine the optimum levels for the key factors determined in the previous step. Choose appropriate low, middle and high levels for each of these factors based on the data analysis results from step 1. Now use software such as JMP to generate appropriate trials (combinations of low, middle and high levels of all the factors) from a central-composite design. Then run duplicate 8-point calibration curves for each trial using a similar plate format as in step 1.

Now obtain the precision profile and the relevant optimization end-points of the calibration curve of each trial. Perform the response-surface analysis of these data to determine the optimal setting of each of the quantitative factors run in this experiment.

Illustration of Experimental Design and Analysis for Sandwich ELISA Optimization

In Table 6, we have the experiment plan from the second step of the optimization process using experimental design for a sandwich ELISA. These four factors (capture antibody, detection antibody, enzyme and volume) were picked out of the six factors considered in the first step of this optimization process (screening phase) for further optimization. We use a statistical experimental design method called central composite design to

generate the appropriate combinations of the high, mid and low levels of the four factors in this second step. For example, trial #6 in this table refers to the middle level of the first, third and the fourth factors, and the low level of the second factor.

Eight-point standard curves in duplicate were generated for each of these trials, in adjacent columns of a 96-well plate. This resulted in six trials per plate, and with 36 trials in 6 plates. We computed the precision profiles of the calibration curves for each of these 36 trials. From these precision profiles, we computed the working range (lower and upper quantification limits), CV and related variability and sensitivity measures. We then used a statistical data analysis method called "response surface analysis" on these optimization endpoints. This resulted in polynomial type models for all the factors. Using the shape of the curve and other features from this model, the optimum levels for these factors were determined. This gave us the most sensitive dynamic working range possible for this assay.

An experiment was then performed for this ELISA to compare these optimized levels to the pre-optimum levels and the assay kit manufacturer's recommendation. The results from this comparison are summarized in Figure 8.

The optimized levels derived from statistical experimental design for this ELISA resulted in the following improvements over the pre-optimum and assay kit manufacturer's recommendation.

- Lower quantification limit decreased more than two-fold to 13.6 nM.
- Upper quantification limit by up to 10-fold to 1662.3 nM.
- Precision area increased by 2-fold and the working range increased by 2-fold to two log cycles.

This improvement is evident from the precision profiles shown in Figure 8.

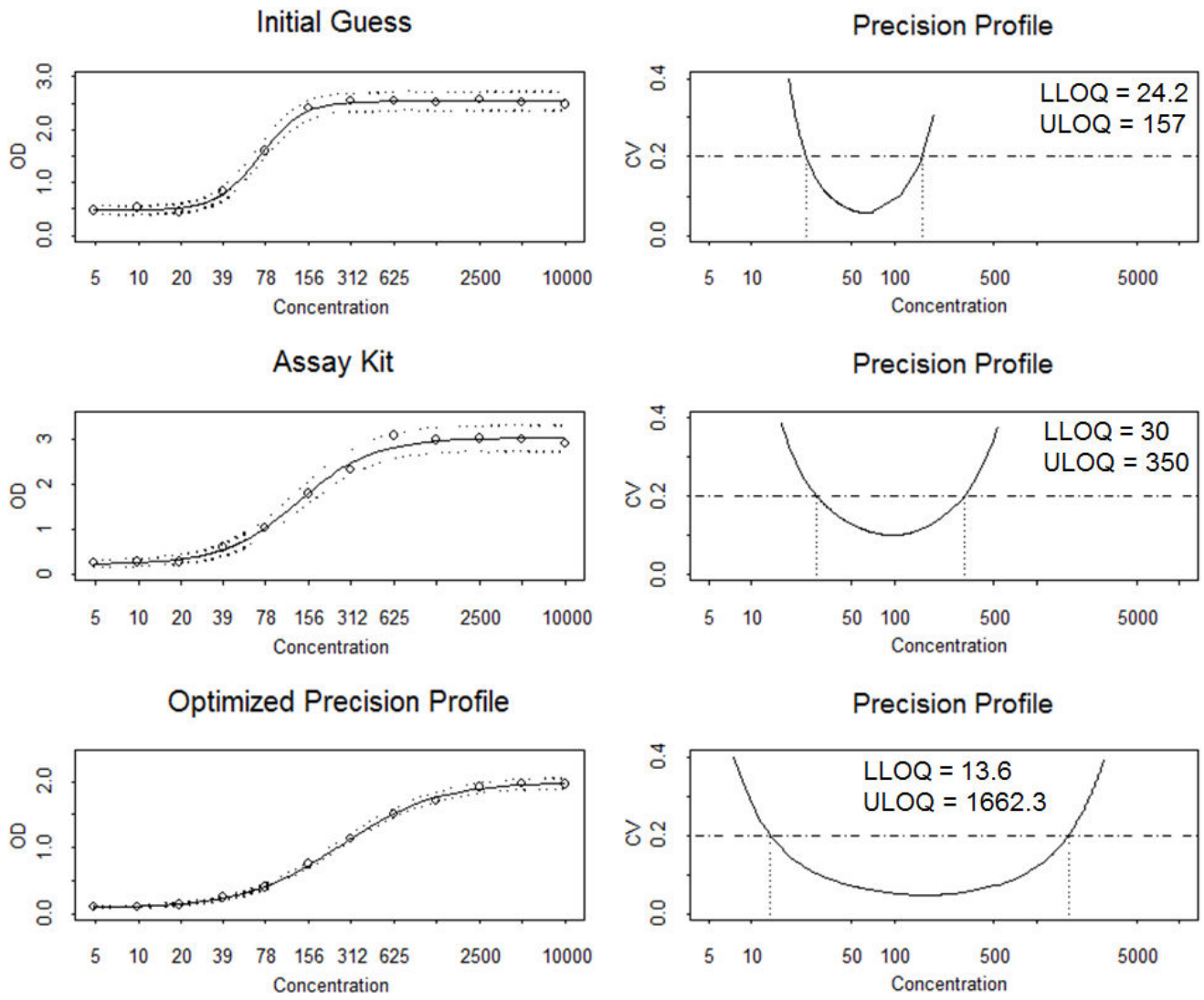


Figure 8: Comparison of optimized levels to pre-optimum levels and those recommended by the manufacturer.

Table 6: Experimental plan from the second step of the optimization process using the experimental design for a sandwich ELISA.

Trial #	Pattern	Capture A	Biotin A	EnzCult	Volume
1	– – – +	250	250	300	100
2	0 0 0 0	500	425	525	75
3	– + + +	250	600	750	100
4	– + + –	250	600	750	50

Table 6 continued from previous page.

Trial #	Pattern	Capture A	Biotin A	EnzCult	Volume
5	– – – –	250	250	300	50
6	0 – 0 0	500	250	525	75
7	0 0 0 0	500	425	525	75
8	0 0 0 0	500	425	525	75
9	0 0 + 0	500	425	750	75
10	– – + +	250	250	750	100
11	+ – – +	750	250	300	100
12	0 0 0 0	500	425	525	75
13	0 0 0 0	500	425	525	75
14	+ + + –	750	600	750	50
15	0 0 0 0	500	425	525	75
16	0 0 0 0	500	425	525	75
17	0 0 0 0	500	425	525	75
18	– + – –	250	600	300	50

Table 6 continued from previous page.

Trial #	Pattern	Capture A	Biotin A	EnzCult	Volume
19	+ 0 0 0	750	425	525	75
20	0 0 0 0	500	425	525	75
21	0 + 0 0	500	600	525	75
22	– + – +	250	600	300	100
23	+ + – –	750	600	300	50
24	0 0 0 –	500	425	525	50
25	0 0 – 0	500	425	300	75
26	0 0 0 +	500	425	525	100
27	0 0 0 0	500	425	525	75
28	0 0 0 0	500	425	525	75
29	0 0 0 0	500	425	525	75
30	+ – + +	750	250	750	100
31	+ + – +	750	600	300	100
32	– – + –	250	250	750	50

Table 6 continued from previous page.

Trial #	Pattern	Capture A	Biotin A	EnzCult	Volume
33	– 0 0 0	250	425	525	75
34	+ – + –	750	250	750	50
35	+ – – –	750	250	300	50
36	+ + + +	750	600	750	100

Initial Concept and Method Development of a Competitive Assay

Competitive Binding Immunoassay

Development and validation of a competition immunoassay requires considerable expertise in reagent characterization and method development. Sandwich and antigen-down immunoassays formats should be explored before attempting the competitive immunoassay format.

Drawbacks Using a Competitive Immunoassay

1. A competitive immunoassay is not as sensitive as a sandwich ELISA.
2. A competitive immunoassay is more sensitive to matrix issues, especially serum matrix, which can affect assay performance.
3. Timing of the various incubation steps is less robust in a competitive assay. That is the IC_{50} of the standard curve will shift with minor changes in incubation of the various steps of the immunoassay.
4. The labeling of the hapten or analyte can change the analyte binding affinity for the antibody. Experiments need to determine the effect of the label on the binding affinity of the antibody to the analyte.

Development of a Competitive Immunoassay

Initial Development Experiment

The goal of the initial development experiment is to determine the optimal coating concentration of the antibody used for capture and the labeled ligand.

Reagents

1. Antibody- mono or polyclonal, specific to the analyte.
2. Buffers- same as for a competitive assay.
3. Labeled ligand- the enzyme or biotin is labeled directly to the analyte or ligand.

Experiment

Coat the ELISA plate with various antibody concentrations to determine the optimal concentration of antibody and labeled ligand.

Protocol

1. Determine the desired analyte working range.
2. The capture antibody is titrated using high, low and zero analyte concentration levels.
3. Dilute the capture antibody in coating buffer at 0.1, 0.5, 1 and 2 µg/ml and add 100 µl to each well of the 96-well microtiter plate. The capture antibody might need to be titrated down further. The amount of antibody coated on the plate will be proportional to the sensitivity of the assay.
4. Incubate the plate containing the capture antibody overnight at 4°C and use the next day.
5. Stability of the capture antibody bound to the plate can be determined in later experiments.
6. Remove the coating antibody solution from the microtiter plates by aspirating or dumping the plate.
7. Add 200 µl of blocking buffer to each well of the 96-well microtiter plate.
8. Incubate the plate for one hour at room temperature.
9. Remove the blocking buffer from the plate by aspirating or dumping the plate.
10. Dilute the labeled standard in antibody dilution buffer over a wide range. The desired result is the condition that gives a readable signal with the least amount of antibody coated, in combination with the least amount of labeled standard.
11. Zero concentration will give you the NSB.
12. Add 100 µl of the labeled standard to each well in the microtiter plate and incubate for 2.5 hours at room temperature. (The standard can either be directly labeled with the enzyme or biotinylated).
13. Wash the plates 3 times with wash buffer.
14. If a biotinylated standard is used, dilute streptavidin-HRP according to the manufacturer's instructions in antibody diluent and add 100 µl to each well in the microtiter plate and incubate for 1 hour at room temperature.
15. For HRP readout, add either OPD or TMB as a substrate to allow color development and incubate for 10-20 minutes at room temperature.
16. Add acid stop reagent to stop the enzyme reaction.
17. Read at 405 nm for TMB/HRP.
18. Determine the linearity of the instrument being used for the readout.

Second Development Experiment

The goal of the second development experiment is to determine the potential dynamic range and sensitivity. Take the conditions established in the initial experiment for the concentration of the antibody and labeled ligand and incubate with a wide range of unlabeled analyte. The resulting standard curve and precision profile calculation will give an estimate of the sensitivity and dynamic range of the assay.

Reagents

Reagents are the same as in the initial experiment.

Protocol

1. Dilute the capture antibody in coating buffer at the concentration determined in the initial experiment. Add 100 µl to each well of the 96-well microtiter plate.
2. Incubate the plate containing the capture antibody overnight at 4°C and use the next day.
3. Remove the capture antibody solution from the microtiter plates by aspirating or dumping the plate.
4. Add 200 µl of blocking buffer to each well of the 96-well microtiter plate.

5. Incubate the plate for one hour at room temperature.
6. Remove the blocking buffer from the plate by aspirating or dumping the plate.
7. Dilute the labeled standard in antibody dilution buffer at the concentration determined in the initial experiment.
8. Dilute the unlabeled ligand in antibody dilution buffer over a wide range of concentrations.
9. Add 100 µl of the labeled standard to each well in the microtiter plate and 100 µl of the various dilution of the unlabeled ligand. Incubate for 2.5 hours at room temperature. This is the competitive part of the assay and will allow for the competition between the labeled and unlabeled ligand for the sites on the antibody.
10. Wash the plates 3 times with wash buffer.
11. If a biotinylated standard is used, dilute streptavidin-HRP according to the manufacturer's instructions in antibody diluent and add 100 µl to each well in the microtiter plate and incubate for 1 hour at room temperature.
12. For HRP readout, add either OPD or TMB as a substrate to allow color development and incubate for 10-20 minutes at room temperature.
13. Add acid stop reagent to stop the enzyme reaction.
14. Read at 405 nm for TMB/HRP.

Third Development Experiment

The goal of the third development experiment is to determine the optimal buffers, incubation periods, temperatures, matrix effects, and other variables that might affect the assay.

Reagents

Reagents are the same as in the initial experiment.

Protocol

Same as in the second development experiment except for the following changes at steps 8 and 9:

8. Dilute the unlabeled ligand in antibody dilution buffer, and the matrix appropriate for the experiment, over a wide range of concentrations. Again the dilution buffer can be varied according to the experimental design.

9. Add 100 µl of the labeled standard to each well in the microtiter plate and 100 µl of the various dilutions of the unlabeled ligand. Incubate for 2.5 hours at room temperature. This incubation time can be varied for longer and shorter periods of time to potentially increase the sensitivity and dynamic range of the assay.

RESULTS

Analysis of the results is by the stasticial software JMP or any other appropriate statistical software can be used to determine the optimal conditions for incubation timing, buffers for dilution, and matrix effects.

Method Validation (Pre-Study)

It is important to note that the precision profile evaluation described earlier in this chapter is based on just the calibration curve. Consequently, only the calibration curve factors (quality and stability of reference standards, quality and stability of reagents, statistical validity of the calibration curve model) are taken into account for deriving these quantitation limits. Sample factors such as analyte (similar physicochemical substances), matrix (other substances that can affect analytical results) and operational factors can affect the performance of the assay/method as well. Thus the quantitation limits derived from the precision profile of a calibration curve is an optimistic assessment of the method performance. If these limits are not satisfactory the assay needs further optimization.

If the quantitation limits from the precision profile are close to the limits desired for the method's intended use, proceed to a full validation experiment as outlined below. This validation experiment is used to establish the method quantitation limits using the analysis of recovery data from validation samples (spiked standards). This experiment will take into account the three major sources of variation described above (calibration curve factors, sample factors and operational factors).

For the full validation experiment, generate the following data in at least three independent runs.

- Calibration curve in each run, preferably in triplicate.
- Validation/Quality Control (QC) samples (independent set of samples spiked with a known amount of standards) in each run at seven or more concentrations with at least two replicates; two concentrations near the precision profile estimates of the lower quantification limit and similarly two more near the upper quantification limit, and three or four that are equally spaced between lower and upper quantification limits.
- Estimate the concentrations of the validation samples of each run using the respective calibration curves. Then compute the % recovery of these validation samples using the following formula:

% Recovery = 100 × (Calibrated Concentration/Nominal Concentration)

- Now compute the average and standard deviation of the % recovery data of the validation samples from all runs for each concentration. The evaluation of standard deviation should be based on a separate variance component analysis of the multiple runs of validation data, and it should include the sources of variability relevant during the use of the assay in production. At the minimum, it will include inter-run and intra-run variability. Some of the other sources to consider might be analyst, plate, equipment, etc. As evident from the above formula for evaluating the percent recovery, note that the standard deviation of percent recovery can be considered as the coefficient of variation (%CV) of the calibrated results. This is essentially the intermediate precision (inter-run %CV) of the assay. We will hereafter refer to this as Intermediate Precision (IP).
- Plot the average % recovery values along with the IP (as calculated above) versus the nominal concentrations. Note that the % recovery along with the intermediate precision as determined above reflects the total error of the assay.
- The % recovery and the IP limits must be within +/- X% of the nominal value. If X is 30%, the percent recovery +/- IP must be within 70 to 130% of the nominal value. This means that the Total Error of the assay must be within 30%. The value of X should be set based on the intended use of the assay. Recommendations on the acceptance criteria are discussed later in this chapter.
- If X is set at 30%, the lower quantification limit is the lowest concentration at which the % recovery +/- IP is within 70% to 130%. The upper quantification limit is the highest concentration at which the % recovery +/- IP is within 70% to 130%.

Method Validation (In-Study)

The in-study validation phase is about making sure that the assay continues to perform according to pre-defined specifications in each study run. During production phase, when the assays are being used for screening the unknowns, it is important to run validation/QC samples in every run, with at least 2 replicates at high, middle and low concentrations (just one or two columns of a 96-well plate). Compute the average % recovery of these samples to make sure that the average recovery is within a reasonable range of accuracy (say, 80% to 120%). This might be adequate for quality control and is a reasonable compromise for any loss in assay throughput. Various methods might be considered for setting criteria for accepting or rejecting an actual assay where samples are being tested during a study (in-study validation). This is addressed in a subsequent section in this chapter.

Example of an Immunoassay Validation Experiment

Set up numerous aliquots of the standard and store frozen at -70°C. If the standard concentration is much higher than the first point on your curve, pre-dilute it so that a single, simple dilution can be made in order to set up the standard curve.

Dilute the standards serially to obtain an 8-point standard curve in the matrix appropriate for the samples that need to be measured. For example, if measuring tissue culture samples then the standards should be diluted in the same tissue culture medium that the samples are in. For serum samples, the standards should be diluted in serum diluted with an optimized buffer to the same dilution that the samples will be diluted.

Set up a series of spiked samples, again in the matrix appropriate for the samples that will be measured. The spiked control samples should not be the same concentration as in the standard curve and should cover the detectable range that the samples are thought to cover.

Follow the immunoassay protocol established during the optimization experiments. Set up the plate with 3-4 replicates of the standard curve and 4 or more replicates of the spiked control samples (See Table 7).

Assay at least 3 plates over 3 different days for a complete validation.

Validation Results from an IL-10 Immunoassay

The percent recovery and the standard error that takes into account the relevant sources of variation are plotted in Figure 9. If X is 30%, then the quantification limits are the lowest and highest concentrations where the % recovery +/- IP is within 70% to 130%. So for this assay, the lower quantification limit is the lowest concentration tested in this validation study (6.2 pg/ml), and the upper quantification limit is 3265 pg/ml (see Table 8)

St1	St1	St1	St1	SP1	SP1	SP1	SP1				
St2	St2	St2	St2	SP2	SP2	SP2	SP2				
St3	St3	St3	St3	SP3	SP3	SP3	SP3				
St4	St4	St4	St4	SP4	SP4	SP4	SP4				
St5	St5	St5	St5	SP5	SP5	SP5	SP5				
St6	St6	St6	St6	SP6	SP6	SP6	SP6				
St7	St7	St7	St7	SP7	SP7	SP7	SP7				
St8	St8	St8	St8	SP8	SP8	SP8	SP8				

Table 7: Validation Plate Layout.

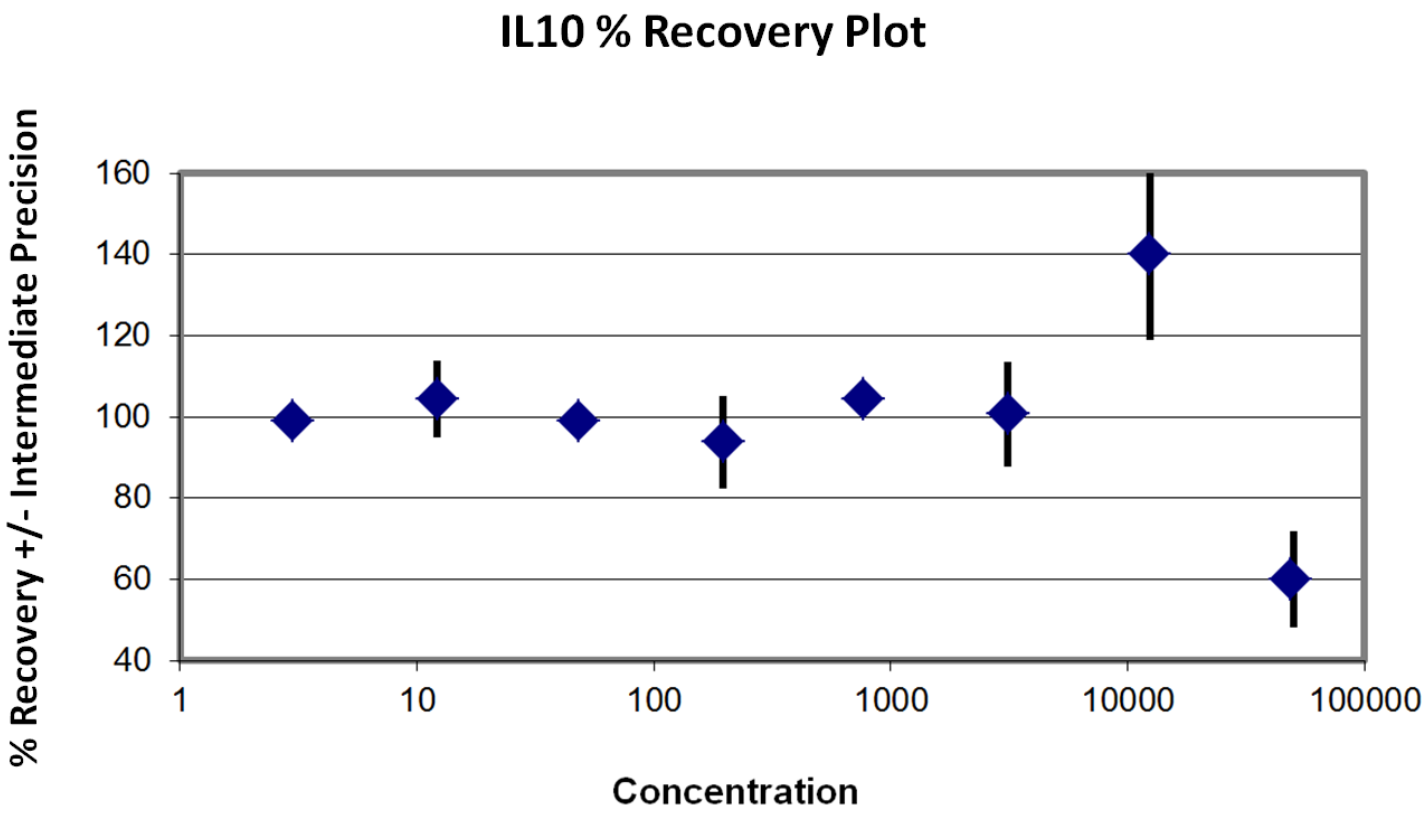


Figure 9: Validation results from IL-10 immunoassay.

Table 8: Validation results from IL-10 immunoassay

IL-10		
Expected Value	Average 4,5,6	% Recovery
40000	36924.96	92.31
11428.57	13846.57	121.16
3265.306	2966.42	90.85
932.9446	892.76	95.69
266.5556	242.70	91.05
76.15874	71.65	94.08

Table 8 continued from previous page.

IL-10		
Expected Value	Average 4,5,6	% Recovery
21.75964	22.32	102.60
6.21704	6.06	97.50

Plate Uniformity and Variability Experiment

It is important to check whether there is any systematic data trend across rows or columns of the 96-well plate and whether there is any significant variability between plates. An experiment with three plates and four concentrations of the standard can be done using the plate-layout in Figure 10. In this layout, C1, C2, C3 and C4 denote the standard concentrations from lowest to highest. For the purpose of illustration, data from one of the plates and a plot of the data from this experiment are given below for a sandwich ELISA. A systematic trend across columns is evident from this plot. For determining the statistical significance of this trend and the plate to plate variability, further statistical analysis of the data can be done with the help of a statistician.

Pre-Study & In-Study Acceptance Criteria

Different methods of quality control are available and routinely used in analytical methods. It is important that the methods used for assessment of method performance are suitable for the intended purpose. Shah et al. (14) proposed the 4-6-X rule for in-study validation phase that has become popular and widely used. This rule states that 4 out of the total 6 samples should be within X% of the nominal/reference value, and at least one out of the two samples at each level must be within X% of the reference value. The choice of X is specified a priori based on the intended use and purpose of the assay, and it was set at 20% by Shah and colleagues. DeSilva et al. (15) proposed a criteria (see Table 9) for pre-study and in-study validation phase of ligand-binding assays for assessing pharmacokinetics of macromolecules.

It should be noted that the acceptance criteria for biomarker assays will depend heavily on the intended use of the assay and should ideally be based on physiological variability as well. According to the criteria listed in Table 9, X is set at 30% for in-study validation, and the total error is set to be within 30% for the pre-study validation, along with the 20% limits for each component of total error (bias and precision). The pre-study criteria (Total Error < X %) and the in-study criteria (4-6-X rule) are not entirely consistent because the variability of the total error estimate and the consequent decision error rates are not taken into account. Thus the uncertainty in these estimates will depend on the magnitude of the errors and the number of measurements, and will in turn impact the level of decision error rates (16). The appropriate value of X in 4-6-X can be determined based on the variability of the total error estimates in pre-study validation. When it is feasible to use more QC samples in each run, 8-12-X or 10-15-X will have much better statistical outcomes than the 4-6-X criteria. In addition, the use of control charts as described by Westgard or tolerance limits based on pre-study validation data might be considered when possible.

The concept of total error as the primary parameter, and with bias and precision as additional constraints, is very useful. This is because total error has a more practical and intuitive appeal as it relates specifically to our primary question of interest about the assay; How far are my observed test results from the reference/nominal value? Because this is the primary practical question in the minds of most laboratory scientists, the criteria on the assay performance for the in-study phase is defined with respect to this question.

If the plate-format is not the same as indicated below, type in the necessary changes below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4
B	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4	C1
C	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2
D	C4	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3
E	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4
F	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4	C1
G	C3	C4	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2
H	C4	C1	C2	C3	C4	C1	C2	C3	C4	C1	C2	C3

Paste the plate-data below:

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.24	2.53	1.23	0.81	2.16	2.61	1.41	1.02	1.99	3.27	1.60	1.10
B	2.31	1.29	0.73	2.12	3.24	1.75	1.00	2.12	3.32	1.81	1.14	2.27
C	1.11	0.91	1.89	3.08	1.74	1.03	1.99	3.61	1.88	1.30	2.43	3.36
D	0.63	1.95	2.92	1.64	1.09	2.30	3.46	1.92	1.21	2.51	3.63	1.90
E	1.84	2.63	1.53	1.06	2.35	3.41	1.70	1.15	2.47	3.45	1.83	1.27
F	2.41	1.90	1.05	2.09	3.29	1.79	1.11	2.35	3.58	1.90	1.24	2.38
G	1.28	0.86	2.02	3.50	1.79	1.13	2.33	3.56	1.91	1.24	2.45	3.50
H	0.65	1.82	2.88	1.72	1.04	2.22	3.05	1.85	1.23	2.33	3.41	1.99

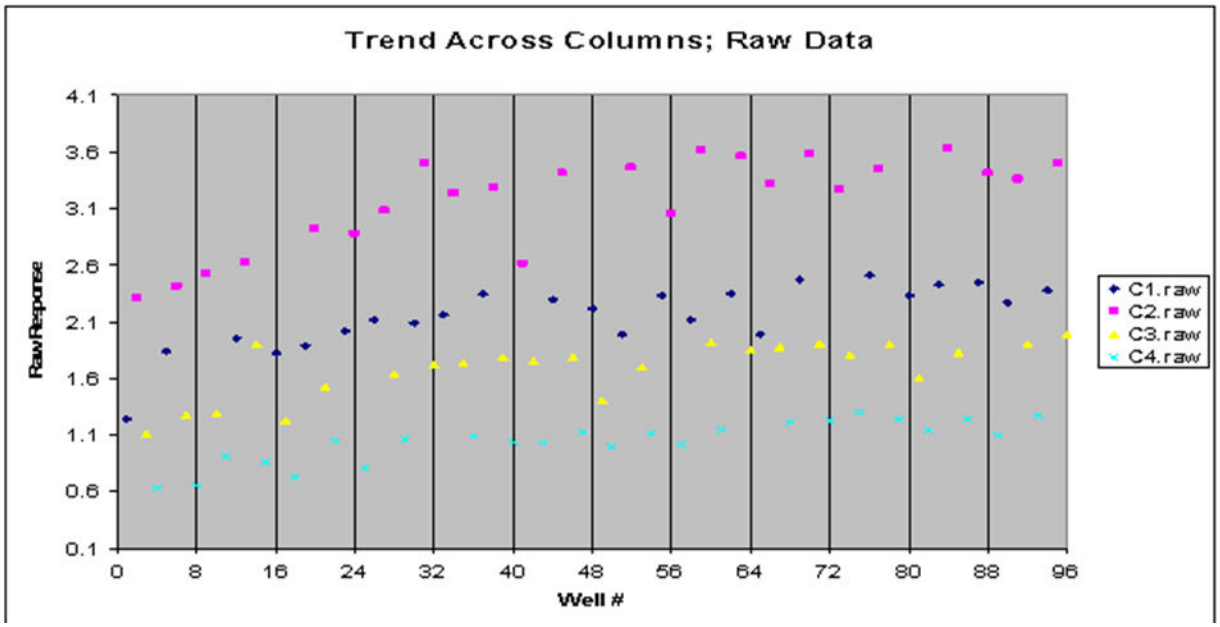


Figure 10: Data from one plate and a data plot from a plate uniformity and variability experiment where C1, C2, C3 and C4 denote the standard concentrations from lowest to highest.

Table 9: Criteria for pre-study and in-study validation phase of ligand-binding assays for assessing pharmacokinetics of macromolecules.

Characteristic	Pre-Study Validation	In-Study Validation
Trueness (%Relative Bias)	≤ ± 20 (± 25 at LQL)	-
Intermediate Precision (%CV)	≤ 20 (25 at LQL)	-
Total Error	≤ 30%	"4-6-30" rule

Consideration of Physiological Variation for Acceptance Criteria

One of the most important considerations for defining the performance criteria of most biomarker methods is the physiological variability in the study population of interest. That is, in order to determine whether a biomarker method is 'fit-for-purpose', we should determine whether it is capable of distinguishing changes that are statistically significant based on the intra-subject and inter-subject variability. The term "subject" here might

refer to animal or human. For example, an assay with 50% total error during pre-study validation might still be adequate for detecting a 2-fold treatment in a clinical trial for a certain acceptable sample size. Thus whenever possible, the acceptance criteria for pre-study validation should be based on physiological variation in the study. An example of the use of intra-subject and inter-subject variation for defining the pre-study acceptance criteria can be found in http://www.westgard.com/guest17.htm (17).

When the relevant physiological data (say, treated patients of interest) are not available during the assay validation phase, then healthy donor samples should be used to estimate the intra-subject and inter-subject variation, and hence the desired specifications on the pre-study assay validation. This can be updated at a later time when there is access to the relevant patient data. If access to healthy donor samples is also not feasible, then other flexible biological rationale should be considered and updated periodically as more information becomes available over time. In the absence of physiological data or other biological rationale, the acceptance criteria for pre-study validation should not be strictly defined. Instead, only the performance characteristics from pre-study validation such as the bias, precision and total error should be reported. Any decision regarding the acceptance of the assay (pre-study acceptance criteria) and consequently the determination of the dynamic range such as the Lower Quantification Limit (LQL) and Upper Quantification Limit (UQL) should be put on hold until adequate information related to the physiological data become available.

As the high, mid and low QC samples are used in the acceptance criteria, it is important to choose their concentrations such that they span the expected range of the study samples. For example, it is of no value to reject batches when large numbers of high concentration quality controls fail, but where the low and medium quality controls are good and when all the study sample results are in the low to medium range. Here the positioning of the high quality control based on expectation before the analysis of incurred samples is flawed – but it does not necessarily make the study sample results invalid.

Precision Profile Tool for Estimating Assay Performance Characteristics

Download the Precision Profile tool here.

Download the Precision Profile data example here.

Download the Precision Profile ReadMe instructions here.

This tool is for the calculation of accuracy, imprecision, sensitivity, dynamic range, etc., for a relative quantitative assay such as Immunoassays or Chromatography assays where the analyte levels are reported in concentration units via calibration from a sample from a reference standard/calibration curve. Data from pre-study validation with the spiked validation/QC samples of different concentrations from multiple assay runs can be pasted in this tool for the calculation of assay performance characteristics listed above. This version can handle any number of runs, replicates, concentrations, etc.

Please see the attached ReadMe for detailed instructions.

Immunogenicity Cut Point Analysis Tool (I-CAT)

Download the I-CAT tool here.

Download the I-CAT data example here.

Download the I-CAT ReadMe instructions here.

We are pleased to provide this Excel-based immunogenicity cut point analysis tool (I-CAT) for the calculation of cut points needed in the assays used for the immunogenicity (anti-drug antibody) assessment of biotherapeutics

in pre-clinical and clinical studies. The different formulae recommended and widely used in practice [Shankar et al. (2008), Shen et al. (2015), Devanarayan et al. (2017), FDA final guidance (2019)] and a simplified version of the analysis method provided in the last paragraph of page 1490 of Devanarayan et al (2017) that is amenable for direct implementation in a program such as Microsoft Excel have been implemented in this tool. This tool also includes the evaluation of distributions, identification of analytical and biological outliers, and several visual aids (graphs) to better understand the assay characteristics related to the analytical and biological factors. A detailed explanation of the criteria to be used for selecting the appropriate cut points is also provided.

Please see the attached ReadMe for detailed instructions.

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Additional References

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