Antibody-Sandwich ELISA Development Kit Catalog #9100

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

This kit provides the liquid components, 96-well plates, plate storage materials, and a template for developing a novel antibody-sandwich format ELISA.

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1. Kit Contents

- Antibody Coating Buffer, 5X, 100 mL (Catalog #644)
- Neptune™ Block, 500 mL (Catalog #63)
- Neptune™ Sample Diluent, 500 mL (Catalog #6125)
- Neptune™ Assay Diluent, 100 mL (Catalog #626)
- HRP Conjugate Stock Stabilizer, 5X, 100 mL (Catalog #667)
- ELISA Wash Buffer, 10X, 500 mL (Catalog #651)
- TMB 1-Component HRP Microwell Substrate, 2 x 100 mL (Catalog #6276)
- Stop Solution for TMB Substrates, 2 x 100 mL (Catalog #6282)
- Costar® 96-Well EIA/RIA Stripwell™ Plates, 10 plates (Catalog #25)
- ELISA Plate Sealing Covers, 1 x 10 pack (Catalog #6287)
- Foil ELISA Plate Storage Bags, 1 x 10 pack (Catalog #6288)
- Desiccant Packets, 1 x 10 pack (Catalog #6289)

2. Key Materials Required But Not Provided

- ELISA plate reader capable of reading 96-well plates at absorbance values of 450 nm
- Washer/aspirator system, or squirt bottle for washing by hand
- Plate-adsorbed capture IgG, high affinity monoclonal or affinity purified polyclonal IgG that is monospecific for the target antigen/analyte

  Minimum quantity: 5-20 mg, depending on intended use following the development phase
- HRP-IgG conjugate (1 mg/mL): horseradish peroxidase (HRP) conjugated, affinity-purified polyclonal or monoclonal IgG specific for the target analyte

  Minimum quantities required will vary, depending upon the intended use of the assay following the development phase. For limited scale ELISA assessment projects, 5 mg of HRP-IgG conjugate may suffice. For on-going projects involving numerous sample assessments per run, 50 mg of HRP-IgG conjugate may be required.

Note: Alkaline phosphatase (AP) may be substituted as the readout enzyme in this format, but this conjugate would require a different colorimetric substrate (pNPP 1-Component AP Microwell Substrate, Catalog #6279) and avoidance of any buffers containing inorganic phosphate salts. These act as reversible inhibitors of the AP enzyme signal generator. If using an AP readout system, substitute the Alkaline Phosphatase Conjugate Stabilizer (Catalog #6271) for the HRP Conjugate Stabilizer included in this kit.

- Target Analyte (antigen standard): purified, biologically isolated and characterized, or recombinant synthesized origin

  Minimum quantity: 10-40 mg, varying by molecular weight of the antigen standard and intended use of the assay. Due to the absolute requirement for the formation of a dual IgG binding event in sandwich ELISA formats, an analyte with a molar weight > 5 kDa is recommended.
- Known positive and negative control samples for verification of ELISA test validity

3. Prerequisite Qualifications for Key Assay Components

- ELISA plate coating antibody (“capture” antibody) must possess high affinity binding kinetics for target analyte being used as the assay standard as well as the native analyte being measured in the assay samples without obscuring epitope(s) necessary for subsequent binding of HRP-IgG conjugate (“up” antibody) to target analyte
- Target analyte standard must be pure and authenticated to be antigenically representative of the target analyte being detected in samples, must contain at least two antigenic epitopes capable of binding antibody
- Affinity purified, target analyte specific HRP-IgG conjugate (up antibody) must possess both high specificity/ high affinity

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binding kinetics for target analyte as well as two to four (2-4) covalently bound, high redox efficiency HRP enzyme molecules for optimal signal generation.

4. Introduction

The Antibody-Sandwich (AS) format ELISA may be the most useful of the immunosorbent assays for detecting antigen because it is both sensitive and robust. An Antibody-Sandwich format ELISA refers to an ELISA plate configuration whereby the analyte being quantified is bound, or "sandwiched" between two layers of antibodies (i.e. capture and detection antibody). In this ELISA format, the plate wells are coated with a monoclonal or affinity purified polyclonal antibody ("capture" antibody). Following the antibody coating step, plate wells are then washed and incubated with a blocking buffer to block the uncoated regions and stabilize the coated antibody. Next, in the capture incubation step, the coated antibody captures target analyte present in samples (see Figure 1). Afterwards, the plate is washed and bound antigen is incubated with the "up" antibody which is used to quantify the amount or concentration of target analyte in the samples. The up antibody may be a monoclonal or affinity purified polyclonal, and it may be directly labeled with enzyme used for detection, such as HRP (in this case, the up antibody and detection antibody are the same). Alternatively, HRP labeled secondary antibody (detection antibody) specific for the unlabeled up antibody may be used in a subsequent incubation step. After the incubation period has ended, ELISA plate wells are washed to remove all non-specifically bound HRP conjugate (detection antibody). The next step is to add a chromogenic HRP substrate, such as TMB, to the plate wells. TMB substrate will allow for the visualization and electronic quantitation of how much target analyte was present within the samples. In general, the more intense the TMB color present within a respective sample-well, the greater the amount of analyte present.

To summarize, the basic AS ELISA format is capable of quantifying the amount or concentration of target analyte present within test samples. This Antibody-Sandwich ELISA Development Kit was created to help guide end users through the common steps and frequently encountered issues associated with building an AS ELISA. The guide describes assay performance milestones that must be achieved to enable progression through the various development stages of the analyte-specific sandwich format ELISA.

5. Basic Considerations to Address Before Starting AS ELISA Development

The overall goal of this AS ELISA development guide is to provide an overview of the steps involved during the assay development process. During the initial planning stages, an immediate determination should be made as to whether or not the necessary quantities of purified antibodies and analyte can be obtained to complete the assay feasibility portion of the project (Section 6). This guide also highlights the six (6) essential assay performance milestones that must be satisfied to assure successful completion of any trustworthy AS ELISA (Table 1). The finalized ELISA should efficiently detect the target analyte within the biological or environmental sample type in which the research project is focused.

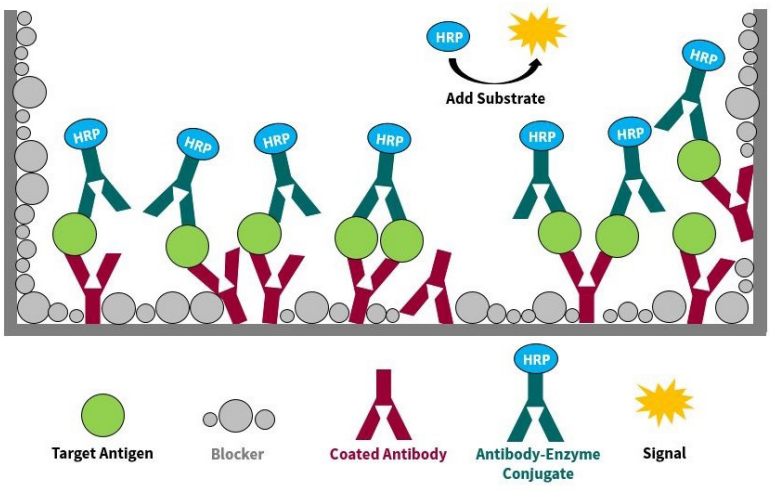
Table 1. Antibody-Sandwich ELISA Development Milestones	
1	Acquisition of Key Assay Components
2	Demonstration of Basic Assay Feasibility
3	Optimization of Capture Antibody Coating
4	Resolution of Sample Matrix Interference Issues
5	Optimization of HRP-IgG Conjugate Concentration
6	Demonstration of ELISA Performance Capabilities

6. Assay Feasibility Assessment

Verification of assay concept feasibility is the single most important milestone of any AS ELISA development project. It is at this development stage that the quality (purity and authenticity) characteristics of the plate coating capture antibody and target analyte standard may be called into question. Additionally, the suitability of the HRP labeled detection antibody or the HRP-IgG secondary detection antibody may be challenged. Fortunately, most commercially sourced HRP-IgG secondary detection antibody conjugates perform well for these purposes.

FIGURE 1: Antibody-Sandwich ELISA

In Antibody-Sandwich ELISAs, a capture antibody specific for the target analyte is coated on the ELISA plate surface. The "up" antibody, also specific for the target analyte, forms the top half of the sandwich. The up antibody can either be directly conjugated to enzyme, allowing it to also function as the detection antibody (pictured, also see Direct Conjugate, Figure 5), or an enzyme conjugated secondary antibody specific for the up antibody may be used as the detection antibody (not shown, see Indirect Conjugate, Figure 5). Antibody-Sandwich ELISAs are used to quantify the amount of target analyte present in a sample.



In the assay feasibility stage, the initial goal is to demonstrate that the assay is capable of showing a simple dose response. Next, focus shifts to optimizing assay sensitivity within the actual biological or environmental liquid matrix environment in which the analyte is typically found. When anticipating the development time for this type of project, it is advantageous to acquire some prior knowledge of the normal concentration range of the target analyte within its sample environment. If the analyte of interest is typically present at > 1 µg/mL concentration, then the need for extensive assay sensitivity enhancement should be minimal. Alternatively, if the normal target analyte concentration levels reside within the low ng/mL to pg/mL range, it will likely be necessary to spend time optimizing assay sensitivity beyond the initial assay feasibility assessment stage.

Finally, assay parameters must be assessed and further optimized to meet acceptable performance criteria within the sample matrix in which the analyte is found. These performance criteria include: dynamic range, sensitivity, linearity, standard recovery, and assay-to-assay precision parameters.

6.1 Prepare an Antibody Coated and Blocked Plate

6.1.1 Day 1 – Coat plate with IgG, leaving blank control wells

A. Add 10 mL of the Antibody Coating Buffer, 5X (Catalog #644) to 40 mL of diH_2O. This provides a 50 mL volume of 1X Antibody Coating Buffer. **Antibody Coating Buffer, 5X may precipitate at refrigerated temperatures. If this happens, gently warm until dissolved.**

B. Transfer 20 mL of 1X Antibody Coating Buffer to a new 50 mL polypropylene tube.

C. Prepare initial antibody coating solution at a concentration of 4 µg/mL by adding 80 µg of the monoclonal or affinity purified polyclonal IgG to 20 mL of 1X Antibody Coating Buffer (this makes the 4 µg/mL solution). For example, if starting with a stock concentration of 1 mg/mL, spike 80 µL into 19.92 mL 1X Antibody Coating Buffer. Mix contents thoroughly by inverting or gently vortexing tube. Do not mix contents in a manner that causes excessive foaming.

D. Remove a new 96-well ELISA plate (Catalog #25) from its packaging. Mark top of the plate with the HRP-IgG conjugate dilutions that will be used to assess useful conjugate concentration levels for future assay development work. Each of the three initial conjugate dilutions will be used within a 4-column section of the plate. A 1:4,000 dilution of the conjugate will be used in well columns 1-4, 1:8,000 dilution in well columns 5-8, and 1:16,000 dilution in well columns 9-12 (Figure 2).

E. Pour the properly mixed 4 µg/mL IgG plate coating solution into a medium-sized solution basin.

F. Pour a small volume of the 1X Antibody Coating Buffer (10 mL) into a medium-sized solution basin. This 1X Antibody Coating Buffer will be used to create the no-antibody blank control wells in rows A and B.

G. Using a calibrated (8 or 12) multi-channel pipettor, carefully dispense a 100 µL volume per well of 1X Antibody Coating Buffer into rows A and B of all columns of the 96-well plate. Carefully dispense a 100 µL volume per well of the 4 µg/mL IgG solution into rows C–H of all columns of the 96-well plate. Always use a 100 µL/well coating volume when beginning any new assay development process. This coating volume is typical for most ELISA development projects.

H. Cover the newly prepared plate with a plate sealing cover (Catalog #6287), Parafilm, or plastic wrap and transfer plate to a humidified, closed environment, such as a sealable plastic container lined with damp paper towels. Incubate the plate in this container at room temperature (RT) overnight in a cabinet or drawer, protected from light to prevent any potential photo-oxidation of assay components.

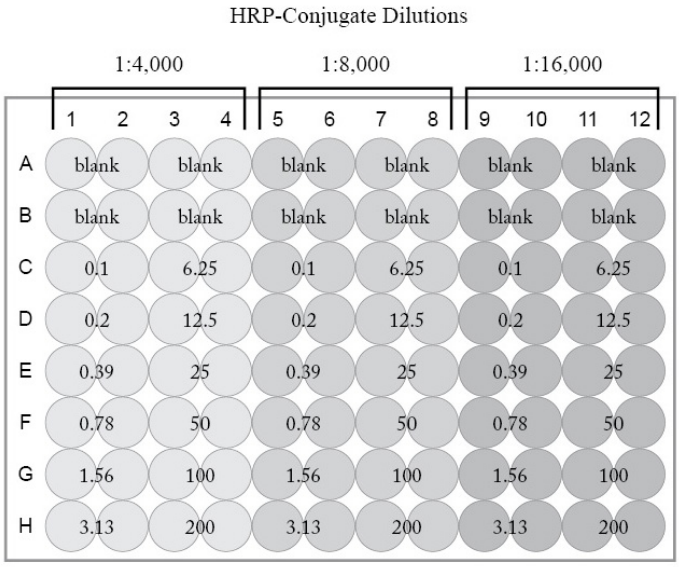


FIGURE 2: Initial Assay Feasibility Plate Map

Plate-wells of initial assay feasibility plate are coated uniformly with 100 µL per well volumes of 4 µg/mL antibody coating solution (capture IgG dissolved in 1X Antibody Coating Buffer), or 1X Antibody Coating Buffer alone to create blank-well controls (rows A and B). Following overnight incubation at RT, plates are washed, blocked with Neptune Block, and either used immediately or dried for long-term storage.

Target analyte standard is subsequently titrated out via serial dilutions and added to three sections of the plate (Section 6.2.1) to obtain a preliminary estimate of assay sensitivity. The suggested initial concentration of the top standard for the assay feasibility trial is 200 ng/mL. Upon completion of the analyte capture step, plates are incubated with three different dilutions of the conjugate (Section 6.2.4), washed, and developed with TMB substrate (Section 6.2.5).

6.1.2 Day 2 – Wash and block plate to reduce background and stabilize capture IgG

A. Prepare a 1 L volume of 1X ELISA Wash Buffer. This is easily prepared by pouring a 100 mL volume of ELISA Wash Buffer, 10X (Catalog #651) into a 1 L glass graduated cylinder containing a magnetic stir bar. Bring the volume up to the 1 L mark with diH_2O. Place the 1 L cylinder on a stir plate and mix for 5-10 minutes. **ELISA Wash Buffer, 10X may precipitate at refrigerated temperatures. If this happens, gently warm until dissolved.**

B. Pour 1X ELISA Wash Buffer into a squirt bottle or into a large reservoir connected to an 8/12 channel washer/aspirator manifold device designed for washing of ELISA plates.

C. Allow blocking buffer to equilibrate at room temperature (~25°C) prior to use. Pour a 35-40 mL volume of Neptune™ Block (Catalog #63) into a new solution basin. Set a multichannel pipette to deliver a 300 µL dispensing volume.

D. Aspirate the well contents using a multi-8/12 channel hand-pipettor, plate washer, or 8/12 channel aspirator manifold. This manifold is connected to a vacuum source, which is separated by a liquid-capturing reservoir of multi-liter capacity.

E. Using the squirt bottle or washer manifold, fill each of the empty wells with 1X ELISA Wash Buffer.

F. Aspirate wash buffer contents as described in step D, or simply dump plate contents into a sink.

G. Repeat wash process (E-F). Pound the plate-wells dry on a small stack of paper towels.

H. Immediately add 300 µL volumes of Neptune Block to each well of the 96-well plate. It is very important that coated wells not be allowed to dry out at this stage. If the multi-channel pipettor cannot be set to dispense 300 µL per delivery, set the pipettor to dispense 100 µL or 150 µL per delivery. Quickly apply blocking buffer across the entire plate and repeat to obtain a total blocking buffer volume of 300 µL/well.

I. Cover the Neptune Block-containing plate with a plate sealing cover, Parafilm, or plastic wrap and place into the humidified, sealable plastic container. Incubate the blocked plate at RT overnight in a light-protected cabinet or drawer.

6.1.3 Day 3 – Complete final steps of plate blocking and optional drying process

A. If planning to begin the initial stage of assay development on day 3, the blocking buffer can be left in the plate, refrigerated, until the initial assay feasibility verification process can begin (Section 6.2). In most cases, these blocker-filled plates can be stored for up to 5 days at 2-8°C without much concern over plate performance.

B. For plates that are intended to be dried down and stored, aspirate the blocking buffer from the plate-wells and pound out any extra blocking solution onto paper towels.

C. Air dry the blocked and stabilized ELISA plate in a lateral flow fume hood or vacuum pump supported vacuum chamber for 4-6 hours at RT.

D. Store blocked and dried plate(s) in the provided moisture-proof Foil Storage Bags (Catalog #6288) at 2-8°C, protected from light, with desiccant pouches (Catalog #6289) to minimize moisture exposure. Plates stored in this manner should retain their antibody derived capture function for over a year.

6.2 Preliminary Assessment of HRP-IgG Conjugate Working Concentration Range

6.2.1 Prepare assay standards

A. Confirm two important criteria regarding the antigen standard: first, the antigen is present in a highly purified form and verified to antigenically represent the target analyte being detected and quantified within this ELISA format; second, an accurate estimate is known for the protein/analyte (standard) concentration or dry weight mass present in the analyte standard vial.

B. Set up a simple 2-fold serial dilution scheme in 12 properly labeled 75 mm glass test tubes (Figure 3).

C. Allow sample diluent to come to room temperature prior to use. Prepare a 2 mL volume of a 200 ng/mL antigen stock using Neptune™ Sample Diluent (Catalog #6125) provided in the kit. For example, if the antigen is supplied at 1 mg/mL:

   a. Make an initial 1:100 dilution by spiking 20 µL of the 1 mg/mL stock into 1980 µL of Neptune Sample Diluent.

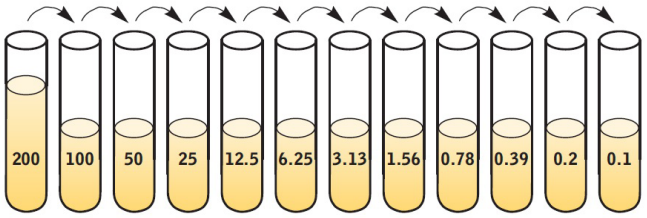
   b. Add 40 µL of this 1:100 dilution to 1.96 mL of Neptune Sample Diluent, which results in a total dilution of 1:5,000 and a final concentration of 200 ng/mL.

D. Add 1 mL Neptune Sample Diluent to each of the remaining labeled test tubes (100 – 0.1 ng/mL) within the serial dilution series.

E. Add 1 mL of the 200 ng/mL antigen stock sample to the tube labeled 100 ng/mL. Mix thoroughly.

F. Repeat serial 2-fold dilution process throughout the remainder of the dilution tube series. Change pipette tips after every dilution in the series to avoid unintended carry over.

FIGURE 3: Serial Dilution of Standard Curve



6.2.2 Load ELISA plate with analyte standards and blanks

A. In a plate that has not been dried and packaged for long-term storage, aspirate blocking buffer from plate-wells and pound out any extra blocking solution onto paper towels. Once the blocking solution has been removed, it is best to get the antigen standards as well as the blanks (Neptune Sample Diluent-Only) into their respective plate locations as soon as possible.

B. Add 100 µL per well of Neptune Sample Diluent-Only to each well in rows A and B for the Blanks (Figure 2).

C. Add 100 µL of the lowest concentration antigen standard (0.1 ng/mL) to each of the three duplicate pair positions on the ELISA plate (Figure 2).

D. Proceed to add, from least concentrated to most concentrated, the remaining antigen standards to the ELISA plate-wells. This allows one to keep the same pipette tip(s) in place over the entire plate loading process.

E. Cover ELISA plate with a plate sealing cover, Parafilm, or plastic wrap and place an empty ELISA plate on top. This covering process will minimize evaporation of well contents during incubation.

F. Incubate ELISA plate for 60 minutes at 37°C or 90 minutes at RT, protected from light.

6.2.3 Prepare three HRP-IgG conjugate dilutions to be examined in this first trial run

A. Label three new 50 mL polypropylene centrifuge tubes with 4,000, 8,000, and 16,000. Note: These numbers represent dilution factors of the conjugate stock assuming the stock concentration is approximately 1 mg/mL. If the conjugate stock is not at 1 mg/mL, the dilution factors should be adjusted proportionally.

B. Allow conjugate stabilizer to come to room temperature prior to use. Dilute 4 mL of the HRP Conjugate Stock Stabilizer, 5X (Catalog #667) 1:5 into 16 mL diH_2O to yield 20 mL of 1X conjugate stabilizer.

C. Prepare a 1:100 dilution of the conjugate by spiking 10 µL of the 1 mg/mL conjugate stock into 990 µL of the 1X conjugate stabilizer. Mix test tube containing the 1:100 dilution of the HRP-IgG conjugate thoroughly.

D. In the tube labeled 4,000, prepare the 1:4,000 conjugate dilution by spiking 250 µL of the 1:100 conjugate dilution into 9.75 mL 1X conjugate stabilizer (1:40 dilution). Mix the tube containing the 1:4,000 dilution of the HRP-IgG conjugate thoroughly by capping and inverting the tube multiple times.

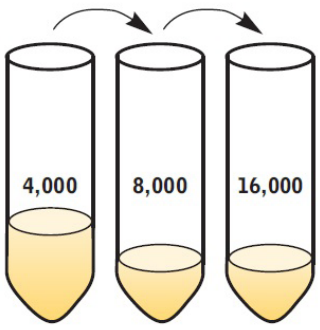
E. Put 4 mL of 1X conjugate stabilizer into the tubes labeled 8,000 and 16,000.

F. Transfer 4 mL from the 1:4,000 dilution tube into the tube labeled 8,000. Cap off tube and mix carefully but thoroughly (Figure 4).

G. Transfer a 4 mL volume of the 1:8,000 dilution to the 4 mL volume of 1X conjugate stabilizer in the tube labeled 16,000. Cap off tube and mix carefully.

H. Set the 1:4,000, 1:8,000, and 1:16,000 conjugate dilution tubes in a drawer, protected from light until ready to use (the porphyrin redox ring of the HRP enzyme tag is light sensitive).

FIGURE 4: Serial Dilution of Conjugate



6.2.4 Wash plate and add the three HRP-IgG conjugate dilutions

A. Pour 1X ELISA Wash Buffer (Section 6.1.2A) into a squirt bottle or into a large reservoir connected to a plate washer, or an 8/12 channel washer/aspirator manifold device that was designed for manual washing of ELISA plates.

B. Label three solution basins as 4,000, 8,000, and 16,000 to represent the HRP-IgG conjugate dilution factors being analyzed.

C. Transfer the contents of each conjugate dilution tube to their respective solution basin.

D. Wash the plate 3X using 1X ELISA Wash Buffer. After the last wash, pound the plate onto a stack of paper towels to assure complete removal of residual wash buffer.

E. Using a multi-channel pipettor, add 100 µL per well of the 1:16,000 conjugate dilution to the designated regions of the ELISA plate (Figure 2). Subsequently, add the 1:8,000 and 1:4,000 conjugate dilutions to their respective locations on the plate. Addition in this manner eliminates the need to change pipette tips after the addition of each HRP-IgG conjugate dilution.

F. Cover plate as directed earlier and incubate at RT for 60 minutes, protected from light.

6.2.5 Wash plate and add TMB substrate

A. Allow substrate to come to room temperature prior to use. Pour approximately 15 mL of TMB 1-Component HRP Microwell Substrate (Catalog #6276) into a new solution basin. Place it in a drawer to protect from light until it is added to the plate.

B. Wash plate 4X in 1X ELISA Wash Buffer after completion of the HRP-conjugate incubation period.

C. Remove any residual wash buffer from plate by tapping onto a stack of clean paper towels.

D. Add 100 µL/well of the TMB substrate to every well using a multi-channel pipettor. Discard tips and load pipettor with new tips.

E. Place the plate in a drawer protected from light and check the level of blue-green color development every five minutes. Substrate is typically incubated for 15-20 minutes at RT.

F. Once the TMB substrate is added to the plate, pour 15 mL of the Stop Solution for TMB Substrates into a new solution basin. This stop solution is not light-sensitive and may be left on the lab bench until needed.

G. Continue to observe TMB color development in ELISA plate. When it is apparent that the wells containing the lower antigen standard concentrations are beginning to take on a slightly blue-green tint, add 100 µL of stop solution to each and every well, using the multichannel pipettor to accelerate the addition process. The addition of stop solution will further oxidize the HRP-oxidized TMB substrate, converting it from blue-green to yellow in color. This stabilizes the reacted product for up to 1 hour, and increases the dynamic range and reproducibility of the assay.

6.2.6 Acquire plate reader results.

A. Set up a 96-well plate reader to quantitate absorbance at 450 nm.

B. Generate standard curves for each set of analyte standards using an analysis model deemed appropriate (examples include Log/Log, 4-parameter regression analysis, 5-parameter regression analysis, absorbance versus concentration line fit, etc.).
Note: which model to select may vary from one assay to the next, and will need to be determined by the end user.

C. Observe the plate absorbance results. Determine the lowest antigen standard concentration yielding average absorbance OD_{A450} values > 0.1 OD units higher than the corresponding blank well controls located within rows A and B of the plate.

6.2.7 Perform an initial macroscopic ELISA performance analysis.

A. Observe the wells and their corresponding OD_{A450} values. What to expect:

a. Absorbance of the blank wells should be < 0.1 OD_{A450} units with very little evidence of yellow color visible. If all of the blank wells have stopped TMB OD_{A450} values > 0.3, then one must assume that there is a plate-blocker or HRP-conjugate dilution issue!

b. Some wells should be visibly yellow.

c. There should be a distinct difference between the observed OD_{A450} values from wells containing the most dilute antigen standard (bottom standard) versus the most concentrated standard (top standard), with proportionate decreases in signal OD that reflect the dilution scheme, i.e., approximately 2-fold numerical decreases in absorbance between adjacent standards.

d. Generally speaking, due to comparison of 3 different conjugate dilutions, a respective antigen standard should yield the highest OD_{A450} signal in columns 1-4 using the 1:4,000 conjugate dilution, a medium signal in columns 5-8 using the 1:8,000 conjugate dilution, and the lowest signal in columns 9-12 using the 1:16,000 conjugate dilution.

B. Perform a quick cursory assessment of these initial assay results. If these four conditions (listed in a-d, above) are observed, proceed to the formal AS ELISA development process. If not, make the necessary procedural adjustments so that these minimal performance expectations are met. Use the Troubleshooting Guide (Table 2) for guidance.

7. Addressing Conjugate Availability Issues

The most common assay development hurdle encountered when developing ELISAs for novel analyte targets is the difficulty in finding a commercial source of an HRP-conjugated antibody that is specific for the captured analyte. This guide briefly summarizes two widely accepted strategies for addressing this common limitation. Both options require that the up/sandwich antibody be identifiable by another component which is covalently bound to a readout enzyme like HRP or Alkaline Phosphatase (AP). The specific aspects of these two methods are addressed in the subsequent sections.

7.1 Use of Anti-Species HRP-IgG or AP Conjugates

In this option, a commercially available anti-species isotype (e.g., rabbit IgG Fc, goat IgG Fc, mouse IgG Fc, etc.) HRP-IgG or AP conjugate can be added as a third tier to label the up/sandwich antibody bound to the upside of the captured antigen on the plate well surface. In this situation, the HRP-IgG or AP conjugate is the detection antibody.

Commercial sources for enzyme-labeled, anti-species isotype antibody are widely available and can easily be found from a simple internet search.

Important: For this scheme to work, the animal source for the up/sandwich IgG (Figure 5, Indirect Conjugate, gray antibody) must be different from the animal host for the plate-adsorbed capture IgG (Figure 5, Indirect Conjugate, red antibody).

Development of a three-tiered sandwich hybrid format would proceed as one would pursue the more classic two-tiered sandwich format. In cases where an additional enzyme-labeled IgG is required to generate the final signal, it is recommended to use a dilution greater than 1:8,000 of a 1 mg/mL HRP-IgG conjugate as the starting point. This would be added to the plate wells after the antigen/analyte has first been captured onto the plate and after the unlabeled up/sandwich IgG has bound the upside of the captured antigen molecule. Upon completion of the requisite wash steps, a 100 µL volume of the conjugate (e.g., HRP-labeled anti-animal IgG isotype) is added to each plate well and allowed to incubate for 60 minutes at RT protected from light. Wash the plate four times in 1X ELISA Wash Buffer, which is compatible with HRP conjugates. Use a non-phosphate wash buffer for AP conjugates. Aspirate or pound out excess wash buffer and add the enzyme-appropriate substrate for color development.

FIGURE 5: Direct versus Indirect Conjugates

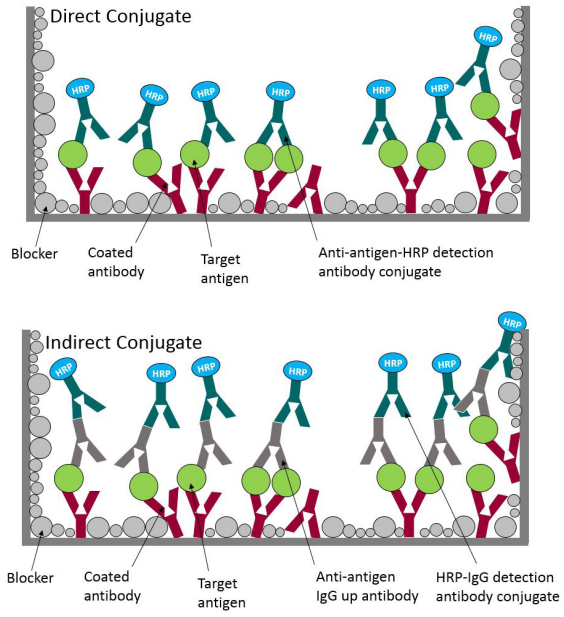


Table 2. TROUBLESHOOTING GUIDE		
Problem	**Cause**	**Action/Solution**
Blank has OD_{A450} > 0.1, easily recognizable yellow colored **Blank Wells** = high background signal problems	Nonspecific binding by conjugate to unblocked or inadequately blocked regions of plate-well surface, or some component within plate-blocker formulation itself.	Ensure blocking procedure was performed correctly. Verify that conjugate was affinity-purified and recognizes antigenic epitope sequences not present within the capture antibody or blocker components adsorbed to the plates.
	Operator or auto-washer error as relates to the proper plate washing process.	Verify that auto-washer is properly aspirating out all of the plate-well contents prior to addition of the next batch of ELISA wash buffer. If plates are manually washed, verify that these same plate washing parameters are being properly carried out.
No signal	Assay set up incorrectly or use of incorrect reagents.	Check plate coating procedure, antigen standard titration, and conjugate dilutions. Was the TMB substrate incubation step performed? Repeat assay.
	Capture or conjugated antibodies not recognizing antigen.	Use an antibody specific for antigen standard.
	Conjugate stored incorrectly or subjected to repeated freeze/thaw cycles.	Use a fresh aliquot of conjugate that has not undergone multiple freeze-thaw events or purchase a new vial of conjugate.
	Not using a 96-well plate that was treated for use in ELISA formats.	Use included ELISA plates, or obtain a brand of 96-well plates that are designated for use in ELISA formats. These plates are factory pre-treated to allow polystyrene surfaces to nonspecifically bind proteins.
Little to no difference between the TMB OD_{A450} signals from plate-wells containing the bottom and top antigen standards	Incorrect placement of standards	Follow plate map as instructed (Figure 2).
	Incorrect standard titration	Follow serial dilution instructions. Change pipette tips between standard dilutions
	Contamination of standards	Change pipette tips between addition of standards to plate or load the standards onto the plate in the order of lowest to highest concentration.
Inconsistent OD_{A450} values between adjacent sample wells.	Incorrect placement of standards and samples.	Follow plate map as instructed (Figure 2).
	Inadequacies in plate washing technique	Examine vacuum portals on plate manifold to verify whether or not there is a partial obstruction in a particular sample uptake port/line.
Entire plate displays a uniform-dark yellow (saturated) color.	Nonspecific binding of conjugate to plate-well or capture antibody	Verify blocking procedure was correctly followed. Verify that conjugate was affinity-purified and recognizes antigenic epitope sequences **not present** on the plate adsorbed capture antibody.
	Nonspecific binding of conjugate to component of blocking buffer.	Examine the binding specificity of the conjugate. Consider a different blocking buffer formulation, possibly synthetic (e.g., SynBlock, Catalog #643).
	Incorrect conjugate dilution.	Follow conjugate dilution instructions exactly. If conjugate was supplied at >1 mg/mL, adjust dilution instructions proportionally.
	Insufficient washing	Increase the number of washing cycles
	Accidental contamination of TMB substrate.	Use fresh substrate and visually confirm that it is colorless prior to addition to plate.
TMB OD_{A450} signal **does not** decrease from higher OD_{A450} levels (2.8-3.3) with increased conjugate dilution.	Conjugate is not diluted enough.	Repeat assay with greater dilutions of the conjugate. Consider diluting out the conjugate another 10-fold.

7.2 Use of Commercial Streptavidin-HRP/AP Conjugates

Alternatively, the up/sandwich antibody can be biotinylated to make it into a detection antibody and subsequently detected with commercially available streptavidin-HRP or -AP conjugate products. One advantage of this option over the HRP-labeled anti-animal IgG isotype readout system is that there are no concerns over using a different host species for the plate-adsorbed IgG versus the up/sandwich IgG. One simply follows a simple, commercially available kit protocol to incorporate 3-6 biotin molecules (tags) per IgG molecule. This allows the up IgG to be easily recognized by a >1:5,000 dilution of streptavidin-enzyme product.

Though the streptavidin-HRP or -AP signal generation option is a straightforward and reliable method of generating a specific signal in cases where the up IgG lacks a readout enzyme component, the hydrophobic structure of the biotin tags will significantly enhance the level of nonspecific binding (NSB) problems. Typically, this sticky problem is addressed through the deliberate limitation of the number of biotin molecules per IgG to a range of 3-6. Incorporation of additional biotin tags per IgG molecule will not necessarily lead to increased streptavidin-enzyme binding due to steric hindrance. However, the extra biotin loading will greatly increase the tendency of the IgG-biotin complex to non-specifically bind to all surface molecules present in the ELISA plate well. Therefore, it is best to only incorporate as many biotin groups as is necessary for achieving good streptavidin-enzyme binding kinetics with the up IgG. Loading biotin at levels exceeding 6 biotins per IgG can lead to future difficulties dealing with the high biotin-associated NSB noise.

Another consideration when seeking to limit the degree of non-specific adherence of IgG-biotin to the plate well surface is to recall the two driving factors behind NSB interactions: exposure time and concentration of the soluble component. To minimize this NSB interaction, the IgG-biotin concentration should be optimized to achieve the maximum signal-to-noise ratio at the lowest IgG-biotin concentration. Limitation of the IgG-biotin exposure time with the plate well surface can also help to reduce the magnitude of the NSB event. By limiting the concentration and exposure time parameters of the IgG-biotin component, the biotin-associated NSB problems can be greatly decreased.

Generally speaking, the same NSB-limiting strategy used for the IgG-biotin component should be applied to the streptavidin-HRP or -AP component. Though the substitution of streptavidin over traditional avidin has decreased the NSB potential of avidin-HRP or -AP conjugates, limiting both the concentration and exposure time of the streptavidin-HRP or -AP component helps maximize specific signal-to-noise ratios. Typical 1 mg/mL commercial streptavidin-HRP or streptavidin-AP conjugate preparations should perform optimally at dilutions of 1:5,000 – 1:10,000.

8. Optimization of ELISA Sensitivity

Antibody-Sandwich ELISA optimization, beyond the initial demonstration of basic assay protocol feasibility, typically focuses on two rather universal performance objectives: 1.) Enhancement of ELISA sensitivity within the biological sample matrix in which the target analyte is to be measured and 2.) Minimization of complex sample matrix effects that lead to the under-reporting of analyte concentration in complex biological samples.

In the subsequent sections, brief descriptions of the tactics most commonly used to address these respective issues are provided, yet there is no universal approach. Every antigen-antibody interaction has its own peculiarities driven by unique factors. Two major considerations are the binding affinity constant and the binding specificity characteristics of the antibody for the target antigen-analyte. Other key factors include sample matrix-associated interference that can suppress the antigen to antibody binding kinetics and sample-associated binding proteins or receptors that compete with capture IgG for binding antigen-analyte. Because of these and other complex interactive and functional relationships, a logical trial and error approach is the best way to meet assay development goals.

Before addressing the parameter of assay sensitivity, the normal concentration range of the target analyte within its sample environment must be considered. If the analyte of interest is typically present at > 1 µg/mL concentrations, there is no need to commit effort toward achieving minimum detectable dose sensitivity in the sub ng/mL analyte concentration range.

For the vast majority of analyte detection projects, maximizing assay sensitivity is essential for obtaining an assay that has any practical relevance to real world situations. In most of these ELISA development situations, emphasis is placed on optimizing the IgG coating and blocking parameters of the assay. This is closely followed by fine-tuning the HRP-IgG conjugate component of the assay. This process optimizes both the conjugate concentration and incubation time. All of the above parameters have direct effects on the specific signal output as well as the amount of non-specific background noise that is generated during the assay. In simple terms, the greater the non-specific background signal, the lower the possibility of obtaining optimal assay sensitivity.

8.1 Optimize IgG Plate Coating Protocol

8.1.1 Evaluate IgG coating concentrations to maximize analyte capture efficiency

Once a working assay format has been achieved, a more sensitive ELISA prototype can be developed. This can be accomplished by performing a simple and straightforward study to determine the optimal plate coating concentration. For best results, all refrigerated liquid components should be equilibrated at room temperature before use.

A. Using the generic plate coating protocol described in Section 6.1.1, prepare four ELISA plates bearing different IgG coating concentrations across the plate surface (Figure 6).
   a. Within the wells in columns 1-2 on each plate, coat with a 2 µg/mL capture IgG concentration.
   b. Coat wells in columns 3-4 on each plate with 4 µg/mL IgG coating concentration.
   c. Repeat the coating process with the 6 µg/mL, 8 µg/mL, and 10 µg/mL IgG coating concentration in columns 5-6, 7-8, and 9-10 respectively. Columns 11-12 can simply be filled with blank 1X Antibody Coating Buffer (no capture IgG).

B. Follow the blocking instructions provided in Section 6.1.2. These plates may be dried after blocking and stored at 2-8°C in ELISA plate storage bags containing desiccant pouches.

C. Using duplicate ELISA plates with multiple IgG coating concentrations prepared above, set up a standard curve of seven standards in Neptune Sample Diluent across each plate (Figure 6). Use the top standard from Section 6 that yielded a OD_{A450} of 2.5-3.0. Row A should be reserved as a sample-diluent-only blank in that it only contains Neptune Sample Diluent without any diluted analyte standard. Use a multichannel pipettor to add 100 µL per well of Neptune Sample Diluent to row A. Next, add the diluted standards starting with the lowest standard concentration (least concentrated in row B) to the highest standard concentration (most concentrated in row H). This plate loading strategy is employed to accelerate and simplify the loading process.

D. Perform the analyte capture incubation step and subsequent washing steps.

E. Select an HRP-conjugate dilution factor from one of the three (3) conjugate dilutions evaluated in the Assay Feasibility Assessment where 1:4,000, 1:8,000, and 1:16,000 dilutions of the HRP-conjugated IgG were examined (Section 6). Ideally, this dilution of the conjugate would lead to a stopped TMB OD_{A450} signal between 2.5 and 3.0 for the high standard and a blank well OD_{A450} < 0.15 units. Note that the finalized HRP conjugate dilution has yet to be determined. The current focus is to determine a suitable conjugate dilution factor range which can be further adjusted as the AS ELISA development process evolves.

F. Carry out the rest of the assay according to the protocol in Section 6.2.5.

G. For each IgG coating concentration, divide the average OD_{A450} value for the highest standard with an OD_{A450} signal < 3.0 units by the average OD value for the respective blank wells (row A) to calculate the signal-to-noise (S/N) ratio for each IgG coating concentration.

$$S/N = \frac{\text{Average } OD_{A450} \text{ high standard}}{\text{Average } OD_{A450} \text{ blank}}$$

H. Examine the S/N ratios as well as their ranking from highest to lowest signal output.

I. Identify the IgG coating concentration that yielded the highest signal-to-noise ratio.

8.2 Address Sample Matrix-Derived Signal Inhibition

Developing sandwich ELISA formats for quantitation of soluble peptide/protein-based target molecules within biological fluids (serum, plasma, urine, etc.) will often require some additional modification of the standard/calibrator well diluent composition. This modification is needed to account for the complex environment (matrix) that the target analyte is dissolved in.

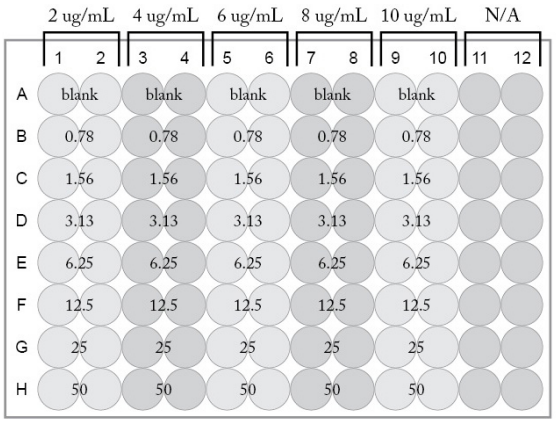
Dealing with the signal-quenching effects arising from sample composition complexity can be very labor-intensive. Included with the Antibody Sandwich ELISA Development Kit is a 100 mL bottle of Neptune™ Assay Diluent (Catalog #626). Assay diluent formulations like Neptune Assay Diluent are incorporated for the sole purpose of increasing the matrix complexity within all wells of the ELISA format assay.

Biological samples are quite complex relative to initial standard/calibrator solution matrices. By including an assay diluent within all ELISA plate wells, the goal is to disproportionately increase the matrix complexity within the standard/calibrator wells relative to the sample well matrix. At issue here is the relative ability of the plate-coated antibodies to bind the target analyte within the standard curve diluent environment versus the complex sample solution environment. When matrix complexity is less within the standard curve wells than within the sample wells, the binding efficiency rates within the standard curve wells are higher than within the sample well environment. When the sample well stopped TMB substrate OD_{A450} absorbance is read against the standard curve OD_{A450} series to extrapolate antigen-analyte concentration in sample wells, the curve extrapolated concentration values of the antigen-analyte within the sample wells will be suppressed or under-reported. To help reduce the matrix complexity disparity between the standard/calibrator diluent and the wells containing the sample solutions, the addition of a 50 µL or 100 µL volume of Neptune Assay Diluent to each and every well of the plate (including the standard curve wells) is recommended.

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

FIGURE 6: IgG Coating Optimization Plate Map

ELISA plate-wells are coated with 2 µg/mL, 4 µg/mL, 6 µg/mL, 8 µg/mL, and 10 µg/mL concentrations of the analyte capture IgG to assess optimal coating concentrations for maximizing capture efficiency, signal-to-noise, and assay sensitivity. Plates are washed, blocked, and either used or dried according to protocol. Analyte standards are set up according to the plate map pictured. This example utilizes a high standard concentration of 50 ng/mL. Plate duplicates are exposed to a pre-determined dilution of the HRP-IgG conjugate. This conjugate dilution was determined from initial assay concept feasibility assays to yield a high absorbance signal between 2.5 and 3.0 OD_{A450} units and blank (0 standard) reading < 0.15 OD_{A450} units.



Alternatively, if assay detection sensitivity is not an issue, one can also dilute the sample solutions that are being analyzed by 1:2, 1:4, or 1:8 in the Neptune Sample Diluent, the diluent used to dissolve the assay standards within the standard curve. When sample dilution is employed to minimize sample matrix complexity differentials, the standard curve derived antigen-analyte concentration will have to be multiplied by the dilution factor used to derive the actual concentration in the sample.

8.3 Minimize Conjugate-Derived Nonspecific Binding

Another important issue to address in AS ELISA development is the need to minimize the nonspecific binding interactions between the HRP-IgG conjugate and the blocked ELISA plate-well surfaces. In most problematic nonspecific binding incidents, the conjugate binds in a nonspecific manner to the blocker present on the blocked plate-well surface. When proteins and/or other chemical additives are incorporated into the diluent formulation used to dilute the anti-isotype HRP conjugate, these additives can passively interact with the dissolved HRP conjugate to reduce its tendency to bind nonspecifically to the blocked plate-well surface. ICT's Antibody-Sandwich ELISA Development Kit includes a 100 mL bottle of HRP Conjugate Stock Stabilizer, 5X (Catalog #667). Dilution of this component 1:5 in diH_2O yields a 1X conjugate stabilizing solution. One of the benefits of this 1X conjugate stabilizing solution is that it can be used to preserve the HRP-IgG conjugate activity at its 1X use concentration. When present within a 1X HRP conjugate stabilizing solution, the sticky, hydrophobic regions of the HRP-IgG conjugate molecules pre-associate with protein and non-protein additives present in the conjugate stabilizing solution, resulting in a reduction of nonspecific interactions between the HRP conjugate and the blocked plate-well surfaces.

Beyond the use of a suitable conjugate stabilizer solution for the conjugate incubation step, two additional strategies can be used to help reduce the nonspecific binding interaction of the conjugate with the immobilized components on the plate-well surface. In general terms, nonspecific binding activity is modulated by two major parameters: concentration of the conjugate and exposure time of the conjugate to the plate-well surface. An increase in either parameter will always lead to increased nonspecific binding of the conjugate. Fortunately, these two major factors driving nonspecific binding are easily manipulated.

8.3.1 Conjugate Concentration

When attempting to maximize assay sensitivity levels, resist the temptation to use greater than necessary conjugate concentrations. Although increasing the concentration of the HRP-conjugate will drive the specific binding kinetics toward shorter equilibrium establishment incubation times, the level of nonspecific binding to plate-well surfaces will also increase with higher conjugate concentrations. When the conjugate concentration exceeds a certain threshold, the level of background signal (noise) will increase at a disproportionally higher rate than the increase in specific signal. This always leads to high signal in the blank, where visible color development is undesirable, and the signal-to-noise ratio drops off precipitously. This will handicap any efforts to maximize the sensitivity of the ELISA.

8.3.2 Conjugate Exposure Time

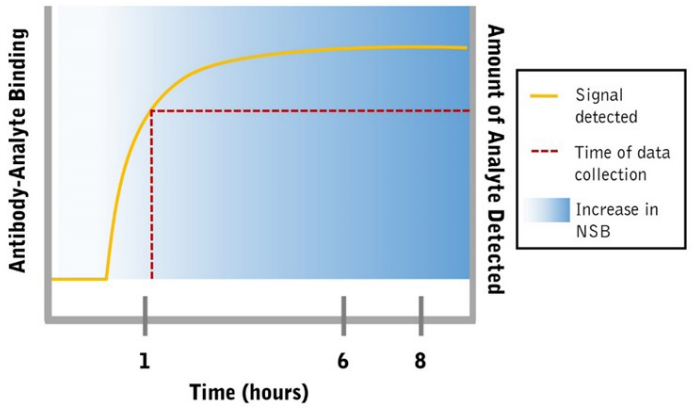
Nonspecific binding can also be modulated by the careful control of the conjugate exposure time within the assay well. Utilization of high-quality HRP-IgG conjugates with high binding affinity constants to the target analyte are essential for any quality AS ELISA. Conjugates that are composed of higher affinity antibodies require less incubation time to reach equilibration. Realistically, most ELISA protocols do not seek to achieve complete antibody/antigen equilibration status. Higher binding affinity kinetics antibody-conjugates will require less plate exposure time to achieve a useful signal. In simple terms, the shorter the conjugate exposure time to the plate-well surface, the less time for the conjugate to bind to the plate-wells in a nonspecific manner (refer to Figure 7 for illustration of this concept).

9. Demonstration of ELISA Performance Capabilities

Upon completion of the ELISA sensitivity optimization steps in Section 8, an overall assessment of ELISA performance capabilities should be performed, including assay linearity, dynamic range, sensitivity, spike (standard) recovery efficiency, and assay-to-assay precision. This final analysis is especially important when the target analyte concentration differentials between a positive and negative clinical event are small, or where overlap of clinically significant target analyte concentration is known to occur, as inadequate performance within these key areas will compromise the accuracy and subsequent interpretation of the experimental results. A detailed discussion of how to perform appropriate statistical

FIGURE 7. Antibody-Specific versus Non-Specific Signal Over Time

Illustration of the two major signal generating processes (antibody-driven specific binding and hydrophobic interaction driven nonspecific binding) was created to show the multi-dimensional signal generation processes occurring within all ELISA formats. Specific, HRP-antibody-conjugate-derived, binding signal levels off as the antibody-antigen-specific binding process approaches equilibrium. Unfortunately, nonspecific, hydrophobic-interaction-facilitated, HRP-antibody-conjugate binding signal accumulation proceeds unabated. As a result, specific signal to background signal (i.e. signal-to-noise) ratios drop off as the amount of nonspecific signal increases over time.



analyses is outside the scope of this manual. Suggested statistical approaches and calculations are included below, but ultimately it is up to the end user to determine what statistical methods are appropriate for their particular assay.

9.1 Assess ELISA Linearity

The linearity of an assay is its ability (within a given range) to obtain test results which are directly proportional to the concentration of target antigen within a sample. When assay linearity has been achieved for any given sandwich ELISA testing format, for example, every two-fold serial dilution of the sample should translate into a two-fold reduction in the assay curve-derived analyte concentration calculation. A linear relationship should be evaluated across the range of the assay (see Section 9.2). Linearity can be evaluated by the visual inspection of a plot of signals as a function of target antigen/analyte concentration. If there is a linear relationship, test results can be subsequently evaluated using statistical methods. Examples of potential statistical methods to employ include the calculation of a regression line by the method of least squares. Additional calculations such as correlation coefficient, y-intercept, slope of the regression line, and residual sum of squares may also provide useful information.

In the vast majority of sandwich ELISA-based analysis of serum and plasma sample types, the need for maximum assay detection sensitivity will often preclude the luxury of being able to dilute the plasma or serum sample at least three or four-fold in the sample/calibrator/standard diluent (note: there are multiple names for this assay component, henceforth referred to as SCS diluent). Because of the typical large disparity in matrix complexity between the less complex SCS diluent and the more complex serum or plasma sample matrix, serious underestimation of target analyte present in serum or plasma samples is likely to occur. A brief overview of the problematic symptoms and the matrix complexity differentials causing these under-recovery problems is discussed below.

In cases where the SCS diluent is not properly matched up with the matrix complexity of the sample solution (e.g., serum, plasma), a simple serial two-fold dilution analysis of analyte-containing serum or plasma samples does not reveal a coordinating two-fold drop off in perceived analyte concentration as read off the standard curve. For example, an artificially positive sample was created at 100 ng/mL by adding standard-analyte to a known negative control plasma sample. A four-point, two-fold serial dilution in SCS diluent was conducted and dilutions were analyzed by ELISA. Protein concentrations were derived from the established standard curve and used to calculate the percent recovery values, i.e. the outcome value divided by the expected value x 100. This hypothetical example reveals under-recoveries and lack of assay linearity (Table 3).

9.2 Assess Dynamic Range of ELISA

The range (or dynamic range) of an assay is the interval between the upper and lower concentration of target antigen in the sample for which it has been demonstrated that the ELISA has a suitable level of precision, accuracy, and linearity. The dynamic range of an ELISA is normally derived from linearity studies and is defined as the range of target analyte concentrations within which an accurate assessment of a given analyte concentration can be determined. To expand on this definition further, the lower concentration limit of a dynamic range parameter is typically set at the lowest concentration that bears an average OD_{A450} value greater than 0.06 above the average OD_{A450} value of the blank (0) standard. This differential between OD_{A450} units is referred to as the low delta. The lower concentration limit should never fall below the lowest standard making up the standard curve. The upper concentration limit of the linear range can be assessed by identifying the top antigen standard that allows an R^2 linear regression correlation coefficient > 0.95. The range can also be established by confirming that the assay provides an acceptable degree of linearity, accuracy, and precision when applied to samples containing amounts of target antigen within or at the extremes of the specified range of the assay.

In most properly constructed ELISA formats, the top end of the dynamic range calculation should never be assumed to be simply the assay standard with the largest absorbance OD. When calculating the upper analyte concentration limit of the assay's dynamic range, a linear regression R^2 calculation must be performed using the concentration data points (x-axis) versus the corresponding OD_{A450} unit data points (y-axis).

When fitting the standard curve data to a regression analysis model, it is important to evaluate the curve fit to determine whether

TABLE 3. Example of Under-Recovery from Standard Curve Generated with SCS Diluent of Lower Matrix Complexity than Sample Matrix

When SCS matrix complexity has been properly matched with the average sample solution complexity, percent recovery values should fall between 90-110% of the expected recovery value. Clearly, the lower percentage recovery values of the more concentrated biological samples in this example fall outside the target range of 100% ±10%. As the biological sample matrix is diluted in the less complex SCS diluent matrix, the overall complexity of the analyte-spiked biological samples decreases, leading to greater percent analyte recovery values. Matching the matrix complexity of the SCS with the sample matrices (e.g., serum or plasma) typically represents the most challenging and labor-intensive development milestone of the ELISA development project. Suggestions on how to resolve this matrix inhibition are addressed in Section 8.2.

Serial Dilution of Spiked Samples	Expected Protein Concentration	Standard Curve-Derived Protein Concentration	Percent Recovery Value
Undiluted	100 ng/mL	70 ng/mL	70%
1:2	50 ng/mL	39 ng/mL	78%
1:4	25 ng/mL	21 ng/mL	84%
1:8	12.5 ng/mL	11 ng/mL	88%
1:16	6.25 ng/mL	6 ng/mL	96%

or not that particular model (4-parameter, 5-parameter, Log-Log, cubic spline, etc.) was appropriate. One approach to evaluating curve fit involves plotting the residuals on an XY scatter graph. A residual plot is a graph that shows the residuals on the vertical axis and the independent variable on the horizontal axis. If the points in a residual plot are randomly dispersed around the horizontal axis, that model was appropriate for the data.

Regardless of what type of curve-fitting option is selected to generate the linear regression fit of the curve, a series of linear regression fits must be performed to determine which high standard data points can be included or excluded from the standard curve fit calculation. As a general rule, as analyte concentrations and OD_{A450} readings increase, a data point will exist where the percent increase in analyte concentration will not result in an equal percent increase in OD_{A450} signal. For example, a two-fold increase in target analyte concentration may only yield a 1.5-fold increase in the OD_{A450} signal. Simple logic dictates that inclusion of these higher data points within the standard curve calculation will lead to an ever decreasing R^2 value. The lower the R^2 value of the curve fit, the less accurate the curve fit will be when estimating a given antigen concentration from a corresponding ELISA-derived OD_{A450} absorbance value.

Completion of this linear fit analysis typically leads to the elimination of the most concentrated antigen standards. Higher data point pairings of antigen-analyte concentration versus OD_{A450} value that no longer reflect the linear relationship between changes in target analyte concentration and the corresponding change in OD_{A450} absorbance signal are simply removed from the curve-fitting calculations. As a general rule, most commercial ELISAs utilize standard curves with R^2 values > 0.98 with many falling in the > 0.99 linear fit range. The top antigen standard that still allows an R^2 linear regression correlation coefficient greater than the assay's accuracy requirement becomes the top end of the dynamic range calculation. Using experimental data that falls outside of the calculated linear dynamic range of the ELISA is ill-advised and definitely not proper scientific protocol.

Every quantitative assay concept has an upper and a lower limit of its ability to provide an accurate estimate of a particular measurement, like concentration. The dynamic range properties of any given chromogenic ELISA are essentially the product of multiple independent factors, including: 1) physical properties (useful surface area) of the ELISA plate, 2) linear absorbance detection range capabilities of the ELISA plate reader, and 3) quality of the chromogenic substrate used for the signal generation.

The restrictions on assay utility to a defined target analyte concentration range are associated with the physical limitations of the internal assay surface area on which the antigen or antibody can be coated. Expansion of useful surface area is the basis for the increased sensitivity and potential dynamic range of antigen or antibody-coated bead assay formats. Relative to an ELISA plate well surface, bead suspension assays present a larger usable surface area on which to coat the antigen or capture antibody components that drive the whole detection process. Despite this comparatively limited surface area, ELISA plate assay formats still offer excellent assay sensitivity potential for detection of most biological or environmental analyte targets. Unlike bead assays, ELISA plate formats do not require special bead accommodation equipment to obtain reliable and quantitative estimations of a soluble target analyte concentration. This feature is likely one of the reasons why the classic, plate-based ELISA concept continues to be the preferred choice of most laboratory, and albeit to a lesser degree, commercial settings.

As stated above, having a greater surface area and concentration of the antibody component responsible for capturing the target analyte from the biological or environmental sample is clearly advantageous to expansion of the linear dynamic range parameter of the assay. Thus, when sample concentrations of the target analyte increase, the plate-adsorbed IgG binding kinetics rate for target analyte also increases within a specific sample incubation time frame. When levels of the adsorbed capture IgG are not rate-limiting, there should be a good linear correlation between increases in target analyte concentrations in the samples and increased absorbance signals upon completion of the substrate development step. Upper constraints on the dynamic range of the assay occur as a result of the saturation of the adsorbed capture IgG binding sites for the target analyte. When this occurs at higher analyte concentrations, a two-fold change in analyte concentration may no longer result in a two-fold increase in bound target analyte. This can easily be observed as a flattening of the curve slope at higher target analyte concentrations. The desired linear relationship between increases in target analyte concentration in samples and corresponding increases in OD_{A450} absorbance after substrate development are diminished or lost. Therefore, the upper limit of the dynamic range of the assay can be defined as the highest standard concentration data point that can be included in the linear regression curve fit with an R^2 linear correlation coefficient > 0.95 or 0.98, depending upon assay accuracy requirements.

Choice of chromogenic substrate used for assay signal generation is a major factor in determining the linear dynamic range of an assay, as well as the linear, electronic absorbance detection span of an ELISA plate reader. Colorimetric, absorbance-based plate readers with linear absorbance reporting capabilities that are limited to OD reading < 2.0 absorbance units or < 2.5 absorbance units electronically limit the upper extent of the linear dynamic range of an assay. High sensitivity substrate formulations that provide detectable and reproducible readings over background absorbance signal levels, even when sampling low concentrations (pg/mL) of target analyte, can extend the useful low-end detection concentration range several-fold. In like fashion, if the solubility of the oxidized chromogenic substrate allows for a higher concentration of this colored substrate to remain in solution before precipitating out, this can expand the useful high-end of the quantitative assay range.

9.3 Assess ELISA Sensitivity

Most ELISA development projects typically place a very strong emphasis on maximizing assay sensitivity. In antigen-down ELISA configurations with the goal of detecting antigen-specific antibodies within a biological fluid like serum or plasma, assay sensitivity is rarely an issue. A typical anamnestic response to an infectious agent will yield enough titer (concentration) of antigen specific antibody isotypes to be easily detected on an antigen coated ELISA plate well. In this particular scenario, the analyte that is being quantitated (serum or plasma antibody) is typically present at high enough concentrations to not present a rate-limiting event.

In contrast to the antigen-down ELISA format, antibody-sandwich ELISA formats are forced to function within environments where the target analyte is usually present at very low (ng/mL or pg/mL) concentrations. At these low target analyte concentrations, the rate of target analyte interaction with the coated capture IgG is often a rate-limiting factor. As a general rule, utilization of mono-specific polyclonal or monoclonal IgG possessing high binding affinity constants for the target analyte will serve to enhance overall assay detection sensitivity. All antibody-sandwich ELISA formats are regulated in terms of assay sensitivity potential by the average binding affinity constant of the adsorbed capture IgG component. Simply stated, sandwich ELISA formats prepared using low affinity capture IgG can never achieve the detection sensitivity limits of those utilizing high antigen-analyte affinity capture antibodies.

Assay sensitivity assessment for any particular antibody-sandwich ELISA format essentially comes down to the determination of the lowest concentration of the target analyte standard at which a clear and reproducible OD_{A450} signal differential is discernable over that of the "No Antigen" (0 standard) assay background signal wells. Commercial ELISA development companies may require that the low, kit-supplied, analyte standard OD_{A450} signal be > 0.04 OD_{A450} units above the mean background signal generated by the negative control samples (e.g., known negative serum or plasma samples, etc.). Limit of blank (LOB), limit of detection (LOD), and limit of quantitation (LOQ) are frequently used terms to describe the lowest concentration of analyte that can be reliably measured by an analytical method. LOB is defined as the greatest analyte concentration expected to be found when replicates of a blank sample containing no analyte are tested. LOD is defined as the lowest amount of target analyte in a sample which can be detected but not necessarily quantitated as an exact value. LOQ is defined as the lowest amount of target analyte in a sample which can be quantitatively determined with suitable precision and accuracy.

Several approaches exist for determining detection limits of an analytical method, such as an ELISA. For example, methods based on standard deviation of the blank, standard deviation of the response of the slope, visual evaluation, and signal-to-noise ratios are all possible approaches. Selection of an appropriate method for limit determination should be based on the analytical procedure being validated. For instance, signal-to-noise ratios can only be applied to procedures which exhibit background noise when no analyte is in solution, while approaches based on standard deviation of the response and the slope are suitable when the method does not exhibit background noise of any magnitude. Complete discussion of the various limit detection approaches is outside the scope of this ELISA development guide. However, for proper evaluation of the limits it is important that the method of limit determination matches the analytical method.

9.4 Assess Spike Recovery Efficiency

Since minimization of the matrix complexity differential between the SCS diluent and the biological or environmental sample solution is essential for the successful development of most antibody-sandwich ELISA formats, the inclusion of an additional matrix equalization assessment step called spike recovery efficiency is recommended. This assessment process is comprised of spiking a known amount of the assay standard into known negative and positive controls (see examples in Sections 9.1 and 9.4.1). Because it is known from the sample spiking process what the expected curve-derived analyte concentration should be for each sample, if the SCS diluent matrix used to generate the standard curve properly matches the serum, plasma, or environmental sample matrices, the curve-derived value for each test sample should fall within 90-110% of the expected concentration. When matrix complexity within the samples exceeds that which exists within the SCS diluent in which the standard curve was run, standard-curve derived analyte concentrations in spiked samples will always show an under-recovery event. These under-recovery values often read at < 50% of the expected value.

When performing the spike recovery efficiency analysis to verify whether or not the SCS diluent has been matched properly with the type of sample matrix displayed by the sample type, several spiked concentrations should be prepared in known negative samples. Analyte spikes should provide analyte concentrations falling at approximately 30% and 75% of the top concentration level of the linear dynamic range determined for this particular assay (see Section 9.2). Set up the sandwich ELISA template to contain the standard curve and quadruplicate reps of each of the artificially positive sample spikes. Since there is some variation between the different sample matrices, if possible, perform these spikes using 10-20 known negative samples from different sources.

9.4.1 Example Assessments of Spike Recovery Efficiency

Scenario 1: A sandwich ELISA was developed to detect a target analyte in serum with a linear dynamic range maximum of 100 ng/mL. The bottom standard had an analyte concentration of 1.56 ng/mL following a typical two-fold serial dilution scheme. The biological samples had higher matrix complexity than the SCS used to generate the standard curve. When the SCS diluent has a less complex, or more permissive matrix complexity relative to the samples, spikes in known negative samples will result in under-recoveries compared to the standard curve. To perform spike recovery efficiency assessment for this assay, spikes of two analyte concentrations, 75 ng/mL and 30 ng/mL, were prepared in multiple samples of known negative serum controls. In this hypothetical example, four spike recovery values for the 75 ng/mL spikes read as 60 ng/mL, 58 ng/mL, 62 ng/mL, and 63 ng/mL from the standard curve, corresponding to under-recoveries of 80%, 77%, 83%, and 84% respectively. The 30 ng/mL spikes read off the standard curve as 23 ng/mL, 25 ng/mL, 22 ng/mL, and 26 ng/mL, corresponding to the spike recovery values of 77%, 83%, 73%, and 87% respectively. With a requirement of spike recoveries between 90-110%, this sandwich ELISA would have failed the spike recovery efficiency parameter (Table 4).

Table 4. Example of Under-Recovery from Standard Curve Generated with SCS Diluent of Lower Matrix Complexity than Sample Matrix

Expected Analyte Concentration	Spike#	Standard Curve Derived Protein Concentration	Percent Recovery Value
75 ng/mL	1	60 ng/mL	80%
	2	58 ng/mL	77%
	3	62 ng/mL	83%
	4	63 ng/mL	84%
30 ng/mL	1	23 ng/mL	77%
	2	25 ng/mL	83%
	3	22 ng/mL	73%
	4	26 ng/mL	87%

Scenario 2: Water-based environmental samples with lower matrix complexity than the SCS used for the standard curve were being analyzed for a certain harmful contaminant. When the SCS diluent has a more complex or restrictive matrix relative to the samples, spikes in known negative samples will result in over-recoveries compared to the standard curve. To perform spike recovery efficiency assessment for this assay, replicate spikes of two analyte concentrations, 75 ng/mL and 30 ng/mL, were each prepared in known negative serum controls. In this hypothetical example, the high spikes yielded standard curve-derived readings of 88 ng/mL, 93 ng/mL, 89 ng/mL, and 91 ng/mL, corresponding to over-recoveries of 117%, 124%, 119%, and 121% respectively. The low spikes read off the standard curve as 45 ng/mL, 40 ng/mL, 41 ng/mL, and 43 ng/mL, corresponding to spike recovery values of 150%, 133%, 137%, and 143%, respectively. Again, with a spike recovery requirement of 90-110%, this sandwich ELISA would have failed the spike recovery efficiency parameter (Table 5).

Table 5. Example of Over-Recovery from Standard Curve Generated with SCS Diluent of Higher Matrix Complexity than Sample Matrix

Expected Analyte Concentration	Spike#	Standard Curve Derived Protein Concentration	Percent Recovery Value
75 ng/mL	1	88 ng/mL	117%
	2	93 ng/mL	124%
	3	89 ng/mL	119%
	4	91 ng/mL	121%
30 ng/mL	1	45 ng/mL	150%
	2	40 ng/mL	133%
	3	41 ng/mL	137%
	4	43 ng/mL	143%

When assays fail the spike recovery efficiency parameter, further laboratory work is needed to equalize the matrix complexity in the standard curve wells with the matrix complexity in the samples wells (Section 8.2). A decision must be made at this point as to whether the extra effort to bring the SCS matrix closer in line with the sample matrix is really necessary to achieve the goal of the research project. In many analyte assessment situations, a result within ±20% of the expected value is sufficient.

9.5 Assess ELISA Precision

The precision of an assay expresses the "closeness" of agreement between a series of measurements. ELISA precision assessment is a way of defining the reproducibility characteristics of the assay. Precision is often evaluated at three levels: repeatability, intermediate precision, and reproducibility. Repeatability (also can be referred to as intra-assay precision) represents the precision under the same operating conditions over short intervals of time. Intermediate precision refers to variations within the laboratory (different days, different analysts, different equipment, etc.). Reproducibility refers to the precision between laboratories (different sites, collaborative studies, etc.).

Precision should not be confused with the related performance terms, accuracy and specificity. The accuracy of an assay expresses the "closeness" of agreement between the value which is accepted as a true value and the value found. A major factor affecting an assay's accuracy resides within its ability to specifically target only the analyte molecules that the test was developed to detect. Specificity is defined as the ability to assess unequivocally the target antigen in the presence of components which may be expected to be present (such as impurities, degradants, matrix, etc.). The specificity of the detection antibody is thus the single most important operational component influencing ELISA accuracy. Accuracy and specificity are included in this discussion on ELISA precision to clearly delineate their functions from what is meant by assay precision.

Assay precision measurements are routinely expressed as the variance, standard deviation, or coefficient of variation (CV) of a series of measurements. For example, the % CV is defined as the standard deviation (SD) of the data population divided by the mean ($\bar{x}$) of the data population x 100 [(SD / $\bar{x}$) x 100]. When sandwich ELISA precision is being evaluated, the % CV calculations should be applied to analyte standard concentrations that were derived from the standard curve. Simply comparing the % CV of the OD_{A450} signals from replicate-to-replicate or plate-to-plate is of little value. By definition, the % CV will increase as the mean value $\bar{x}$ (denominator) of the standard curve-derived protein concentration decreases and vice versa: % CV will decrease as the mean value increases within the linear dynamic range of the assay, or, more specifically, within the linear range of the standard curve. Hypothetically, once the analyte standard concentrations exceed the linear dynamic range, where an increase in analyte concentration corresponds to a linear increase in OD_{A450} signal, the potential for an increasing SD / $\bar{x}$ ratio is the likely outcome due to eventually reaching the solubility limits of the substrate.

The level of assay-to-assay precision stringency should be driven by the eventual performance needs of the end user. An assay used for clinical diagnostics would require a higher standard of assay-to-assay precision than an identical assay format that may only be used on an occasional basis to assess single experiment laboratory results. For most non-commercial, research-grade sandwich ELISA formats, average intra-assay % CV values < 15% for analyte concentration run on the same plate may be deemed acceptable. Average inter-assay (between different assay plates) % CV precision values < 20% may also be acceptable for research purposes. A major emphasis driving the maximization of precision (minimizing % CV) occurs when the assay intent falls into the realm of clinical diagnostics, or when the analyte concentration differential between a posi-

tive and negative response in subtle. Under these conditions, or for commercial grade assays, acceptable average intra-assay (within plate) % CV values of the target analyte concentration assessment could well be < 10%. Therefore, the intended use for the final ELISA data will drive the necessity for maximum precision requirements.

9.5.1 Example Assessment of ELISA Precision

Scenario 1: A sandwich ELISA format was developed for the detection of a novel melanoma cell inhibitor protein secreted into the cell culture media by a virally transfected, adherent monocyte precursor cell line. Four cell culture flasks with different growth conditions were used to cultivate this potentially chemotherapeutic product. The protein concentrations present within the cell culture flasks were determined using a typical eight-point, two-fold serial dilution standard curve with a top inhibitor protein standard concentration of 1000 ng/mL. To assess intra-assay precision levels, 20 replicates of each of the four pools of cell culture supernatant were assessed (Table 6). To calculate the intra-assay precision (% CV) for each of the different pools, the SD for each pool was divided by the respective mean $\bar{x}$ of the 20 replicates and multiplied by 100. Therefore, in this hypothetical research purposes ELISA, these % CV values would be acceptable levels of intra-assay precision.

Table 6. Calculating the percentage coefficient of variation for hypothetical ELISA samples

	Standard Curve-Derived Protein Concentrations with Standard Deviations	Equation for Percentage Coefficient of Variation and Hypothetical % CV
Pool 1	$\bar{x}$ = 800 ng/mL; SD = 60	60/800 x 100 = 7.5% CV
Pool 2	$\bar{x}$ = 500 ng/mL; SD = 50	50/500 x 100 = 10.0% CV
Pool 3	$\bar{x}$ = 600 ng/mL; SD = 70	70/600 x 100 = 11.7% CV
Pool 4	$\bar{x}$ = 900 ng/mL; SD = 70	70/900 x 100 = 7.8% CV

10. Quality Assessment of Plate Coating Process

Once the general operational parameters of the AS ELISA have been established, and the timeline dynamics of the project have been clearly defined, then it is prudent to consider performing an ELISA-based antibody coating precision analysis of the present plate coating process. Even if the study is just designed to perform a simple qualitative "yes/no" analysis, a cursory pre-screening run using a mid-level antigen standard should still be a mandatory practice. It is important to verify that the target antibody was successfully adsorbed to the ELISA plate-wells while still retaining the ability to bind to the target analyte being quantified in samples.

10.1 Frequent Sources of AS ELISA Variability Problems

Antibody coating irregularities can arise from a variety of environmental factors. A non-comprehensive short list of these would include: 1.) irregularities within the ELISA plate supplier's manufacturing process, 2.) improper selection of the antibody coating buffer leading to the precipitation or partial denaturation of key antigen-binding paratope content on the plate coating antibody, 3.) inconsistencies in the plate-well to plate-well liquid volume used for antibody-coating, blocking, and washing steps, and 4.) antibody denaturation resulting from liquid surface tension disruption (shearing) of antigen binding paratope structure during initial plate coating process (may be associated with automatic plate coating equipment).

If the project is limited to a one or two sample time-point assessment (e.g. a one-time 6-12 total ELISA plate production batch size), the risk of serious plate-to-plate coating irregularities would be minimal compared to what could occur with a large production batch-size (> 100 plates) plate production event. When this additional potential for plate coating variability factor is combined with the fact that the large production batch of AS ELISA plates must be stable over the course of a year or more, performance of some type of plate coating precision assessment takes on a greater level of importance.

10.2 Plate Coating Precision Study Setup

A. Remove a predetermined sampling percentage of the coated/blocked/packaged AS ELISA plate batch inventory. For example, this initial plate screen sampling protocol could call for plate precision analysis to be performed on 5% of the total production lot packaged and refrigerated AS ELISA plate inventory. However, if there is some prior evidence of a potential for antibody coating inconsistencies, then it may be necessary to increase the plate precision screening protocol to initially sample a greater portion of the packaged AS ELISA plate inventory. There may be time associated factors within the plate coating process itself, which may arise from the antibody's physical composition characteristics. Changes in antigen binding properties of the capture antibody could lead to an increase or reduction of antigen binding capabilities over the course of the plate coating process. To a much lesser degree, there may be variations in antibody coated and blocked plate-well performance dynamics that are related to plate processing order. This can be observed by assigning each plate a number based on the order in which it was prepared. Numbering and processing production-batch plates in numerical-order can enable the detection of time-dependent antibody coating features that would otherwise be non-discernable.

B. Prepare an appropriate quantity of a mid-level antigen standard diluted into the Neptune Sample Diluent (Catalog #6125) using a dilution factor previously determined to give a stopped TMB OD_{A450} value around 0.6 – 0.8 OD units. This raw OD_{A450} signal value target is recommended because it is 25% to 33% of the customary 2.4 OD unit upper limit for ELISA curve linearity within most ELISA formats.

   For example, if the plate coating precision study was designed to evaluate five (5) AS ELISA plates, the recommended volume of mid-level antigen standard would be 60 mL. Each plate requires approximately 10 mL volume if using a conventional 100 µL per well fill volume, using a total of 50 mL (5 plates x 10 mL per plate). The process of dispensing liquid into ELISA plate-wells is commonly performed by first placing the liquid into a reservoir and then dispensing into the ELISA plate-wells using a multichannel pipettor. The remaining 10 mL volume (of the 60 mL total volume prepared) of antigen standard pool will assure that there will be sufficient volume remaining in the reservoir to easily accommodate the proper loading of 5 ELISA plates with a multichannel pipettor. If using a multi-channel pipettor to load the same standard across all plates, there is no need to change the pipette tips between plates.

C. Perform the AS ELISA plate screening analysis using the most current AS ELISA protocol (optimized in Sections 6 and 8).

D. Obtain the raw OD_{A450} readings using the software available on the colorimetric ELISA plate reader.

10.3 Plate Coating Precision Study Analysis

Many visible-absorbance plate readers are equipped with an ELISA analysis software package capable of performing plate precision calculations. In the event of using a plate reader that is not equipped with software capable of such analysis, please see the following section for brief guidelines. The following section builds on the aforementioned example where 5 plates were reserved for the coating precision study.

A. All five AS ELISA precision testing plates should have similar stopped, raw, TMB OD_{A450} score values for each well. Any deviations from the calculated mean ($\bar{x}$) of the total (5 x 96 wells = 480 wells) raw OD_{A450} values must originate from improper end-user assay performance technique or plate-coating irregularities.

B. Plate precision screening analysis will typically reveal the more macroscopic plate-to-plate variability (inter-plate variability) as well as the subtler within-plate (intra-plate variability) elements. Coefficient of Variation (CV) is probably the most commonly used statistical term when addressing ELISA plate precision topics. It is typically expressed in the form of a percent CV (% CV) of a particular set of ELISA generated raw plate-well OD values. % CV is defined by the formula [(SD / $\bar{x}$) x 100] where $\bar{x}$ is the mean of a selected set of raw plate-well derived OD values and SD is the Standard Deviation of that particular set of plate derived OD values.

C. To perform a more generalized plate-to-plate variability analysis, copy and paste raw plate reader OD_{A450} values into an Excel sheet and then setup the Excel formula macros to calculate the mean ($\bar{x}$) of all 96 plate-well OD_{A450} output values as well as the standard deviation (SD) for these 96 OD_{A450} readings. Calculate the % CV for that particular AS ELISA plate. Repeat this process for the four remaining AS ELISA plates being screened for plate coating precision.

Establishing a reasonable upper % CV plate precision limit for any new batch of coated and blocked plates can be complex and varies depending on the nature of the project. Realistically, it may not always be practical to mandate that all plate coating projects have % CV values less than 5%, 10%, or 15% variability.

In situations where the study has more modest aspirations, a higher degree of plate coating variability, such as % CV values of < 20% may be acceptable. Alternatively, there may be scenarios that require a lower degree of variability, such as situations where the differences in target analyte concentrations between positive versus negative test results are small, or in ongoing studies involving multiple assays run on multiple days. Here, the upper limit for an acceptable % CV within plate-well to plate-well (intra-plate) precision likely should be << 10%. On a plate-to-plate (inter-plate) basis, a < 15% CV precision score would likely be acceptable.

As each project is unique, the end user is ultimately responsible for determining what is considered to be an acceptable % CV precision score. Plate batches found to have a % CV score greater than the predetermined upon upper limit would be deemed unusable for any AS ELISA based project. Encountering such a situation should immediately trigger a reassessment of the plate coating and blocking protocol before any attempts to create another batch of antibody coated and blocked plates be undertaken.

11. Conclusion

Each ELISA development project will present its own unique display of antigen versus antibody binding dynamics. Antibody versus antigen binding relationships are highly variable, and therefore can only be identified and understood through implementation of an educated trial and error assessment process. It is best to approach the AS ELISA development process through a logical series of antigen and antibody exposure events. Binding event outcomes associated with each antigen + antibody exposure session will vary based on factors such as the average antigen binding affinity constant of the participating antibody components, the length of the individual component exposure times, and composition of the liquid matrix in which these antibody-to-antigen binding events are occurring. The goal, therefore, should be to establish a limited and clearly defined set of experimental protocol modifications for each new ELISA condition run. Knowing the cause (e.g., component alteration or procedure modification) and effect (e.g., change in ELISA performance parameter) should create a logical pathway for development of virtually any antibody-sandwich format ELISA that a research project may require.

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