

Antigen-Down ELISA Development Kit

Catalog #9101

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

This kit provides the liquid components, 96-well plates, plate storage materials, and a template for developing a novel 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, 100 mL (Catalog #6102)
- ELISA Wash Buffer, 10X, 500 mL (Catalog #651)
- TMB 1-Component HRP Microwell Substrate, 2 x 100 mL (Catalog #6276)
- Stop Solution for TMB Substrates, 2 x 100 mL (Catalog #6282)
- Costar® 96-Well EIA/RIA Stripwell™ Plates, 20 plates (Catalog #25)
- ELISA Plate Sealing Covers, 2 x 10 pack (Catalog #6287)
- Foil ELISA Plate Storage Bags, 2 x 10 pack (Catalog #6288)
- Desiccant Packets, 2 x 10 pack (Catalog #6289)

2. Key Materials Required but Not Provided

- ELISA plate reader capable of reading 96-well plates at 450 nm absorbance
- Washer/aspirator system, or squirt bottle for washing by hand
- Known Positive and Negative Control serum/plasma sample pools (hereafter identified as Known Positive or Negative Controls). The majority of samples routinely monitored using an Antigen-Down 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 Controls must have undergone a prescreening process, preferably via Antigen-Down ELISA, to properly validate the presence or absence of a specific antibody titer toward the target antigen. Acquisition of these controls is critical for proper assay development.

Minimum quantities of Positive and Negative Control sample pools needed: 20-50 mL of at least a high titer Known Positive Control sample or pool, depending on the intended use of the assay following the development phase. If possible, acquire 10-50 mL pool volumes of different human/animal origin low, medium, and high titer control pools. To minimize the potential of antibody titer depletion, all serum or plasma controls should be aliquoted and frozen in 0.5-1 mL aliquots. Use high-quality 1.2-1.8 mL cryovials for frozen sample storage. Store at $< -25^{\circ}\text{C}$.

- Cell culture-derived Positive and Negative Controls (optional): these controls would only be relevant if 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 or 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 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 Antigen-Down HRP Conjugate Stabilizer included in this kit.

- Target Antigen for plate coating: purified, biologically isolated and characterized, or recombinant synthesized origin. Store plate coating antigens at $< -25^{\circ}\text{C}$.

Minimum antigen quantity requirements vary with the expected size of the sample screening project. Smaller projects will require lower amounts, while larger projects will require greater amounts. The amount needed will likely fall between 5 to 20 mg of plate coating antigen.

Due to the loss of epitope accessibility arising from steric hindrance of epitope regions directly adsorbing to the polystyrene ELISA plate well surface, or the loss of epitope structure from the physical forces associated with adsorption to the well surface, generally only antigens with molecular weights $> 8\text{ kD}$ should be used for direct onto plate coating. Exceptions can be made to these minimum molecular weight guidelines if prior studies clearly demonstrate that these smaller molecular weight antigens retain enough of their key epitope integrity to adequately represent the native form of the target antigen.

When working with small molecular weight (hapten-like) targets, 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 Control pools. Qualification typically involves an ELISA or other orthogonal immune-titer assessment assay.
- Affinity purified, Ig isotype-specific HRP-IgG conjugate (detection 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 per IgG molecule.
- 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

An Antigen-Down (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 (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 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. 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. Examples of different types of Target Antigens include oligopeptides, polypeptides, and certain polysaccharide molecules. After the Target Antigen has been adsorbed to the ELISA plate wells, the entire 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. To do so, samples are incubated in the coated wells, after which the ELISA plates are washed to remove unbound sample. Next, anti-animal isotype specific IgG-HRP conjugate is added to each well and incubated. After the conjugate 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, such as TMB, to the plate wells. This will allow for the visualization and electronic quantitation of how much Target Antigen-specific antibodies were present within the samples. In the presence of the HRP-IgG conjugate (which is specifically bound to Target Antigen that was previously 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 sample.

To summarize, the basic AD ELISA format is capable of providing 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. This Antigen-Down ELISA Development Kit was created to help guide end users through the common steps and frequently encountered issues associated with building an AD ELISA. The guide describes the assay performance milestones that must be achieved to enable progression through the various development stages of the antigen-down format ELISA (Table 1). The finalized ELISA should efficiently detect isotype-specific, low to high positive titer, humoral antibody responses in serum or plasma samples.

Table 1. Antigen-Down ELISA Development Milestones

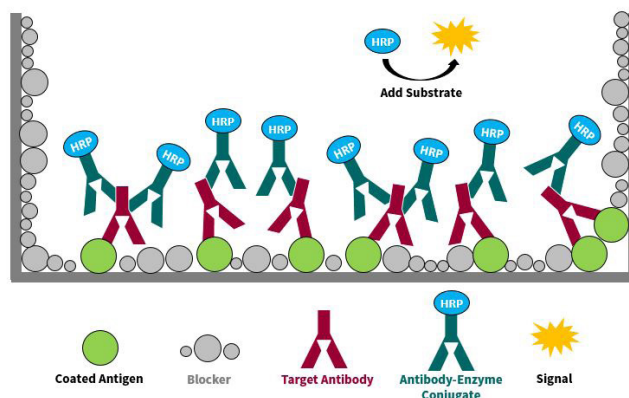
1	Define Project Scope/Objectives and Construct Assay Development Plan
2	Acquisition of Key Assay Components
3	Demonstration of Basic Assay Feasibility
4	Optimization of Assay Performance Parameters
5	Accounting for Natural Variation in Biological Samples and Assay Conditions

5. Basic Considerations Before Starting AD ELISA Development

When considering the possibility of developing an AD ELISA format to detect a humoral antibody response, it is critical to first clearly define the scope of the project. Is the goal to generate simple “yes/no” determinations for the presence of a particular antigen-specific antibody within hybridoma cell supernatants or test-bleed serum

FIGURE 1: Antigen-Down ELISA

In Antigen-Down-ELISAs, a Target Antigen coated onto the ELISA plate surface is used to quantify specific antibody concentrations present within samples. When the sample is added (such as human serum), the antigen on the plate is bound by antibodies (IgE, for example) from the sample, which are then retained in the well. A species-specific antibody (anti-human IgE, for example) labeled with HRP is added next, which binds to the antibody bound to the antigen on the plate. The next step is to add a chromogenic HRP substrate, such as TMB, to the plate wells. After the substrate incubation, a stop solution is added to stabilize reacted product and increase the dynamic range of the assay. Finally, the absorbance is read in an ELISA plate reader. The higher the signal, the more antibodies there are in the sample.



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. 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 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 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 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 estimate of a serological concentration of a particular antigen-specific antibody in serum or plasma samples. When used in conjunction with the term “ELISA”, it simply represents a relative approximation of the antigen specific antibody concentration as revealed via the intensity of the AD ELISA OD_{A450} signal for any given serum/plasma sample dilution factor.

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 assay is capable of detecting known medium to high humoral-response serum or plasma samples, the proposed 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, the suitability of the HRP-labeled anti-animal-isotype IgG conjugate may be challenged. Fortunately, most commercially sourced HRP-IgG 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 low antibody titer samples. When anticipating the development time needed for this type of project, it is helpful 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 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 > 50-fold. 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 Target Antigen, leaving blank control wells**

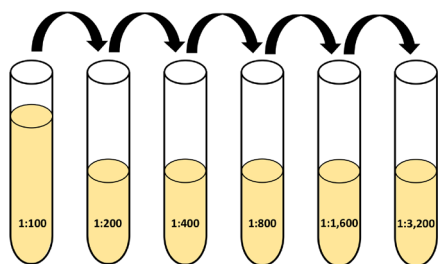
- Add 10 mL of the Antigen Coating Buffer, 5X (Catalog #6247) to 40 mL of diH₂O. This provides a 50 mL volume of 1X Antigen Coating Buffer. **Antigen Coating Buffer, 5X may precipitate at refrigerated temperatures. If this happens, gently warm to room temperature (RT) until dissolved.**
- Transfer 20 mL of 1X Antigen Coating Buffer to a new 50 mL polypropylene tube.
- 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 agent that the screening is intended to monitor. This protein, carbohydrate, or glycoprotein

6.1.3 Day 3 - Complete plate blocking and optional drying

- 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 assessment can begin. 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.
- 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.
- 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.
- Store blocked and dried plate(s) in the provided moisture-proof Foil Storage Bags (Catalog #6288) at 2-8°C, protected from light, with desiccant pouches (Catalog #6289) to minimize moisture exposure. Plates stored in this manner should retain their 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 Control**

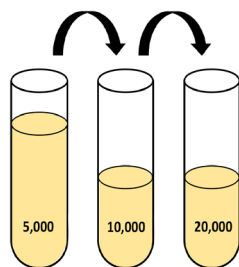
- Confirm two key criteria regarding the positive control: first, it has been authenticated by serological assessment to contain specific antibodies to the plate-adsorbed Target Antigen; second, antibody titers generated within these human/animal-derived serum or plasma sources were the direct result of immune system exposure to a relevant event.
- Allow General Serum Diluent (Catalog #648) to come to RT before use. Set up a simple 2-fold serial dilution scheme in six labeled tubes (Figure 3). Prepare a 6 mL volume of a 1:100 dilution of a Known Positive Control in General Serum Diluent.
- Make an initial 1:100 dilution by spiking 60 µL of the Known Positive Control into 5.94 mL of General Serum Diluent.
- Add 3 mL volumes of General Serum Diluent to each of the labeled tubes within the serial dilution series (1:200, 1:400, 1:800, 1:1,600, and 1:3,200).
- Add 3 mL of the 1:100 sample to the tube containing 3 mL General Serum Diluent labeled 1:200. Mix thoroughly.
- Repeat 2-fold dilution process throughout the remainder of the series. Change pipette tips after every dilution in the series to avoid unintended carry over.

FIGURE 3: Serial Dilution of a Known Positive Control**6.2.2 Load ELISA plate with Known Positive Control and Blanks**

- 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 Controls as well as the blanks (General Serum Diluent Only) into their respective plate locations as soon as possible.
- Add 100 µL per well of General Serum Diluent to each well in rows A and B for the blanks.
- Add 100 µL of the lowest concentration (1:3,200) Known Positive Control to each well in row C on the plate (Figure 2).
- Proceed to add, from least to most concentrated, the remaining dilutions of the Known Positive Control to the plate wells. This allows one to keep the same pipette tips in place over the entire plate loading process.
- Cover plate with a plate sealing cover, Parafilm, or plastic wrap to minimize evaporation.
- Incubate plate for 45 minutes at 37°C or 75 minutes at RT, protected from light.

6.2.3 Prepare the HRP-IgG conjugate dilutions

- Label three new 50 mL tubes 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, adjust the dilution factors proportionally.
- Allow conjugate stabilizer to come to room temperature prior to use. Dilute 8 mL of the Antigen-Down HRP Conjugate Stabilizer, 5X (Catalog #6101) 1:5 into 32 mL diH₂O to yield 40 mL of 1X conjugate stabilizer.
- 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 thoroughly.
- In the tube labeled 5,000, prepare the 1:5,000 conjugate dilution by spiking 200 µL of the 1:100 conjugate dilution into 9.8 mL 1X conjugate stabilizer (1:50 dilution). Mix the tube containing the 1:5,000 dilution of the HRP-IgG conjugate thoroughly by capping and inverting the tube multiple times.
- Put 10 mL of 1X conjugate stabilizer into the tubes labeled 10,000 and 20,000.
- Transfer 10 mL from the 1:5,000 dilution tube into the tube labeled 10,000. Cap the tube and mix carefully but thoroughly (Figure 4).
- Transfer a 10 mL volume of the 1:10,000 dilution to the 10 mL volume of 1X conjugate stabilizer in the tube labeled 20,000. Cap the tube and mix.
- 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).

FIGURE 4: Serial Dilution of Conjugate**6.2.4 Wash plate and add the three HRP-IgG conjugate dilutions**

- Pour 1X ELISA Wash Buffer into a squirt bottle or into a large reservoir connected to a plate washer, or an 8/12 channel washer/aspirator manifold device that was designed for manual washing of plates.
- Label three solution basins as 5,000, 10,000, and 20,000 to represent the HRP-IgG conjugate dilution factors being analyzed.
- Transfer the contents of each conjugate dilution tube to its respective solution basin.
- 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.
- Using a multi-channel pipettor, add 100 μ L per well of the 1:20,000 conjugate dilution to the designated regions of the plate (Figure 2). Subsequently, add the 1:10,000 and 1:5,000 conjugate dilutions to their respective locations on the plate. Addition in this manner eliminates the need to change pipette tips after the addition of each HRP-IgG conjugate dilution.
- 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

- Allow substrate to come to room temperature prior to use. 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.
- Wash plate 4X in 1X ELISA Wash Buffer after completion of the HRP-conjugate incubation period.
- Remove any residual wash buffer from plate by tapping plate onto a stack of clean paper towels.
- Add 100 μ L/well of the TMB substrate to every well using a multi-channel pipettor. Discard tips and load pipettor with new tips.
- 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.
- Once the TMB substrate is added to the plate, pour 15 mL of Stop Solution for TMB Substrates (Catalog #6282) into a new solution basin. This stop solution is not light-sensitive and may be left on the lab bench until needed.
- Continue to observe the TMB color development in the plate

wells. When it is apparent that the wells containing the lower Known Positive Control concentrations are beginning to take on a slightly blue-green tint, use the multichannel pipettor to add 100 μ L of stop solution to all wells. The addition of stop solution will further oxidize the HRP-oxidized TMB substrate, converting it from blue-green to yellow in color. This stabilizes the reacted product for up to 1 hour and increases the dynamic range and reproducibility of the assay.

6.2.6 Acquire plate reader results

- Set up a 96-well plate reader to quantitate absorbance at 450 nm and read the stopped ELISA plate.
- Examine the range of OD_{A450} values and select a sample and HRP-conjugate dilution factor that yields a stopped TMB OD_{A450} value between 2.5 and 3.0 OD_{A450} units. There may be multiple combinations of sample dilutions, conjugate dilutions, and plate coating concentrations which yield stopped TMB OD_{A450} values within this target 2.5-3.0 range. It is too early in the ELISA development process to select the best assay component combination at this point in time, however 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 further.
- When multiple sets of assay conditions yield stopped, high titer, Known Positive Control associated TMB OD_{A450} values falling in the 2.5-3.0 range, select the set of conditions that yields the lowest OD reading in the blank wells (rows A and B, exposed to General Serum Diluent only). Ideally the adsorbance in the blank wells should be < 0.15 OD_{A450} units. Keep in mind that at this early stage of the assay development process, there is no absolute best set of assay conditions. Simply choose a set of conditions that will allow the assay optimization phase in Section 7 to begin.

6.2.7 Perform an initial ELISA performance analysis

- Observe the wells and their corresponding OD_{A450} values. What to expect:
 - Absorbance of the blank wells should be < 0.15 OD_{A450} units with very little evidence of yellow color visible. If all of the blank wells have stopped TMB OD_{A450} values > 0.3, then one must assume that there is a plate-blocker or HRP-conjugate dilution issue.
 - Some wells would be expected to display very bright yellow colored stopped TMB substrate solutions (OD_{A450} > 3.0).
 - There should be a discernable difference between the OD_{A450} values in columns coated with 1 μ g/mL versus 4 μ g/mL Target Antigen coating solutions. That is, for a given Known Positive Control and HRP conjugate dilution pairing, the OD_{A450} values should be higher in wells coated with the higher concentration of Target Antigen coating solution.
 - There should be a distinct difference between the observed OD_{A450} values from wells containing the most dilute Known Positive Control samples (largest dilution factor) versus the most concentrated Known Positive Control samples (smallest dilution factor).

- e. Generally speaking, due to comparison of 3 different conjugate dilutions, a respective Known Positive Control dilution should yield the highest OD_{A450} 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_{A450} values.
- B. Perform a quick cursory assessment of these initial assay results. If these five conditions (listed in a-e, 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) for guidance.

7. Optimization of Antigen-Down ELISA Performance Parameters

AD ELISA optimization, beyond the initial demonstration of basic assay protocol feasibility, typically focuses on two universal 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 false positive signal in Known Negative Control 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. 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.) Antigen-Down 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 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 Target Antigen is 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. 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.

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 Controls and clearly defined positive signals with Known Positive Controls, then the optimization strategy can move on to 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, 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 controls. Both medium and high-titer ELISA controls must be diluted to a point where their stopped TMB OD_{A450} signal falls between 1.0 and 3.0 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 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 (Section 6), 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.

- A. Using the generic plate coating protocol described in Section 6.1.1, prepare **duplicate ELISA plates** bearing different Target Antigen coating concentrations across the plate surface (Figure 5). This will enable the concurrent titration of a Known Positive and Negative Control sample across plate-wells pre-coated with a range of antigen coating concentrations.
- B. 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 μ L per

Table 2. TROUBLESHOOTING GUIDE

Problem	Cause	Action/Solution
Blank has OD ₄₅₀ > 0.3, easily recognizable yellow colored Blank Wells = high background signal problems	Nonspecific binding by conjugate to unblocked or inadequately blocked regions of plate well surface, or some component within plate blocker formulation itself	Ensure blocking procedure was performed correctly. Verify that conjugate was affinity-purified and recognizes antigenic epitope sequences not present within the 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.
	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 Note: in some cases, certain animal origin sample types may display more nonspecific binding problems when compared to other animal sources	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.
	Operator or auto-washer error as relates to the proper plate washing process	Verify that auto-washer is properly aspirating out all of the plate well contents prior to addition of the next batch of ELISA wash buffer. If plates are manually washed, verify that these same plate washing parameters are being properly carried out.
No signal	Assay set up incorrectly or use of incorrect reagents	Check plate coating procedure, conjugate dilutions, and dilutions and authenticity of Known Positive Control. Was the TMB substrate incubation step performed? Repeat assay.
	Serum/plasma antibodies not recognizing plate coated antigen	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 plates.
	HRP-conjugated anti-isotype readout antibodies not recognizing antibody isotype binding to plate wells	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.
	Conjugate stored incorrectly or subjected to repeated freeze/thaw cycles	Use a fresh aliquot of conjugate that has not undergone multiple freeze-thaw events or purchase a new vial of conjugate.
	Not using a 96-well plate that was treated for use in ELISA formats	Use included ELISA plates, or obtain a brand of 96-well plates that are designated for use in ELISA formats. These plates are factory pre-treated to allow polystyrene surfaces to nonspecifically bind proteins.
Little to no difference between the TMB OD ₄₅₀ signals from plate-wells containing the greatest dilution of serum or plasma samples versus those same samples that underwent the least dilution factor	Incorrect positive control dilution protocol	Follow plate map as instructed (Figure 2).
	Questionable authenticity of known positive control serums/plasmas	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.
	HRP-conjugated anti-isotype readout antibodies not recognizing antibody isotype binding to plate-wells	Purchase a new vial of anti-isotype HRP readout conjugate and start over.
Inconsistent OD ₄₅₀ Values between adjacent known-positive control sample wells	Incorrect placement of positive control sample	Follow plate map as instructed (Figure 2).
	Inadequacies in plate washing technique	Examine vacuum portals on plate manifold to verify whether or not there is a partial obstruction in a particular sample uptake port/line.
Entire plate displays a uniform dark yellow (saturated) color	Nonspecific binding of conjugate to plate well or 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 antibodies to plate well surface.
	Nonspecific binding of conjugate to component of blocking buffer	Examine the binding specificity of the conjugate. Consider a different blocking buffer formulation, possibly synthetic (e.g., SynBlock, Catalog #643).
	Incorrect conjugate dilution	Follow conjugate dilution instructions exactly. If conjugate was supplied at >1 mg/mL, adjust dilution instructions proportionally.
	Accidental contamination of TMB substrate	Use fresh substrate and visually confirm that it is colorless prior to addition to plate.
TMB OD ₄₅₀ signal does not decrease from higher OD ₄₅₀ levels (2.8-3.3) with increased conjugate dilution	Conjugate is not diluted enough	Repeat assay with greater dilutions of the conjugate. Consider diluting out the conjugate by another 10-fold.

well. Each of the duplicate plates should contain this 2-column antigen-blank control section (Figure 5).

- C. Within columns 3-4 of each of the two (2) plates, add 100 μ L per well of the 0.25 μ g/mL Target Antigen concentration (dissolved in the 1X Antigen Coating Buffer).
- D. Coat wells in columns 5-6 on each of the two (2) plates with a 100 μ L per well volume of the 0.5 μ g/mL IgG coating concentration.
- E. Continue plate coating process by adding 100 μ L per well of the 1 μ g/mL, 2 μ g/mL, and 4 μ g/mL Target Antigen coating concentration in columns 7-8, 9-10, and 11-12, respectively.
- F. Follow the blocking instructions in Section 6.1.2. Plates may be dried after blocking and stored at 2-8°C in plate storage bags containing desiccant pouches.
- G. Prepare dilutions of Known Positive and Negative Controls.
 - a. Set up a 2-fold dilution scheme in 7 properly labeled tubes (1:1,000, 1:1,200, 1:4,000, 1:8,000, 1:16,000, 1:32,000, and 1:64,000) to be used for generating the Known Positive and Known Negative Control dilution series. Prepare 6 mL of a 1:1,000 dilution by spiking 6 μ L of the Known Positive Control into 5.994 mL of General Serum Diluent.
 - b. Add 3 mL volumes of General Serum Diluent to each of the remaining tubes within the dilution series.
 - c. Add 3 mL of the 1:1,000 sample to the tube containing 3 mL of the General Serum Diluent labeled 1:2,000. Mix thoroughly.
 - d. Repeat serial 2-fold dilution process throughout the remainder of the dilution series. Change pipette tips after every dilution.
 - e. Repeat steps a-d using the Known Negative Control.
- H. Using duplicate plates (one plate for the Known Positive Control dilutions, and one plate for the Known Negative Control dilutions), load all samples (refer to Figure 5 for plate layout).
 - a. Row A should be reserved as a sample diluent-only blank that contains General Serum Diluent only without a diluted Known Positive or Known Negative Control. Use a multichannel pipettor to add 100 μ L per well of General Serum Diluent to row A.
 - b. Add 100 μ L per well of 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).
- I. Perform the sample incubation steps and subsequent washing steps.
- J. Select the HRP-conjugate dilution factor determined to be optimal in Section 6.2.6 (ideally the dilution factor that led to a Positive Control with a stopped TMB OD_{A450} signal between 2.5-3.0, and a blank with an OD_{A450} that is < 0.15 units). Add the HRP-IgG conjugate to the ELISA plate, cover, and incubate for 60 minutes, protected from light.
- K. Carry out the rest of the assay according to the protocol in Section 6.2.5.
- L. Read the plate at 450 nm absorbance. Examine the outcome of the parallel serial dilution analysis of a Known Negative Control in General Serum Diluent. One reason for running a Known Negative Control sample at this point is to demonstrate a clear dif-

ference in signal output between a Known Positive and Known Negative sample type.

- M. For each Target Antigen coating concentration, divide the average OD_{A450} values for the Known Positive and Negative Control samples by the average OD_{A450} value for the corresponding sample-type dilution run in the no-antigen control wells coated with coating buffer only (columns 1-2 on each plate). The focus should be on the Known Positive sample dilution wells with stopped TMB OD_{A450} 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 Control dilution factor) and the quality of the signal to noise ratios (S/N).

$$S/N = \frac{\text{Average OD}_{A450} \text{ for a given Target Antigen coating concentration and sample dilution}}{\text{Average OD}_{A450} \text{ for a no antigen/blank coating concentration and same sample dilution}}$$

- N. 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.
- O. Paying particular attention to the Known Positive Controls, identify the Target Antigen coating concentration that yielded the highest S/N ratios.
- P. Obtain a consensus as to what amount of Known Negative Control sample signal constitutes a legitimate concern. Elevated Known Negative Control sample signal can be a direct result of the plate blocker/stabilizer selection. 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.
- Q. All 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.
- R. 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 label to these 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?
- S. 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.

FIGURE 6: Establishing a Dilution Factor Range for Samples

ELISA titer screening plates were prepared according to the plate preparation and subsequent assay protocol format described in Sections 6 and 7 of this Antigen-Down ELISA Development Kit manual. As a recommended starting point, Known Positive Control serum or plasma samples were diluted out according to the plate map pictured using a 5-fold serial dilution scheme (e.g., 1:500, 1:2,500, 1:12,500, etc.). In this example, a single plate will accommodate testing of up to 6 samples at 5 dilutions. It is recommended that all sample dilutions be screened in duplicate. Upon completion of the assay protocol, an end-point analysis is conducted to determine whether or not this 5-dilution factor range resulted in stopped TMB OD_{A450} signals between 0.3 and 3.0 OD units in all the Known Positive Control serum or plasma samples that were evaluated.

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:500	1:500	1:500	1:500	1:500	1:500	1:500	1:500	1:500	1:500	1:500	1:500
B	1:2,500	1:2,500	1:2,500	1:2,500	1:2,500	1:2,500	1:2,500	1:2,500	1:2,500	1:2,500	1:2,500	1:2,500
C	1:12,500	1:12,500	1:12,500	1:12,500	1:12,500	1:12,500	1:12,500	1:12,500	1:12,500	1:12,500	1:12,500	1:12,500
D	1:62,500	1:62,500	1:62,500	1:62,500	1:62,500	1:62,500	1:62,500	1:62,500	1:62,500	1:62,500	1:62,500	1:62,500
E	1:312,500	1:312,500	1:312,500	1:312,500	1:312,500	1:312,500	1:312,500	1:312,500	1:312,500	1:312,500	1:312,500	1:312,500
F	SD only	SD only	SD only	SD only	SD only	SD only	SD only	SD only	SD only	SD only	SD only	SD only
G	SD only	SD only	SD only	SD only	SD only	SD only	SD only	SD only	SD only	SD only	SD only	SD only
H	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty

7.3 Minimize Conjugate-Derived Nonspecific Binding

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 plate well surfaces. In most problematic nonspecific binding incidents, the conjugate binds in a nonspecific manner to the blocker present on the blocked 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 well surface. This kit includes a 100 mL bottle of Antigen-Down HRP Conjugate Stabilizer, 5X. 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 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 well surfaces.

Beyond the use of a suitable conjugate stabilizer, two additional strategies can also be used to help reduce the nonspecific binding interaction of the conjugate with the immobilized components on the well surface. In general terms, nonspecific binding activity is modulated by two major parameters: 1.) concentration of the conjugate, and 2.) exposure time of the conjugate to the well surface. An increase in either parameter will always lead to increased nonspecific binding of the conjugate. Fortunately, these two key 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. Although increasing the concentration of the HRP-conjugate will drive the specific binding kinetics toward shorter equilibration times, the level of nonspecific binding to 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 disproportionately 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 Control as well as a Known Negative Control sample on the same plate. Selection of a Known Low-Positive Control for this analysis is a deliberate attempt to avoid selecting a conjugate dilution factor providing low nonspecific binding signal in the negative serum or plasma wells but failing to detect the known low positive samples present in their respective wells. Upon completion of the required wash steps, the plate is exposed to multiple dilution factors (e.g., 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 within the negative control samples as well as an easily discernable positive signal within 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 nonspecific binding to a minimum.

7.3.2 Conjugate Exposure Time

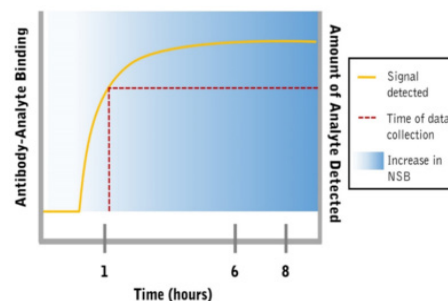
Nonspecific binding can also be modulated by the careful control of the conjugate exposure time within the assay well. Utilization of high-quality HRP-IgG conjugates with high binding affinity constants to the Fc portion of the antigen-specific serum/plasma antibody are essential for any quality AD ELISA. Conjugates that are composed of higher affinity antibodies require less incubation time to reach equilibrium. Realistically, most ELISA protocols do not seek to achieve complete antibody/antigen equilibration. 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 it to bind to the plate-wells in a nonspecific manner (refer to Figure 7 for illustration of this concept).

8. Quality Assessment of the 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

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

This illustration of the two major signal generating processes: 1.) antibody-driven specific binding, and 2.) hydrophobic interaction-driven nonspecific binding, was created to show the multi-dimensional signal generation processes occurring within all ELISA formats. Specific signal levels off as the antibody-antigen binding process approaches equilibrium. Unfortunately, signal resulting from nonspecific binding of the HRP-conjugate (background signal) continues to accumulate. As a result, specific signal to background signal (i.e. signal-to-noise) ratios drop off as the amount of nonspecific signal increases over time.



an 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 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

Antigen coating irregularities can arise from a variety of environmental factors. A 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(s) on the plate coating antigen, 3.) inconsistencies in the well-to-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).

If the project is limited to a one or two sample time-point assessment (e.g., a one-time, 6-12 total ELISA plate production batch size), the risk of serious plate-to-plate coating irregularities would be minimal compared to what could occur with a large production batch-size (e.g., > 100 plates). When this additional potential for plate coating variability 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.

8.2 Plate Coating Precision Study Setup

A. Remove a predetermined sampling percentage of the coated/blocked/package 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 AD ELISA plate inventory. However, if there is some prior evidence of a potential for antigen coating inconsistencies, a larger percentage may be necessary. 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 buf-

fers 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 the 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 Control pools. Dilute the selected Known Positive Control into General Serum Diluent using a dilution factor previously determined to give a stopped TMB OD_{A450} value around 0.6-0.8 OD_{A450} units, which should be dilute enough to place the Known Positive Control within the lower portion of the assay’s linear range. 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.

If the plate coating precision study was designed to evaluate five (5) AD ELISA plates, the recommended volume of appropriately diluted Known Positive 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 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).
- D. Obtain the raw OD_{A450} readings using the software available on the colorimetric ELISA plate reader.

8.3 Plate Coating Precision Study Analysis

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

- A. All five AD ELISA precision testing plates should have similar stopped, raw, TMB OD_{A450} values for each well. Any deviations from the calculated mean (\bar{x}) of the total (5 x 96 well = 480) raw OD_{A450} values must originate from improper end-user assay performance technique or plate coating irregularities.
- B. Plate precision screening analysis will typically reveal the more macroscopic plate-to-plate variability (inter-plate variability) as well as the subtler within-plate (intra-plate variability) elements. Coefficient of Variation (CV) is probably the most commonly used statistical term when addressing ELISA plate precision topics. It is typically expressed in the form of a percent CV (% CV) of a particular set of ELISA generated raw well OD values. % CV is defined by the formula $[(SD / \bar{x}) \times 100]$ where \bar{x} is the mean of a selected set of raw well derived OD values and SD is the Standard Deviation of that particular set of plate derived OD values.
- C. To perform a more generalized plate-to-plate variability analysis, copy and paste raw plate reader OD_{A450} values into an Excel sheet and then setup the Excel formula macros to calculate the mean (\bar{x}) of all 96 OD_{A450} output values as well as the standard deviation (SD) for these 96 OD_{A450} readings. Calculate the % CV for that particular plate. Repeat this process for the four remaining plates being screened.

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

In situations where the serological screening study has more modest aspirations, a higher degree of plate coating variability may be acceptable. For example, a small screening study to verify whether or not a new, two-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 injection, hyper-immunization 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 likely still be effective in assessing whether or not the immunization protocol was successful.

Alternatively, there may be scenarios that require a lower degree of variability. An example of one such 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 antigens are evenly and equally incorporated into their designated ELISA plates or pre-assigned regions within a particular ELISA plate grouping. Here, the upper limit for an acceptable % CV within plate-well to plate-well (intra-plate) precision likely should be << 10%. On a plate-to-plate (inter-plate) basis, a < 15% CV precision score would likely be acceptable.

As each project is unique, the end user is ultimately responsible for determining what is considered to be an acceptable % CV precision score. Plate batches found to have a % CV score greater than the acceptable 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 before any attempts to create another batch of plates be undertaken.

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

9.1 Comparison over Time and Multiple Bleed Dates

Interpretation of data derived from multiple date test bleed results over a multi-month time frame presents a formidable data interpretation challenge. One option for addressing this is to utilize a Sample OD_{A450} to Known Positive OD_{A450} (S/P) ELISA OD Ratio Method. Utilization of this technique requires an adequate supply (25-30 mL pool volume) of a high or medium ELISA-titer Known Positive Control, which should be aliquoted into 0.5 mL volumes within high quality type commercial cryovials (1 mL volume size) and stored at < -70°C. Implementation of an S/P ratio comparison method is an easy and efficient way to assign a comparative numerical value to the raw OD_{A450} values generated within the AD ELISA format. Refer to Figure 8 for an example of how to set up such a plate. To employ this technique, 2 fixed dilutions of Known Positive Control serum/plasma sample must be permanently included within the assay protocol. Ideally, both a medium and high titer Known Positive Control would be sampled on each 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.

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 titer controls are 1:10,000 and 1:30,000, respectively, and the predetermined unknown sample

	Sample 1		Sample 2		Sample 3		Sample 4		Sample 5		Sample 6	
	1	2	3	4	5	6	7	8	9	10	11	12
A	1:500		1:500		1:500		1:500		1:500		1:500	
B	1:5,000		1:5,000		1:5,000		1:5,000		1:5,000		1:5,000	
C	1:25,000		1:25,000		1:25,000		1:25,000		1:25,000		1:25,000	
D	←								Empty Wells			→
E	Medium Titer Positive Control				←				Empty Wells			
F	High Titer Positive Control				←				Empty Wells			
G	Negative Control				←				Empty Wells			
H	Sample Diluent Only				←				Empty Wells			

ELISA production plates, TMB substrate, etc. When switching over to a new assay component lot, unexpected performance differences can typically be minimized by performing a side-by-side comparison of the new lot to that of the old lot. 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 relationship existing between, for example, the progress of a hyper-immunization protocol and the generation of a serological titer of antigen-specific Ig isotype.

9.5 Sources of OD_{A450} Variability not Resolved by S/P Ratio

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 control on the ELISA plate, the S/P OD_{A450} ratio conversion strategy will not be helpful in attenuating variability.

9.6 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 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 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. The stopped TMB signal was mistakenly read at a suboptimal absorbance wavelength.

Even in the presence of suboptimal assay operation conditions, these factors would likely have an equal effect on the medium or high positive control. Thus, the actual change in overall S/P OD_{A450} 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 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 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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