Antigen-Down ELISA Development Kit
Catalog Number 9101

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

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1. Kit Contents

- Antigen Coating Buffer, 5X, 100 mL (Catalog # 6247)
- General Block, 500 mL (Catalog # 633)
- General Serum Diluent, 500 mL (Catalog # 648)
- Antigen-Down HRP Conjugate Stabilizer, 5X, 25 mL (Catalog # 6169)
- ELISA Wash Buffer, 10X, 500 mL (Catalog # 651)
- TMB 1-Component HRP Microwell Substrate, 100 mL (Catalog # 6276)
- Stop Solution for TMB Substrates, 100 mL (Catalog # 6282)
- 96-Well ELISA Plates, 20 per kit (Catalog # 261)
- ELISA Plate Sealing Covers, 20 per kit (Catalog # 6287)
- Foil ELISA Plate Storage Bags, 20 per kit (Catalog # 6288)
- Desiccant Packets, 20 per kit (Catalog # 6289)

2. Materials Required but Not Provided

- ELISA plate reader capable of reading 96-well plates at absorbance values of 450 nm or 650 nm
- Washer/aspirator system
- Polypropylene centrifuge tubes (PCT): 15 mL and 50 mL, disposable, screw cap
- 75 mm glass test tubes for serial dilution of serum/plasma samples (unknowns) and negative and positive serum controls
- Known (Positive and Negative) Reference-Control serum/plasma sample pools (hereafter identified as Known Positive or Negative Reference-Controls): the majority of samples routinely monitored using
an AD ELISA format are of serum or plasma origin. These will, for the purpose of this protocol, simply be referred to as samples. All Positive and Negative Reference-Controls must have undergone a prescreening process, preferably via Antigen-Down (AD) ELISA format, to properly validate the presence or absence of a specific antibody titer toward the target antigen (TA). Acquisition of these two reference controls is critical for proper assay development.

- Minimum volume quantities of Positive and Negative Reference-Control sample pools needed: 20 mL-50 mL of at least a high titer Known Positive Reference-Control sample or pool, depending on the assay’s intended use following the development phase. If possible, try to acquire 10 mL-50 mL pool volumes of different human/animal origin low, medium, and high titer control pools. To minimize the potential of multiple freeze/thaw associated antibody titer depletion, all serum or plasma controls should be aliquoted and frozen in 0.5 mL to 1 mL aliquots. Use high-quality 1.2-1.8 mL cryovials for frozen sample storage. Store Reference-Controls or plate-coating antigens at < -25°C.

- Optional cell culture-derived Positive and Negative Control media pairings: these controls would only be relevant if AD ELISA format cell culture screenings were being performed. In many hybridoma clonal screening scenarios, only a negative cell culture media control is readily available. Because these are often Yes/No type assays, the availability of a positive control cell culture media is not an absolute necessity.

- HRP-IgG conjugate (1 mg/mL): horseradish peroxidase (HRP) conjugated, affinity-purified polyclonal IgG specific to the human/animal Ig isotype being targeted in this ELISA. Minimum quantities required will vary, depending upon the intended use of the assay following the development phase. For limited scale ELISA assessment projects, a 1-2 mg quantity of HRP-IgG conjugate anti-human/animal isotype may suffice. For on-going projects involving numerous sample assessments per run, a 10-20 mg quantity of the 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 Antigen for plate coating: purified, biologically isolated and characterized, or recombinant synthesized origin. Minimum antigen quantity requirements vary with the expected size of the sample screening project. Smaller scale specific antibody titer assessment projects involving < 500 samples (total) would only require up to 5 mg of plate coating antigen. Larger scale studies involving > 100 samples per sampling date and run at multiple time points over a 6-12 month (or longer) time course, would require a minimum of 20 mg of the plate coating antigen.

Due to the inevitable loss of epitope accessibility arising from steric hindrance of epitope regions directly adsorbing to the polystyrene ELISA-plate-well surface, or the loss of tertiary (noncontiguous) epitope structure from the physical forces associated with adsorption to the well surface, generally only antigens with molecular weights > 8kD should be used for direct-onto-plate adsorption coating
strategies. Exceptions can be made to these minimum molecular weight limitation guidelines if prior direct-onto-plate absorption studies clearly demonstrate that these smaller molecular weight antigens retain enough of their key epitope integrity to adequately represent the native form of the peptide-molecule.

When working with small molecular weight (hapten-like) target peptides, the routine plate coating strategy must be dramatically altered. It is often necessary to present these small molecular weight antigen/hapten-like targets by covalent attachment to larger carrier protein or other artificial support molecules.

3. Prerequisite Qualifications for Key Assay Components

- Obtain qualified Known Positive and Negative Reference-Control pools. Qualification typically involves an ELISA or other orthogonal immune-titer assessment assay.
- Affinity purified, Ig isotype-specific HRP-IgG conjugate (up antibody) must possess both high specificity/high affinity binding kinetics for target antibody-associated Fc chain epitopes as well as two to four (2-4) covalently bound, high redox efficiency HRP enzyme molecules for optimal signal generation.
- ELISA plate coating antigen must be pure and authenticated to be antigenically representative of the native or field strain target or organism that is responsible for generating host antibody titers being detected by this ELISA.

4. Introduction

What is an Antigen-Down (AD) ELISA, and how does one develop an AD format ELISA? An AD format ELISA refers to an ELISA plate configuration whereby the plate wells are pre-coated with a highly purified target antigen (hereafter called Target Antigen) that will subsequently be used to quantify specific antibody concentrations that are present within serological or cell culture origin samples (see Figure 1). Having the ability to detect and quantitate a population of specific antibodies to a particular pathogen, allergen, or self-antigen within serological samples is an absolute essential for any successful epidemiological, allergic hypersensitivity, or autoimmune focused monitoring study.

Briefly, the specific antibody detection and quantification attributes of the AD ELISA format result from its simplistic design. Commercial ELISA plate manufacturers each utilize their own in-house polystyrene plastics modification process. Their goal is to create an ELISA plate containing 96 individual sample wells. Inner surfaces of each sample-well possess the modified plastics surface chemistry to enable passive binding of the Target Antigen to the individual plate well surfaces. Several examples of different Target Antigen iterations include; oligopeptides, polypeptides, and certain polysaccharide molecules. After the Target Antigens have been adsorbed to their respective ELISA plate wells, the entire ELISA plate well surface is treated with a blocking agent that will minimize unwanted non-specific binding signal. Reduction of non-specific binding signal is a universal goal of all types of ELISA format.

Once the Target Antigen-coated plate has been created (blocked and stabilized), screening of serum, plasma, or cell culture samples for the presence of a Target Antigen-specific poly or monoclonal antibody concentration or titer can begin. Obviously, there are more individual ELISA development parameters requiring optimization during the plate coating and blocking/stabilizing process. However, these will be discussed in more detail later on in the protocol. For now, the focus is to provide a basic background
discussion describing what an AD ELISA is composed of, as well as how they work. Once the plates have been coated with target antigen and then blocked, it is now possible to detect (and in some cases, to quantify) Target Antigen-specific antibody present within serological or cell culture samples. Dilutions of serum, plasma, or cell culture media are added to respective target antigen coated wells on each AD ELISA plate. Samples are then incubated in the coated wells, after which the ELISA plates are washed to remove unbound sample. Then, anti-animal isotype specific IgG-HRP conjugate is added to each well that was previously exposed to the serological or cell culture origin sample and incubated. After the incubation period has ended, ELISA plate wells are washed to remove all non-specifically bound HRP conjugate. The next step is to add a chromogenic HRP substrate like TMB to your plate wells. This TMB-substrate will allow for the visualization and electronic quantitation of how much Target Antigen-specific antibodies were present within the serological or cell culture origin test samples. In the presence of the IgG-HRP conjugate (which is specifically bound to Target Antigen previous absorbed onto plate well surfaces), the horseradish peroxidase (HRP) will cause a color change in the initially clear TMB substrate solution. In general, the more intense the TMB color present within a respective sample-well, the greater the amount of Target Antigen-specific antibody in that particular serological or cell culture sample.

To summarize, the basic AD ELISA format is capable of providing both a relative estimation of the Target Antigen-specific antibody titer/concentration present within the serological test samples as well as a means by which to determine the identity of the isotype(s) that are participating in the humoral immune response. ICT’s motivation for creating a manual devoted to the topic of how to develop a research-specific AD ELISA is to help guide end users through the common steps and stages of (and frequently encountered issues associated with) the building of an AD ELISA.

**Figure 1.** Graphic representation of an Antigen-Down (AD) format ELISA. In an AD-ELISA, a Target Antigen coated on the ELISA plate surface is used to quantify specific antibody concentrations present within serological or cell culture origin samples.

5. **Basic Considerations to Address Before Starting AD ELISA Development**

When considering the possibility of developing an AD ELISA format to detect and/or semi-quantify a scientifically relevant humoral antibody response, it is critical to first clearly define the scope and magnitude of the project. Is the goal to generate simple yes/no determinations for the presence of an antigen-specific antibody production event within hybridoma cell supernatants or
test-bleed serum samples obtained from a limited number of human or animal sources? In most situations, this type of limited-scope serological or cell culture antibody screening process should easily be completed within a 2-week time frame (including the time required to complete sample testing). If developing a more comprehensive serological titer assessment study that is designated to take place over a 6, 12, 18 month or longer time frame, additional assay-variability-enhancement-factors will also need to be considered. Such factors include the effects of the plate coating/blocking/Target Antigen-stabilizing dynamics on assay performance and reagent stability during long term storage. If multiple analysts, or different laboratories/sites will be responsible for performing the assay, this can introduce additional variability. Despite the complexity, these multi-source assay variability situations can still be quite manageable through the implementation of an ELISA development strategy capable of addressing the various performance-critical steps.

The AD ELISA format is commonly used to monitor the current and ongoing serological antibody-titer status of an antigen-specific antibody or specific antibody-isotype population within the host subject group. The term “titer” as it is used in this protocol refers to a “generalized indicator estimate” of a serological concentration of a particular antigen-specific antibody component in serum or plasma samples. When used in conjunction with the term “ELISA”, it simply represents a relative approximation of the antigen/epitope(s) specific antibody concentration as revealed via the intensity of the AD ELISA OD_{450} signal for any given serum/plasma dilution factor. Acquiring the necessary ELISA components/reagents is a critical early step in the assay development process. Some important considerations are: does the project starting point involve the need to purchase purified Target-Antigen from commercial sources or must the Target Antigen first be isolated and purified from a living biological system? Does the assay require the inclusion of positive and negative titer serum or plasma pools? Lastly, has a reliable commercial source for the HRP or alkaline phosphatase (AP) labeled IgG anti-Ig isotype conjugate been identified?

The overall goal of this AD ELISA development guide is to provide a slightly more comprehensive 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 plate coating antigen and Positive and Negative Reference-Control pools can be obtained to complete the assay feasibility portion of the project (Section 6). This guide also highlights the four (4) essential assay performance milestones (see Table 1 below) that must be satisfied to assure successful completion of any trustworthy AD format ELISA. The finalized ELISA should efficiently detect isotype-specific, low to high positive titer, humoral antibody responses in serum or plasma samples.

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6. **Assay Feasibility Assessment**

Verification of assay concept feasibility is the single most important milestone of any AD ELISA development project. Unless the early-development-stage AD ELISA is capable of detecting known medium to high humoral-response caliber serum or plasma samples, the proposed AD ELISA concept is a non-starter. It is at this development stage that the quality (purity and authenticity) characteristics of the plate coating antigen may be called into question. Additionally, but usually to a lesser extent, would the HRP labeled anti-animal-isotype IgG conjugate be called into question. Fortunately, most commercially sourced IgG-HRP secondary detection antibody conjugates perform well for these purposes.

In the assay feasibility stage, the initial goal is to demonstrate that the assay is capable of detecting at least the known positive high titer serum/plasma pool samples. Next, focus shifts to optimizing assay sensitivity levels to enable detection of medium and subsequently low antibody titer samples. When anticipating the development time requirement for this type of project, it is advantageous to acquire some prior knowledge of previously documented humoral antibody response levels observed in other related situations. If published data on the magnitude of a typical serological response to an analogous antigen stimulating event (pathogen, autoimmune stimulus, etc.) indicates that high antibody titers tend to be the norm, then the need for extensive assay sensitivity enhancement should be minimal. Alternatively, if humoral antibody responses to this particular antigenic stimulating event are minimal, it will likely be necessary to push the sensitivity of the assay to at least assure the detection of the top 50% of the potential low-positive responders.

Fortunately, matrix inhibition problems associated with complex samples like serum and plasma, which tend to be problematic during most antibody-sandwich ELISA development projects, are rarely an issue in AD ELISA formats. Serum or plasma samples commonly used in these types of antigen-specific antibody detection assays can often be diluted at least 50-fold and still perform well. Such dilution means that nearly all of the problematic matrix inhibition effects are simply diluted out of existence. Assay precision and sensitivity are the two main ELISA performance metrics that should be evaluated upon completion of the Optimization of Assay Performance Parameters milestone.

### 6.1 Prepare an Antigen Coated and Blocked Plate

#### 6.1.1 Day 1 – Coat plate with antigen, leaving blank control wells.

A. Add 10 mL of the Antigen Coating Buffer, 5X (Catalog # 6247) to 40 mL of diH₂O. Typically a 50 mL Polypropylene Centrifuge Tube (PCT) works well for these limited scope dilution processes. This provides a 50 mL volume of 1X Antigen Coating Buffer.

B. Transfer 20 mL of 1X Antigen Coating Buffer to a new 50 mL PCT.

C. Prepare initial trial antigen coating concentrations of 1 µg/mL and 4 µg/mL by adding 80 µg of the Target Antigen to 20 mL of 1X Antigen Coating Buffer (this makes the 4 µg/mL solution). The Target Antigen used for serum/plasma screening must be antigenically representative of the wild type virus, spirochete, or bacterial agent that the screening is intended to monitor. This protein, carbohydrate, or glycoprotein antigen component will be used to detect Target Antigen-specific antibodies within the samples. Mix contents thoroughly by inverting or gently vortexing tube. Do not mix contents in a manner that causes excessive foaming. Exposure of
proteins to the altered surface tension environment of air-bubbles can become a source for unwanted protein denaturation damage. Transfer an additional 9 mL of the 1X Antigen Coating Buffer into a second 50 mL PCT and add 3 mL of the 4 µg/mL antigen coating solution to create a 1 µg/mL antigen coating solution.

D. Remove a new 96-well ELISA plate (Catalog # 261) 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 (Figure 2). Each of the three initial conjugate dilutions will be used within a 2-column section of the plate. A 1:5,000 dilution of the conjugate will be used in well columns 1-2, 1:10,000 dilution in well columns 3-4, and 1:20,000 dilution in well columns 5-6. On the right half of the plate-wells 7-12, plan to repeat the same 1:5,000, 1:10,000, and 1:20,000 conjugate dilution assessment pattern.

E. Pour the properly mixed 1 µg/mL and 4 µg/mL antigen solution into two medium-sized plastic weigh boats.

F. Pour a small volume of the 1X Antigen Coating Buffer (4 mL) into a 15 mL PCT. This 1X Antigen Coating Buffer will be used to create the no-antigen control wells in rows A and B.

G. Add a 100 µL volume/well of the 1X Antigen Coating Buffer into all the wells in rows A and B.

H. Using a calibrated (8 or 12) multi-channel pipettor, carefully dispense a 100 µL volume per well of the 1 µg/mL antigen solution into rows C – H of all columns on the left side of the 96-well plate (Columns 1 – 6) and add 100 µL volume/well of 4 µg/mL into rows C - H of all remaining well columns on right side of plate (Columns 7 – 12). 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. Later on, if the Target Antigen supply is limited by one’s ability to acquire and purify this critical component, or if commercial acquisition becomes cost prohibitive, smaller coating volumes (50 µL) may be quite acceptable.

I. 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: Plate-wells of initial assay feasibility plate are coated uniformly with 100 µL per well volumes of either the 1 µg/mL (depicted as light gray wells) or 4 µg/mL (depicted as dark gray wells) antigen coating solution (Target Antigen dissolved in 1X Antigen Coating Buffer), or 1X Antigen Coating Buffer alone to create Blank-well controls (depicted as white wells). Blank wells (no Target Antigen coating) controls are positioned across the top two rows of the plate. These wells receive only the 1X Antigen Coating Buffer that does not contain any Target Antigen component. All remaining plate-wells in rows C-H receive either the 1 µg/mL or 4 µg/mL antigen coating solution.

6.1.2 Day 2 – Wash and block the plate to reduce background and further stabilize coated Target Antigen component.

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 diH₂O. Place the 1 L cylinder on a stir plate and mix for 5-10 minutes.

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 manual washing of ELISA plates.

C. Pour a 35-40 mL volume of General Block (Catalog # 633) into a clean plastic basin. Set a multichannel pipette to deliver a 100 µL or 150 µL dispensing volume.

D. Aspirate the well contents using a multi-8/12 channel hand-pipettor 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 using washer manifold 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 General 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 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 General Block-containing plate with a plate sealing cover (Catalog # 6287), Parafilm, or plastic wrap and place ELISA plate 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 will retain their antigen integrity for over a year.

6.2 Preliminary Assessment of HRP-IgG Anti-Isotype Conjugate Working Concentration Range

6.2.1 Prepare serial dilutions of a Known Positive Reference-Control.

A. Confirm two important criteria regarding the positive control: first, the Known Positive Reference-Control has been authenticated (by an accepted serological assessment protocol) to contain specific antibodies directed to the plate adsorbed antigen/analyte target molecules; second, antibody titers generated within these human/animal derived serum or plasma sources were the direct result of immune-system exposure to wild type (virus or microbial) infection or autoimmune reaction driven event.

B. Set up a simple 2-fold serial dilution scheme in six properly labeled PCT tubes (Figure 3).
Figure 3: Two-fold Serial Dilution of a Known Positive Reference-Control.

C. Prepare a 6 mL volume of a 1:100 dilution Known Positive Reference-Control using the General Serum Diluent (Catalog # 648) provided in the kit.

D. Make an initial 1:100 dilution by spiking 60 µL of the Known Positive Reference-Control into 5.94 mL of General Serum Diluent.

E. Add 3 mL volumes of sample diluent to each of the labeled (1:200, 1:400, 1:800, 1:1,600, and 1:3,200) PCTs within the serial dilution series.

F. Add 3 mL of the 1:100 sample to the tube containing 3 mL General Serum Diluent labeled 1:200. Mix thoroughly.

G. Repeat serial 2 fold dilution process throughout the remainder of the dilution tube series to eventually obtain the 1:3,200 dilution factor.

6.2.2 Load ELISA plate with Known Positive Reference-Controls 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 Known Positive Reference-Controls as well as the Blanks (General Serum Diluent-Only) into their respective plate locations as soon as possible.

B. Add 100 µL per well of General Serum Diluent-Only to each well in Rows A and B for the Double Parameter Assay Blanks.

C. Add 100 µL of the lowest concentration (1:3,200) Known Positive Reference-Control to each well (Row C) on the ELISA plate (See Figure 2).

D. Proceed to add, from least concentrated to most concentrated, the remaining Known Positive Reference-Controls to the ELISA plate-wells. Note: The highest concentration (smallest dilution factor) should end up going into row H at the bottom of the plate. This allows you to keep the same pipette tip(s) in place over the entire plate loading process.
E. Cover ELISA plate with a plate sealing cover (Catalog # 6287), 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 45 minutes at 37°C or 75 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 (PCT) with 5,000, 10,000, and 20,000. 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. Dilute 8 mL of the Antigen-Down HRP Conjugate Stabilizer, 5X (Catalog # 6169) 1:5 into 32 mL diH₂O to yield 40 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 PCT labeled 5,000, prepare the 1:5,000 conjugate dilution by spiking 500 µL of the 1:100 conjugate dilution into 24.5 mL 1X conjugate stabilizer (1:50 dilution). Mix the PCT containing the 1:5,000 dilution of the HRP-IgG conjugate thoroughly by capping and inverting the tube multiple times.

E. Put 10 mL of 1X conjugate stabilizer into the tubes labeled 10,000 and 20,000.

F. Transfer 10 mL from the 1:5,000 dilution PCT into the PCT labeled 10,000. Cap off tube and mix carefully but thoroughly (Figure 4).

![Figure 4: Serial Dilution of Conjugate](image)

G. Transfer a 10 mL volume of the 1:10,000 dilution to the 10 mL volume of 1X conjugate stabilizer in the PCT labeled 20,000. Cap off tube and mix carefully.
H. Set the 1:5,000, 1:10,000, and 1:20,000 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).

**6.2.4 Add three conjugate dilutions to predetermined well column locations on plate.**

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

B. Label three solution basins as 5,000, 10,000, and 20,000 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 1:20,000 conjugate dilution to the designated regions of the ELISA plate (See Figure 2 above). Subsequently, add the 1:10,000 and 1:5,000 conjugate dilutions to their respective locations on each plate (See Figure 2). 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 the TMB substrate.**

A. Pour about 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 plate onto a stack of clean paper towels.

D. Add 100 µL/well of the TMB substrate to every well using a multichannel 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 plastic weigh boat. 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 Known Positive Reference-Control 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.
6.3 Analyze Initial ELISA Data

6.3.1 Acquire plate reader results.

A. Set up a 96-well plate reader to quantitate absorbance at 450 nm and, if possible, use a 540 nm absorbance subtraction to reduce noise from ELISA-plate plastics.

B. Determine the sample (serum/plasma) dilution-factor values that yield stopped TMB substrate absorbance (OD\textsubscript{450}) values < 0.1 OD\textsubscript{450} units within the Assay Blank Well Controls located within Rows A and B of the plate.

C. Examine the range of OD\textsubscript{450} values and select a sample and HRP-conjugate dilution factor that yields a stopped TMB OD\textsubscript{450} value between 2.4 and 2.8 OD\textsubscript{450} units. There may be multiple combinations of sample dilutions, conjugate dilutions, and plate coating combinations which yield stopped TMB OD\textsubscript{450} values within this target 2.4-2.8 range. Although, it is too early in the ELISA development process to select the best assay component combination at this point in time, it is critical that there is some detectable level of TMB substrate development. If the ELISA plate does not contain any yellow colored wells at this juncture, then there has been an assay component failure which must be identified before progressing any further with the assay development process.

D. Select an assay condition providing stopped, high-titer, Known Positive Reference-Control associated TMB OD\textsubscript{450} values falling between 2.4 and 2.8 OD\textsubscript{450} units range. Out of these different assay conditions, select a condition that yields the lowest OD reading in the Blank wells (rows A and B, exposed to serum diluent only). Keep in mind once again that at this early stage of the assay development process, there is no absolute best set of assay condition iterations. Simply choose a single set of conditions that will allow the assay optimization phase in Section 7 to begin.

6.3.2 Perform an initial macroscopic ELISA performance analysis.

A. Observe the wells and their corresponding OD\textsubscript{450} values. What to expect:
   a. Absorbance of the blank wells should be < 0.1 OD\textsubscript{450} units with very little evidence of yellow color visible. If all of the blank wells have stopped TMB OD\textsubscript{450} values > 0.3, then one must assume that there is a plate-blocker or HRP-conjugate dilution issue!
   b. Some wells would be expected to display very bright-yellow colored stopped TMB substrate solutions (OD\textsubscript{450} > 3.0).
   c. There should be a distinct difference between the observed OD\textsubscript{450} values from wells containing the most dilute Positive Reference-Control samples (largest dilution factor) versus the most concentrated Positive Reference-Control sample containing wells.
   d. Generally speaking, due to comparison of 3 different conjugate dilutions, a respective Known Positive Reference-Control dilution should yield the highest OD\textsubscript{450} signal in columns 1-2 and 7-8 using the 1:5,000 conjugate dilution. Columns 3-4 and 9-10 using the 1:10,000 conjugate dilution, and columns 5-6 and 11-12 using the 1:20,000 conjugate dilution, would be expected to display progressively lower OD\textsubscript{450} values.
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 AD ELISA development process. If not, make the necessary procedural adjustments so that these minimal performance expectations are met. Use the Troubleshooting Guide (Table 2, below) for guidance.

**Table 2. Troubleshooting Guide**

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<th>Problem</th>
<th>Cause</th>
<th>Action/Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank has $OD_{450} &gt; 0.3$, easily recognizable yellow colored <strong>Blank Wells</strong> = high background signal problems</td>
<td>Nonspecific binding by conjugate to unblocked or inadequately blocked regions of plate-well surface, or some component within plate-blocker formulation itself.</td>
<td>Ensure blocking procedure was performed correctly. Verify that conjugate was affinity-purified and recognizes antigenic epitope sequences not present within the target antigen or blocker components adsorbed to the plates. Increase the dilution factor used to dilute out serum or plasma samples. If serum/plasma samples were only diluted 1:100, this may not be a large enough dilution factor to prevent nonspecific binding interactions with coated and blocked, plate-well surfaces. Consider reducing the concentration of the HRP labeled, anti-Ig isotype conjugate and/or the conjugate incubation exposure time.</td>
</tr>
<tr>
<td>Nonspecific binding event may originate from nonspecific interaction between generic (non-target) IgG and a plate blocker-component or inadequately blocked regions of plate-well surface. <strong>Note:</strong> in some cases, certain animal origin sample types may display more nonspecific binding problems when compared to other animal sources.</td>
<td>Repeat assay feasibility protocol using Neptune Sample Diluent (Catalog # 694). This more complex buffer is better suited for assays that require greater assistance in preventing nonspecific binding of generic (non-target) sample IgG to the blocked plate-well surfaces.</td>
<td></td>
</tr>
<tr>
<td>Operator or auto-washer error as relates to the proper plate washing process.</td>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>No signal</td>
<td>Assay set up incorrectly or use of incorrect reagents.</td>
<td>Check plate coating procedure, conjugate dilutions, and authenticities of Positive Reference-Control samples. Was the TMB substrate incubation step performed? Repeat assay.</td>
</tr>
<tr>
<td>Issue</td>
<td>Correction/Action</td>
<td></td>
</tr>
<tr>
<td>----------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
<td></td>
</tr>
<tr>
<td>Serum/plasma antibodies not recognizing plate coated antigen.</td>
<td>Verify that the plate coating antigen is representative of the wild type/field strains of the viral or bacterial pathogen or target protein analyte that was responsible for generation of the hypothetical humoral-immune response being targeted by the ELISA. If plate coating antigen is incorrect, identify another source of the proper antigen and coat new ELISA plates.</td>
<td></td>
</tr>
<tr>
<td>HRP-conjugated anti-isotype readout antibodies not recognizing antibody isotype binding to plate-wells.</td>
<td>Verify specificity of commercially sourced HRP-IgG anti-isotype readout antibody. Is the detection antibody specific for the animal Fc isotype of serum/plasma antibodies being monitored? Obtain a reliable source of HRP-IgG conjugate.</td>
<td></td>
</tr>
<tr>
<td>Conjugate stored incorrectly or subjected to repeated freeze/thaw cycles.</td>
<td>Use a fresh aliquot of conjugate that has not undergone multiple freeze-thaw events or purchase a new vial of conjugate.</td>
<td></td>
</tr>
<tr>
<td>Not using a 96-well plate that was treated for use in ELISA formats.</td>
<td>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.</td>
<td></td>
</tr>
<tr>
<td>Little to no difference between the TMB OD&lt;sub&gt;A450&lt;/sub&gt; signals from plate-wells containing the greatest dilution of serum or plasma samples versus those same samples that underwent the least dilution factor.</td>
<td>Incorrect positive control dilution protocol. Follow plate map as instructed (Figure 2).</td>
<td></td>
</tr>
<tr>
<td>Questionable authenticity of known positive control sera/plasmas.</td>
<td>Establish the fact that the plate coated antigen is representative of the wild type pathogen which generated these animal serum/plasma antibody titers. Is the positive control really a negative or very low antibody titer positive? Obtain a reliable source of positive controls.</td>
<td></td>
</tr>
<tr>
<td>HRP-conjugated anti-isotype readout antibodies not recognizing antibody isotype binding to plate-wells.</td>
<td>Purchase a new vial of anti-isotype HRP readout conjugate and start over.</td>
<td></td>
</tr>
<tr>
<td>Inconsistent OD&lt;sub&gt;A450&lt;/sub&gt; values between adjacent known-positive control sample wells.</td>
<td>Incorrect placement of positive control sample. Follow plate map as instructed (Figure 2).</td>
<td></td>
</tr>
<tr>
<td>Inadequacies in plate washing technique</td>
<td>Examine vacuum portals on plate manifold to verify whether or not there is a partial obstruction in a particular sample uptake port/line.</td>
<td></td>
</tr>
<tr>
<td>Entire plate displays a uniform-dark yellow (saturated) color.</td>
<td>Nonspecific binding of conjugate to plate-well or to nonspecifically bound host antibody. Verify blocking procedure was correctly followed. Verify that conjugate was affinity-purified and recognizes antigenic epitope sequences not present on the plate adsorbed antigen. Further dilute-out the positive control samples to minimize nonspecific binding of host antibody.</td>
<td></td>
</tr>
</tbody>
</table>
7. Optimization of Antigen-Down ELISA Performance Parameters

Antigen-Down (AD) ELISA optimization, beyond the initial demonstration of basic assay protocol feasibility, typically focuses on two rather universal AD ELISA-format performance criterion: 1.) Enhancement of ELISA sensitivity within the biological sample matrix in which the target analyte (Ig) is to be measured and 2.) Minimization of the probability of any false positive data readings from Known Negative Reference Control serum/plasma samples.

Before addressing the sensitivity aspects of the AD ELISA, the topic of sample dilution factors must be addressed. Most anamnestic serological antibody responses are robust enough in terms of the levels of antigen-specific-antibody generation to accommodate at least a 1:100 dilution of the samples. Even with the inclusion of this 100-fold dilution step, a majority of low-positive antibody response events should still be easily detected. Occasionally, when attempting to detect and qualitatively monitor a low titer IgE response in serum or plasma, it may become necessary to only use a 1:25 dilution factor. Given the universal quest to minimize nonspecific binding signal while correspondingly maximizing specific signal generation, the further out a particular sample can be diluted into the sample diluent, the lower the potential passive nonspecific binding signal will be. At this point, it is also necessary to emphasize the fact that the HRP-IgG anti-isotype component of the AD ELISA will also be a major contributor to unwanted nonspecific binding signal generation (See Section 7.3 for additional details).

In contrast to Antibody-Sandwich ELISA configurations, most AD ELISA formats do not require much effort toward achieving the requisite assay detection sensitivity levels necessary for their routine performance proficiency. There are two (2) reasons for this: 1.) Typical anamnestic immune responses generally excel at the process of amplifying the serological antibody titer to a particular antigenic stimulus, and 2.) AD ELISA formats essentially get to bypass the more rigorous antigen/analyte target capture step that must take place within the Antibody-Sandwich ELISA platform. AD ELISA plate-wells already contain the target antigen, in a pre-coated state, on the ELISA-plate-well surfaces. To state this another way, the antigen-analyte adsorption to plate-well step has already been completed during the AD ELISA plate coating.
process. All that remains is for the humoral Ig components (analyte) to bind to their respective pre-coated antigen-epitope-targets. Within this scenario, the rate limiting step is the specific interaction of host Ig isotypes (in serum or plasma samples) with the target antigen. However, as this typically occurs under conditions where plate adsorbed antigen-target components are presented in excess molar density levels on plate-well surfaces, this is a far less challenging task than asking plate-well adsorbed capture Igs to pull out target antigen-analyte at < nanogram per mL concentrations as must occur within an Antibody-Sandwich format assay. In essence, it is by far easier to capture quantifiable serum, plasma, or cell culture associated Ig antibodies within the AD ELISA platform when compared to what is required of the plate-adsorbed IgG antibody within the Antibody-Sandwich ELISA format. When anticipating the development time requirement for this type of project, it is advantageous to acquire some prior knowledge of previously documented humoral antibody response levels observed in other related situations. If published data on the magnitude of a typical serological response to an analogous antigen stimulating event (pathogen, autoimmune stimulus, etc.) indicates that high antibody titers tend to be the norm, then the need for extensive assay sensitivity enhancement should be minimal. Alternatively, if humoral antibody responses to this particular antigenic stimulating event are minimal, it will likely be necessary to push the sensitivity of the assay to at least assure the detection of the top 50% of the potential low-positive responders.

Optimization of plate antigen coating concentrations and the selection of an optimal blocker/stabilizer formulation are always the first two parameters addressed during the AD ELISA format development process. Once the coating/blocking conditions have been adjusted to yield minimal nonspecific binding signal with Known-Negative Reference Control samples and clearly defined positive signals with Known-Positive Reference Control samples, then the optimization strategy can move on the HRP mediated signal generation aspects of the assay. Selection of the appropriate HRP-IgG anti-isotype concentration in conjunction with the proper conjugate diluent formulation are usually the last of the major development tasks within the AD ELISA construction process. All of the above parameters will directly impact both the specific signal output as well the amount of nonspecific binding background noise generated by the assay. Regardless of whether the desired ELISA format is an Antibody Sandwich format or AD configuration as this protocol document addresses, the common goal will always be to create an assay mechanism capable of creating the highest positive signal to background noise ratio (S/N) as is reasonably attainable.

Included within this assay development milestone would be a determination of an optimal dilution factor range for the positive, medium and high ELISA titer reference controls. Both medium and high-titer ELISA reference controls must be diluted to a point where their stopped TMB O.D. signal falls between 1.0 and 2.8 OD units. Since humoral antibody responses will vary by the individual subject being evaluated, these dilution-factor calculations should be pre-determined in advance for each Known-Positive Reference Control.

7.1 Optimize Target Antigen Plate Coating Protocol

7.1.1 Evaluate target antigen coating concentrations to enhance assay sensitivity.

Once a working assay format has been achieved, a more sensitive ELISA prototype can be developed. As discussed above, most AD ELISA format assays do not require a great deal of detection level sensitivity enhancement. This is especially true when the main goal is focusing
more on a simple yes/no determination for the presence of a specific antibody titer in a serum or plasma sample.

E. Using the generic plate coating protocol described above in section 6.1.1, prepare duplicate ELISA plates bearing different Target Antigen coating concentrations across the plate surface (Figure 5, below). This will enable the concurrent titration of a Known Positive and Negative Reference-Control sample across plate-wells pre-coated with a range of antigen coating concentrations.

F. Prepare a no antigen coating control section containing 1X Antigen Coating Buffer without any Target Antigen present. Add this antigen-blank control solution to columns 1-2 at 100 µL per well. Each of the duplicate ELISA plates should contain this 2-column antigen-blank control section (See Figure 5, below).

G. Within the columns 3 and 4 of each of the two (2) plates, add 100 µL per well of the 0.25 µg/mL Target Antigen concentration (dissolved in the 1X Antigen Coating Buffer).

H. Coat wells in columns 5 and 6 on each of the two (2) plates with a 100 µL per well volume of the 0.5 µg/mL IgG coating concentration.

I. Continue plate coating process by adding 100 µL per well of the 1 µg/mL, 2 µg/mL and 4 µg/mL Target Antigen coating concentration in columns 7-8, 9-10, and 11-12 respectively.

J. Follow the blocking instructions provided within Section 6.1.2, above. These plates may be dried after blocking and stored at 2-8°C in sealed bags containing desiccant pouches.

K. Using duplicate ELISA plates with multiple Target Antigen coating concentrations prepared above, set up a serial dilution series in General Sample Diluent with the Known Positive Reference-Control to be run on one plate and the Known Negative Reference-Control dilution series to be run on the other plate (Figure 5). Row A should be reserved as a sample-diluent-only blank in that it only contains General Sample Diluent without any diluted Positive/Negative Reference-Control. Use a multichannel pipettor to add 100 µL per well of General Sample Diluent to Row A, Next, add the serially diluted Positive/Negative Controls to their respective designated plates, starting with the largest Positive/Negative Control dilution (least concentrated in row B) to the smallest Positive/Negative Control dilution (most concentrated in row H). This plate loading strategy is employed to accelerate and simplify the loading process.

L. Perform the sample incubation steps and subsequent washing steps.

M. Select an anti-isotype, HRP-conjugate dilution factor from one of the three (3) conjugate dilutions evaluated in Section 6.2 (above), where 1:5K, 1:10K, and 1:20K dilutions of the HRP-conjugated IgG anti-isotype were examined for their ability to provide a strong positive stopped TMB OD₄₅₀ signal in antigen-coated plate-wells incubated with a range of dilutions of the Known Positive Reference-Control. Ideally, this dilution of the conjugate would lead to a stopped TMB OD₄₅₀ signal between 2.5 and 3.0 OD₄₅₀ units when using a 1:100, 1:500, or some other dilution factor of the Known Positive Reference-Control. At some point, these positive well OD signals will be compared with the signal generated by a Known Positive Reference-Control (prepared using the same dilution factor) that was incubated within a no-antigen coated control well in columns 1 and 2. Once again, be reminded that the finalized
anti-isotype 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 AD ELISA development process evolves.

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

O. Examine the outcome of the parallel serial dilution analysis of a Known Negative Reference-Control, in General Sample Diluent. One reason for running a Known Negative Reference-Control sample at this point is to demonstrate a clear TMB signal output differential between a Known-Positive and Known Negative sample type.

P. Divide the average OD_{A50} values for the Known Positive and Negative Reference-Control samples by the average OD_{A50} value for the corresponding sample-type dilution run in the no-antigen control wells coated with coating buffer only (columns 1 and 2 on each plate). The focus should be on the Known Positive sample dilution wells with stopped TMB OD_{A50} Signal values < 3.0 OD units. This exercise will provide some insight into the relationships that exist between two independent assay environment variables (antigen coating concentration and Known Positive or Negative Reference-Control dilution factor) and the quality of the signal to noise ratios (S/N).

Q. Examine and rank the S/N ratios from highest to lowest for both the Known Positive and Known Negative Control sample types. Some of the observed Known Negative stopped TMB signal may actually represent a low level specific interaction in addition to the anticipated non-specific interaction events that can be minimized but are unavoidable.

R. Paying particular attention to the Known-Positive Reference Control samples, identify the Target Antigen coating concentration that yielded the highest signal-to-noise ratio.

**Figure 5: Target Antigen Coating Optimization Plate Map**

![Plate Map](image)
plasma is diluted out according to plate map above. 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 OD₄₅₀ units and blank reading < 0.15 OD₄₅₀ units.

S. Obtain a consensus as to what amount of Known Negative Control sample signal constitutes a legitimate concern. Elevated (e.g. > 0.4 OD units) Known Negative Control sample signal can be a direct result of the plate-blocker/stabilizer product type/formulation selected. Additionally, elevated Known Negative Control sample signal can be indicative of some form of a pre-exposure event or exposure to an antigenically similar target or pathogen.

T. All “hypothetically authenticated” Known Negative Control samples should be included during the early stages of any new AD ELISA development project. This allows for better assessment of the prevalence of low positive responder samples. Survey these Known Negative Control samples using assay conditions that seemed to be optimal (i.e. best S/N ratio) for the Known-Positive Control.

U. Concurrent with running a more comprehensive serological screening of all Known-Negative Control samples, review what types of assessment qualifications were used to assign the Known-Negative Control sample label to these negative control samples. In other words, what was the nature of the immunological testing protocol that lead to the conclusion that these negative control samples were really negative?

V. After completion of the pre-designated Known-Negative Control sample evaluation, determine whether the initial Known-Negative Control sample was an outlier. If the results of the future serological screening project impact a wide range of other ongoing assumptions and projects, obtaining and testing a larger number of hypothetical negative control samples (50-100 samples) is recommended.

7.2 Establishing a Consensus Dilution Factor Range for Samples (Optional)

Identification of a defined dilution factor range for assessment of the majority of serum or plasma samples in the study represents an important first-step toward streamlining the overall ELISA titer screening process. Typically, AD ELISA screening protocols represent a simple straightforward approach to making direct comparisons of specific antibody titers between different bleed dates taken from a common human or animal subject over the course of a structured immunization protocol or pathogen associated disease state. When it is known in advance that a specific Ig ELISA titer assessment project will be of a very limited scope, one-time event (e.g. < 30 bleed samples), it may not be worth taking the time to establish a formalized consensus 3 or 4 dilution factor protocol for use in all future AD ELISA titer screenings.

When selecting a consensus dilution factor, it is important to avoid sample dilution-factors which are not diluted out far enough to bypass most generic nonspecific Ig binding issues. Occasionally, problematic serums containing a “sticky” variant of a nonspecific serum IgM isotype or other serum components can also occur. These serum components have a tendency to adhere to blocked plate-well surfaces. Due to the sticky nature of these plate-well-bound aberrant protein molecules, elevated nonspecific binding levels of both generic serum/plasma Ig and subsequent HRP-IgG anti-isotype conjugate can create unexpected background problems.
If such nonspecific background issues are encountered, switching over to an alternate sample diluent formulation may help minimize this problem. In addition to the General Serum Diluent provided in this kit, ICT also offers two other sample diluent formulations. All three sample diluent formulations are included in the Sample Diluent Optimization Pack (Catalog # 959). Though use of the optimal sample diluent formulation for a particular assay can certainly help minimize the amount of nonspecific binding signal, the most effective way to deal with nonspecific binding issues is to simply dilute them out while relying on higher affinity, specific antibodies to generate a strong signal readout.

7.2.1 Select the plate coating concentration that was shown above in section 7.1, to yield a stopped TMB OD₄₅₀ value of 1.0-2.8 whilst presenting the lowest nonspecific binding signal levels in the General Serum Diluent-Only sample blank wells (Row A in Figure 5).

7.2.2 Using the plate preparation protocol described above in section 5.1, prepare a multiple plate batch (e.g. 6 plates) of the coated and blocked AD ELISA plates. These can be used for determining whether or not a consensus sample dilution factor range can be established going forward.

7.2.3 Prepare 5-fold serial dilutions of Known Positive Control samples (in order to function as a reliable Known Positive Control, it is recommended that the sample material being used be prequalified by an orthogonal industry-recognized immune titer screening method). Titer screening of up to 6 samples could be accommodated using just 1 plate. Ideally, it is best to perform an initial AD screening survey on as large a Known Positive Control sample population as is accessible. Every 6 additional Known Positive Control samples will require an additional antigen coated and blocked ELISA plate. As an example, ELISA titer screening of 7-12 known positive samples would require the use of two ELISA plates (see Figure 6, below). Since the range of the potentially elevated immune-titer for any particular humoral Ig response will be initially unknown, the initial dilution-factor should start out down at the 1:500 level (see Figure 6, below). After making the initial 1:500 dilution, go on to prepare the 5-fold serial dilutions, and then load each dilution in duplicate as depicted below in Figure 6. Note that the dilution scheme described here and depicted in Figure 6 is just a recommended starting point. As all samples are unique, alternative dilution schemes may need to be evaluated to determine the ideal dilution factor range for a particular sample.
7.2.4 Perform AD ELISA screening of these Known Positive Control sample pools following the parameters established during the optimization steps performed in Section 7.1.

7.2.5 Evaluate the stopped TMB OD<sub>A450</sub> signal ranges from this initial small sampling pool. Determine whether or not this type of dilution factor range provides OD<sub>A450</sub> signals falling between 0.3 OD units and 2.8 OD units for all Known Positive samples tested.

7.2.6 Record the various dilution factor and OD<sub>A450</sub> output pairings that were obtained for each Known Positive Control sample evaluated in this AD ELISA format.

7.2.7 Consider the possibility of having a new generic sample-titer classification labeling-group. For example, designation of any AD ELISA sample generating an OD<sub>A450</sub> signal > 2.8 units at a 62,500 dilution factor as a high titer sample. Once the ELISA OD signal exceeds a certain upper absorbance threshold at a predesignated dilution factor, for simple “yes/no” determinations, the need to perform additional dilutions to find one falling within the linear-quantitative range of the plate reader instrument fails to merit the extra work needed for additional screening.

7.3 Minimize Conjugate-Derived Nonspecific Binding Signal Generation

Another important issue to address in AD 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 Antigen-Down ELISA Development Kit includes a 25 mL bottle of Antigen-Down HRP Conjugate Stabilizer, 5X (Catalog # 6169). Dilution of this component 1:5 in diH2O 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.

Two other strategies can also be enlisted to help reduce the conjugate nonspecific binding interaction 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-associated conjugate binding problems. Fortunately, these two major factors driving nonspecific binding events are easily manipulated.

### 7.3.1 Conjugate Concentration

When attempting to maximize assay sensitivity levels, resist the temptation to use greater than necessary conjugate concentrations. Increasing the concentration of the HRP-conjugate will drive the specific binding kinetics toward shorter equilibrium establishment incubation times. Unfortunately, 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/ 0 control standard where visible color development is undesirable, and the signal-to-noise ratio drops off precipitously. To best approximate an optimal HRP-IgG anti-isotype concentration, it is recommended to run a simultaneous comparison of several dilutions (1:500, 1:1,000, 1:2,000, and 1:4,000) of a Known Low-Positive Reference-Control as well as a Known Negative Reference-Control sample on the same ELISA plate. Selection of a Low Known Positive Reference-Control for this analysis is a deliberate attempt to avoid selecting a conjugate dilution factor providing low nonspecific binding signal in the negative serum/plasma wells but failing to detect the known low positive samples present in their respective wells. Upon completion of the required wash steps, the ELISA plate is exposed to multiple dilution factors (1:2,500, 1:5,000, and 1:10,000) of the conjugate. This step should reveal which conjugate dilution factor provides the desirable minimal background signal with the negative control samples as well as an easily discernable positive signal readout on the low titer positive samples. Although, it is inherently preferable to identify a single conjugate dilution factor capable of recognizing >90% of the harder to detect, low positive samples, this emphasis on assay sensitivity must be counterbalanced by a desire to keep excess conjugate concentration driven nonspecific binding activity to a minimum.

### 7.3.2 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 Fc portion of the antigen-specific serum/plasma antibody are essential for any quality AD-configuration 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 6 for illustration of this concept).

Figure 7. Accumulation of 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 AD and AS 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 non-specific signal increases over time.

8. Quality Assessment of Plate Coating Process

Once the general operational parameters for this AD ELISA have been established and the timeline dynamics of the serological sample screening project have been clearly defined, then it is prudent to consider performing an ELISA-based antigen coating precision analysis of the present plate coating process. Even if the serological titer screening study is just designed to perform a simple “yes/no” analysis, a cursory pre-screening run using a diluted mid-level positive reference control sample should still be a mandatory practice. It is important to verify that the target antigen was successfully adsorbed to the ELISA plate-wells while still retaining a useful level of antigenicity relative to the antibody population being analyzed in the animal or human host subjects.

8.1 Frequent Sources of AD ELISA Variability Problems
As stated above, 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 still 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 AD 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.

Antigen 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 antigen coating buffer leading to the precipitation or partial denaturation of key antigenic epitope content on the plate coating antigen, 3.) inconsistencies in the plate-well to plate-well liquid volume used for antigen-coating, blocking, and washing steps, and 4.) antigen epitope denaturation resulting from liquid surface tension disruption (shearing) of antigen epitope structure during initial plate coating process (may be associated with automatic plate coating equipment).

8.2 Setting up a Generic ELISA Plate Coating Precision Study

When large (e.g. >50 AD ELISA plate batch-size) runs are the norm and the antigen coating on these ELISA plates must retain its antigenic epitope properties for > 12 months (refrigerated 2-8°C), then the time and money invested in plate preparation and serological sample collection activities over the course of the study clearly justify the comparably small time investment put toward assuring that the antigen coating process was successful.

8.2.1 Generic AD ELISA Plate Coating Precision Screening Protocol Setup

A. Remove a predetermined sampling percentage of the coated/blocke/packaged AD 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 AD ELISA plate inventory. Obviously, if there is some prior evidence of a potential for antigen coating inconsistencies, then it may be necessary to increase the plate precision QC screening protocol to initially sample a greater portion of the packaged AD ELISA plate inventory. There may be time associated factors within the plate coating process itself, which may arise from the antigen’s physical composition characteristics. Some of these characteristics would include:

1. The antigen’s molecular solubility properties in aqueous buffers ranging from pH 7 - 9.5.
2. Molecular aggregation tendencies leading to auto-precipitation in aqueous/neutral pH/isotonic salt environments. This event is modulated by the relative protein surface presentation-ratio of hydrophobic to hydrophilic amino acid concentration regions.
3. How robust are the key antigen epitope properties of the coating antigen? Changes in antigen epitope properties could lead to an increase or reduction of the antigenic presentation features over the course of the plate coating process. To a much lesser degree, there may be variations in antigen 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 antigen coating features that would otherwise be non-discernable.

B. Prepare an appropriate quantity/volume of one of the Known Positive Reference-Control pools. This Known Positive Reference-Control should be diluted into the General Serum Diluent (Catalog # 648) 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. However, it should be noted that in many instances the legitimate positive sample ELISA OD signal can run down into the 0.2 – 0.3 OD unit range. The motivation for conducting routine plate coating precision screening analysis would then be to avoid instances where a plate coating irregularity issue resulted in missed low positive sample detection.

For example, if the plate coating precision study was designed to evaluate five (5) AD ELISA plates, the recommended volume of appropriately diluted Known Positive Reference-Control 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 diluted positive control pool 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 the diluted positive control 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 diluted Known Positive Reference-Control across all plates, there is no need to change the pipette tips between plates.

C. Perform the AD ELISA plate screening analysis using the most current AD ELISA protocol (optimized in Sections 6 and 7, above).

D. Obtain the raw OD$_{A450}$ – OD$_{A540}$ readings using the software available on the colorimetric ELISA plate reader.

**8.2.2 AD ELISA Plate Precision Screening 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 to performing such an analysis. The following section builds on the aforementioned example where 5 plates were reserved for the coating precision study.

A. All five AD 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 well = 480) raw OD 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 \( [(\text{SD or } \sigma / \bar{X}) \times 100] \) where \( \bar{X} \) is the mean of a selected set of raw plate-well derived OD values and SD or \( \sigma \) are acronym and statistical symbols respectively, for 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 values into an Excel sheet and then setup the Excel formula macros to calculate the mean \( (\bar{X}) \) of all 96 plate-well OD\(_{450}\) output values as well as the standard deviation (SD) for these 96 OD\(_{450}\) readings. Calculate the % CV for that particular AD ELISA plate. Repeat this process for the four remaining AD 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 the plates were created for. 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 serological screening study has more modest outcome assessment aspirations, a higher degree of plate coating variability may be acceptable. An example of such a situation is provided in the following example: The goal of the screening study is to verify whether or not a new, limited number (2) subcutaneous injection, hyper-immunization protocol was capable of generating a useful anamnestic serological IgG response in a small number (12) of New Zealand White rabbits. In this scenario, only a small number of coated plates will be needed (most likely < 10 AD ELISA plates), as the project does not involve a long-term, multiple hyper-immunization injection protocol. In this example, the serological assessment expectations of the AD ELISA are a “yes” or “no” outcome determination. Based on these expectations, even coating precision % CV variability of 20% would not immediately render the plates incapable of correctly assessing whether or not the immunization protocol was successful.

Alternatively, there may be scenarios that require a lower degree of variability (i.e. lower upper limit for an acceptable % CV value). An example of such a situation would be a study involving a long-term ongoing serological analysis for veterinary diagnostic purposes. Such assays are used to monitor everything from herd or flock exposure incidents to an Influenza A virus to small companion animal (dog or cat) food allergy screening. In antibody screening services such as these, it is imperative that the various specific coating antigen iterations are evenly and equally incorporated into their designated ELISA plates or pre-assigned sections within a particular ELISA plate grouping. Here, the upper limit for an acceptable % CV within plate-well to 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 (as are the antigens and other reagents used to build an AD ELISA), 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 AD ELISA based serological screening project. Encountering such a situation should immediately trigger a
reassessment of the plate coating and blocking protocol. Only after completion of this necessary thought process should any attempts to create another batch of Target Antigen coated and blocked plates be undertaken.

9. Accounting for Natural Variation in Biological Samples and Assay Conditions

9.1 Comparison of ELISA Sample(s) Signal over Time and Multiple Bleed Dates

Interpretation of AD ELISA format data derived from multiple sample date test-bleed results over a multi-month time frame, presents a more formidable data interpretation challenge than analyzing data obtained from a more defined and straightforward quantitative Antibody-Sandwich ELISA format. One option for addressing this is to utilize a Sample OD₄₅₀ to Known Positive OD₄₅₀ (S/P) ELISA OD Ratio Method. Utilization of this technique is dependent at the very least, on the availability of an adequate supply (25-30 mL pool volume) of a high or medium ELISA-titer Known Positive Reference-Control. These serum/plasma pools should be aliquoted into 0.5 mL volumes within high quality type commercial cryovials (1 mL volume size) and stored at < -70⁰ C. These can serve as assay reference calibrators for performing S/P OD₄₅₀ signal conversion normalization.

Implementation of an S/P ratio comparison method is an easy and efficient way to assign some type of comparative numerical value to the raw OD₄₅₀ value readings generated within the AD ELISA format. Refer to Figure 8 for an example of how to set up such a plate. To employ this raw OD₄₅₀ score normalization strategy, 2 fixed dilutions of Known Positive Reference-Control serum/plasma sample must be permanently included within the assay protocol. Ideally, both a medium and high ELISA titer Reference-Control would be sampled on each AD ELISA plate. Care must be taken to verify whether or not legitimate low positive samples are still detectable within one of the 3-4 chosen serum/plasma dilution ranges. Obviously, selection of a sample dilution factor that dilutes out the low positive samples to the point where the assay no longer detects them, should be avoided. If available, evaluate multiple dilution-factor AD ELISA screenings on a small number of low positive serum or plasma samples in advance, to assure that low positive samples, when diluted out into the consensus chosen dilution factor range (determined in Section 7.2, above) still remain within the detectable range of the assay.

Typical serum or plasma screening AD ELISA templates would contain 3 sets of reference controls:

1. Four (4) replicates of the selected dilution factor medium titer reference control.
2. Four (4) replicates of the selected dilution factor high titer reference control.
3. Four (4) replicates of the selected dilution factor negative reference control.

Test-samples should be plated out using 2 replicates of each predetermined dilution factor. For example, if the predetermined dilution factors for the medium and high ELISA titer reference controls are 1:10,000 and 1:30,000 respectively and the predetermined unknown-sample dilution factors are 1:500, 1:5,000, and 1:25,000, one might consider setting up a plate template structure resembling what is shown in figure 6, above. Without prior knowledge of the magnitude of the potential humoral immune response that will be encountered in these unknown test samples, it will be necessary to examine each new test-sample only after it has been diluted out 1:500, 1:5,000, and 1:25,000 in the sample diluent (see Figure 8).
Figure 8. Plate set-up example for normalizing plate-to-plate variations by S/P ratio. To normalize plate-to-plate variability during routine testing of unknown samples (6 unknown samples are shown in this example), include a Medium Titer and High Titer Positive Reference-Control, and a Negative Reference-Control on each ELISA plate (to be run in quadruplicate, and one previously determined dilution). All unknown samples will be tested (at least in duplicate) at 3 different dilutions. The dilution factors for the unknown samples should be based on previous screening of Known Positive Reference-Control samples. Any unknown sample that produces an OD$_{450}$ value greater than 2.8 when diluted 1:25,000 can either be retested using a larger dilution factor or can be labeled as “High Titer” and not retested. All unknown samples will be normalized by comparison to the OD$_{450}$ values collected from the Medium and High Titer Reference-Controls and the Negative Reference-Control.

Should an occasional very high ELISA titer test-sample be encountered which would produce ELISA TMB substrate OD$_{450}$ values $>>$ 2.8 when diluted at the 1:25,000 level, this particular sample would need to be re-tested using a higher dilution factor (e.g. 1:75,000). Trial antigen-down ELISA titration of the Positive (high ELISA titer) Reference-Controls in conjunction with re-running those excessively-high ELISA titer sample(s) using a higher dilution factor is a simple way to establish an additional consensus higher sample dilution strategy. To summarize, there needs to be some form of historical ELISA titer evidence-based justification for incorporating additional higher dilution factor steps into the ongoing ELISA protocol.

9.2 Streamlining Ongoing AD ELISA Screening Process Going Forward

Once AD ELISA derived, sample-specific, raw OD$_{450}$ values have been obtained from the animal or human subject population, subsequent sample bleed ELISA titers from that particular animal or human subject should become more predictable. It is typically a given that the humoral Ig response will be subject to a varying degree of upward or downward change over the course of the specific immune response period. The magnitude of these serological specific Ig antibody concentration changes between 2 respective bleed dates (< 4 weeks apart) is not likely to exceed a 20-fold
concentration differential. That would suggest that the number of required dilutions per test sample could be reduced to no more than 3, or even 2, if sampling the antigen-specific ELISA titers during the humoral response plateau period.

Establishing an arbitrary in-house dilution factor cutoff (as detailed above in section 7.2.7) would further simplify the periodic serological sample evaluation workload. In this scenario, any sample presenting a stopped TMB ELISA OD\text{A\textsubscript{450}} signal > 2.5 OD\text{A\textsubscript{450}} units at a designated sample dilution factor (e.g. Figure 6, choosing a 1:62,500 dilution) would simply be assigned a generic “high titer” designation. Once the ELISA OD\text{A\textsubscript{450}} signal exceeds a certain upper absorbance threshold at a predesignated dilution factor, there would be no need to perform additional dilutions to find one falling within the linear semi-quantitative range of the plate reader instrument. Many AD ELISA screening projects, once they enter the mature (humoral Ig production plateau) stages of the immune response to a particular antigenic stimulus event, can get by using only a 1 or 2 dilution factor testing protocol, thus further reducing the sample evaluation workload.

**Rationale for Adopting S/P Method for ELISA OD\text{A\textsubscript{450}} Signal Comparison**

As is the case when evaluating all types of AD ELISA format data output (particularly when data collection events occur multiple times over the course of a 3, 6, or 12-month time frame), a common recurring data analysis challenge is how to interpret raw OD\text{A\textsubscript{450}} signal variation between common target specificity assays run within a single day, as well as between assays run on different days. Signal variation dynamics may be exacerbated when assays are run months apart, or by several different people, or both. Conversion of raw OD\text{A\textsubscript{450}} data into an S/P transformed hybrid-OD\text{A\textsubscript{450}} Score creates an opportunity to interject a signal normalization scheme into the process of comparing different raw ELISA OD\text{A\textsubscript{450}} value data from assays evaluating different bleed-date samples, which may have also been assayed on different days. By including one or two Known Positive Controls within each ELISA analysis run, these inevitable raw OD\text{A\textsubscript{450}} score variations become less problematic.

**9.3 Common Sources of Raw OD\text{A\textsubscript{450}} Score Signal Variation**

When examining the root cause(s) of AD ELISA signal variation, the most common source of signal variability occurs when different people are tasked with running a common antigen-down serological screening assessment. Variation in ELISA performance expertise will greatly influence such assay performance parameters as incubation time consistency, time required to complete sample/component additions to plate, and plate washing proficiency. Additionally, if the person performing the ELISA protocol has also been tasked with running 3, 4, or 5 ELISA plates per day (each possibly having a different assay protocol), additional signal variation can ensue. Commonly, these “Busy Day” associated raw OD\text{A\textsubscript{450}} signal generation inconsistencies tend to favor the likelihood of there being protocol deviations in the time-duration of the various incubation steps within the assay. Multi-plate runs (> 3 assays per day per person), when coupled with having several different people performing this activity concurrently, can be another source of assay-to-assay variability. Other factors contributing to signal variation include daily laboratory temperature inconsistencies during assay performance periods, as well as variations in TMB substrate incubation times prior to stopping the reaction with stop solution.

Expanding a bit further on TMB substrate step associated variability, AD ELISA protocols containing TMB incubation periods << 15 minutes in duration can, with only a slight delay in adding the stop solution, produce assay OD\text{A\textsubscript{450}} levels well above the expected OD\text{A\textsubscript{450}} range for that particular serum or
plasma sample. Furthermore, variability can be introduced when switching to a new lot of AD ELISA production plates, TMB substrate, etc. Different production lots of ELISA plates may perform differently. Often, performing some sort of side-by-side comparison to compare performance of the new lot to that of the old lot can minimize the likelihood of encountering unexpected performance differences. When performing such a comparison, all other reagents should be held constant. For example, to evaluate a new lot of TMB, compare the performance of the new lot while simultaneously evaluating the performance of the old/current lot of TMB. Both assays should be performed using a common pool of reagents (same lots of all other materials) that have been prepared at the same time, by the same analyst, and following the same ELISA protocol. Without the ability to normalize the multiple ELISA generated raw OD_{A450} values, it is difficult to make any credible statement regarding the temporal relationship existing between, for example, the progress of a hyper-immunization protocol and the generation of a serological titer of antigen-specific Ig isotype.

9.4 Sources of OD_{A450} Data Variability not Resolved by S/P Ratio Conversion

Unfortunately, not all operator error associated problems can be repaired using the S/P OD_{A450} data conversion strategy. For example, isolated incidences of bad pipetting technique leading to “random” reduced or elevated sample OD_{A450} readings will not benefit from the use of the S/P OD_{A450} ratio conversion. Unless the impact of a protocol deviation error has an equal enhancement or suppression effect on every sample and reference control on the ELISA plate, the S/P OD_{A450} ratio conversion strategy will not be helpful in attenuating variability.

9.5 How S/P Ratio OD_{A450} Data Conversion Works

When an S/P OD_{A450} data conversion method replaces simple raw-score ELISA OD_{A450} value comparison for monitoring humoral response trends over time, much of the operator or environment associated variation factors are removed from the data sets. Assay-to-assay data variation events can be attenuated by inclusion of known positive medium and/or high ELISA titer reference controls within each ELISA plate run. In essence, when assay operating conditions, regardless of the source, create situations where test sample OD_{A450} values are artificially too high or too low, these same factors would correspondingly affect the reference control sample OD_{A450} values. Situations causing sample OD_{A450} values to run abnormally high or low include:

1. The ELISA was performed when lab temperatures were elevated or decreased by at least 5°C for the same bleed sample(s) comparison.
2. The HRP–IgG anti-isotype conjugate incubation step was allowed to run > 10 minutes longer or shorter than designated in the assay protocol.
3. The HRP-IgG anti-isotype conjugate solution was unknowingly prepared at a greater or lower concentration than prescribed by the assay protocol.
4. The TMB substrate incubation step was allowed to run > 5 minutes longer or shorter than prescribed by the assay protocol.
5. The TMB substrate was switched to a formulation with higher or lower signal-sensitivity.
6. Reading the stopped TMB signal at a suboptimal absorbance wavelength will greatly diminish the raw ELISA OD_{A450} readings on all ELISA plates run on that particular day.
Even in the presence of suboptimal assay operation conditions, these factors would likely have an equal effect on the medium or high positive reference control denominator. Thus, the actual change in overall S/P ratio values on these plates, on these particular days, may not be significant enough to cause a misinterpretation of the serological-status of the anti-antigen Ig concentration.

10. Conclusion

Each ELISA development project will present its own unique display of antigen versus antibody binding dynamics. These highly variable antibody versus antigen binding relationships can only be identified and understood through implementation of an educated trial and error assessment process. It is best to approach the AD-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 antigen-down format ELISA that a research project may require.

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