Optimizing the Immobilization of Protein and Other Biomolecules for ELISA Assays

Application Note

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Introduction

The first and probably the most important step in an Enzyme Linked Immunosorbent Assay (ELISA) is the initial immobilization of the biomolecule to the solid surface. In the Corning Application Note (CLS-DD-AN-454) *Immobilization Principles — Selecting the Surface for ELISA Assays*, we discussed the interactions that take place between an immobilized biomolecule and the surface. These interactions can be affected by factors such as buffer composition and pH, biomolecule concentration and purity, and incubation time and temperature. Other assay factors can affect the precision of binding, many of which are of particular concern when using 96-well microplate formats. In designing a Certification Test for our 96-well microplate and 8-well strip products, we encountered many steps in our assay that, unless optimized and controlled, caused incremental (and sometimes additive) precision problems. In this bulletin, we focus on the immobilization step of an ELISA and discuss methods to (i) improve biomolecule-surface interactions and (ii) improve well-to-well precision.

Assay Environment

Particulate Contamination

The environment surrounding an assay microplate can play an important part in the assay itself. We, as manufacturers, are acutely aware of the microenvironment associated with the bioassay products that we produce, which is why all of these products are produced in a clean room environment to minimize airborne contamination. Polystyrene is an electrostatic material that will attract airborne particles like a magnet. To reduce surface contamination, it is recommended that plates and strips be kept in their original packaging until used. Surfaces that have particulate contamination will exhibit variations in biomolecule immobilization. Molecules can attach to the particulates as well as to the surface, and can be washed away in subsequent steps, leaving a void area on the surface itself which compromises assay precision. Particulate contamination can also interfere with some enzyme/substrate reactions by introducing enhancement or inhibition agents.

Oil Contamination

Oils are also surface contaminants that can affect precision by reducing the surface area available for biomolecule immobilization. Oils readily form films on plastic surfaces. Biomolecules, such as proteins, can attach hydrophobically to these oil films. When washed with detergent containing solutions, these oil films — and their attached proteins — are removed from the surface, leaving a void area behind. Oils are usually present in the atmosphere in unnoticeable mists that are produced by laboratory vacuum pumps and/or compressed air systems. Laboratories that share a ventilation system with another lab employing equipment such as vacuum pumps may also have oil mist in the air even though these devices are prohibited from being used in the assay area. The best method to combat oil contamination is prevention.

Oxidation and UV Degradation

Many of the newer, more specialized ELISA surfaces available today require additional handling precautions. Pre-activated covalent surfaces, such as the N-oxysuccinimide and Hydrazide surfaces, are susceptible to oxidation when exposed to the air. These products are packaged in low vapor transmission foil pouches for stability and should be used within 30 minutes after removal from the package. The Universal Covalent surface, which is used to covalently immobilize biomolecules through abstractable hydrogen via UV activation of the functional group on the surface, must be protected from UV light prior to the activation process. These plates and strips are packaged in metal foil pouches that eliminate UV exposure prior to use. Upon opening, the plates or strips should be used within 30 minutes to reduce direct or prolonged exposure to UV light.

Regardless of the surface type, protecting assay plates and strips from the environment is beneficial. It reduces the chance of surface contamination from airborne particles and oil and maintains covalent functionality of preactivated surfaces by reducing the likelihood of premature oxidation and/or UV activation.

Precise immobilization can only occur on a surface that is clean and functionally active.

Pipetting Optimization

One of the most common sources of assay imprecision is pipetting error. It is important that manual pipetting techniques be optimized prior to the development of an assay. Practice makes perfect. The following is a simple test for pipetting precision that can be used for both manual and automated pipetting procedures.

1. Prepare the following dye: To 1,000 mL of reagent grade water, add 12.1 g Trizma® base (MilliporeSigma Cat. No. T6791) and 20 mg phenol red. Mix well.
2. Using the pipets or pipetting apparatus set at the volumes that are normally used for assays, pipet the phenol red solution into each well of a 96-well microplate or 8-well strip plate. Immediately read the optical density in each well at 540 nm to reduce the effect of evaporation.
3. Calculate the mean optical density, standard deviation, and coefficient of variation. The goal is a CV of 2% or less.
4. Use these results to develop both technique and timing, which will lead to enhanced precision.

Additional helpful hints to improve manual pipetting proficiency include:

1. Use a multi-channel pipettor whenever possible to reduce the number of pipetting steps (less steps lead to reduced chance for error).
2. Be sure that the pipet tip is properly positioned and sealed to each channel of a multi-channel pipettor. A loose tip will lead to inaccurate volume pick up and delivery (Table 1).
3. Avoid using the blowout step when manually pipetting. This function can introduce a bubble into the fluid in the well, which will effectively reduce the surface area available for biomolecule immobilization to the surface. By eliminating the blowout step, a volume setting of 100 µL will deliver a consistent 98 µL of volume each time. (This can be verified using a calibrated pipet set at 100 µL.) If using the blowout step is unavoidable, be sure that bubbles adhering to the assay surface are burst (puncture with a sharp needle) to allow complete surface availability for the biomolecule being immobilized. Be careful to avoid well-to-well cross-contamination when eliminating bubbles in this manner.
4. Always pipet below the fluid line or against the sidewall of the well to remove the last drop that adheres to the pipet tip. The volume in this last drop can vary from tip to tip and with each pipetting maneuver.
5. Always pre-wet pipet tips with the solution being pipetted. Pipet tips are typically hydrophobic and will immobilize biomolecules. By pre-wetting, the inner and outer surfaces of the pipet tips become saturated and little to no biomolecule loss is detected with subsequent pipetting steps. If pre-wetting is not employed, the first well(s) may receive a less concentrated solution than the following wells.
6. Before beginning any pipetting routine, warm all reagents to room temperature. Temperature affects pipetting precision. The physical act of pipetting (friction) cold solutions tends to warm the solution around the microenvironment of the pipet tip that can cause fluctuations in the volume aspirated and dispensed. By starting at room temperature, these temperature fluctuations are minimized. We do not recommend warming solutions above room temperature for assays that are eventually incubated at elevated temperatures. Room temperature is sufficient and the easiest to maintain during the pipetting process.
7. Finally, use high quality pipet tips that have a uniform bore size and exhibit reliable sealing to the pipettor.

As with manual pipetting, automated pipetting needs optimization. Equipment should receive routine maintenance, such as cleaning after each assay, and performance checks, such as the exercise using the dye solution described above. Remember: assay precision can only be as good as one's pipetting precision.

Biomolecule Concentration and Purity

Concentration

All assay components need to be titered during the assay development process to ensure reliability. The most precise method for optimizing biomolecule concentrations is the Checkerboard Titration Method (Figure 1). This method, originally designed to determine antigen/ antibody concentration for ELISA tests, can be used to determine optimal coating concentrations for any type of immobilized biomolecule. This method also allows one to select an optimal concentration for any or all reactants in the assay. It is critical that the immobilized or capture molecule be coated to the

Wells >10% of the Average	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
	0	0	0	0	0	0	0	0	0	0	0	0
Wells <10% of the Average	0	0.973	0	0	0	0	0	0	0	0	0	0
	0	0.909	0	0	0	0	0	0	0	0	0	0
	0	0.816	0	0	0	0	0	0	0	0	0	0
	0	0.798	0	0	0	0	0	0	0	0	0	0
	0	0.771	0	0	0	0	0	0	0	0	0	0
	0	0.769	0	0	0	0	0	0	0	0	0	0
	0	0.747	0	0	0	0	0	0	0	0	0	0
	0	0.728	0	0	0	0	0	0	0	0	0	0

Table 1. Example of Pipetting Error. Loose pipet tip on the second channel of a 12-channel pipettor resulting in lower than average ODs. Zeros (0) represent ODs lying within 10% from the mean of all 96 wells.

surface as a monolayer to ensure the greatest precision. A monolayer coverage reduces void areas on the surface (concentration too low) or unstable multi-layer formation from protein-protein interactions (concentration too high).

Purity

Many biomolecules denature upon adsorption to a surface and require the addition of stabilizing reagents. Many monoclonal antibodies, enzymes, and antigens fall into this category. To alleviate this problem, the addition of an inert protein to the stock reagent is typical. Many "purified" antibodies are pure only in the sense of their affinity for an antigen; they normally contain an inert protein, such as BSA, to aid in stability. Our experience is that a virtually pure antibody will denature when immobilized and result in precision problems. We suggest the use of antibody preparations that contain a standardized amount of inert protein (between 8 to 15 mg/mL BSA or equivalent). Note: Due to the increased stability of biomolecules bound to covalent surfaces, "pure" reagents (without a stabilizer) typically can be used.

Another purity related issue lies with the type of buffer used with enzyme preparations. For example, the use of peroxidase precludes the inclusion of sodium azide as a preservative for assay buffers (including the wash buffer solution). Sodium azide is a strong peroxidase inhibitor. Likewise, the use of phosphate buffers for alkaline phosphatase enzyme systems should be avoided. Inorganic phosphates are competitive inhibitors for alkaline phosphatase (they reduce enzyme conversion of the substrate into product by competing for the active site of the enzyme). We recommend either Tris- or carbonate-based buffers for alkaline phosphatase.

Removal of Protein Aggregates

Proteins stored as aqueous solutions will form aggregates. If not removed, these aggregates will bind to surfaces in a manner different from single protein molecules and adversely affect precision. There are two methods that we recommend to remove protein aggregates: (i) centrifuging the stock solution prior to making test dilutions or (ii) filtering the test dilution through a 0.45 µm cellulose acetate, polyethersulfone, or other low protein binding membrane.

Proper Mixing of Dilute Samples

Typical biomolecule concentrations destined for immobilization to a hard plastic surface are in the µg/mL range. These concentrations are usually made by diluting a more concentrated stock solution by at least 1,000-fold. It is important that these test dilutions be properly mixed to ensure homogeneity of the solution. Slight variations in concentration throughout the solution can cause large errors in precision. Although thorough mixing is essential, it is also important not to over mix. If foaming occurs, there may be irreparable damage (denaturation) to the biomolecules. Swirling or gentle vortexing is recommended.

Storage Conditions

Proteins and other biomolecules require low temperature storage, typically 4°C or lower. Proteins stored frozen are usually buffered by the addition of stabilizing compounds; inert proteins, sugars, or glycerol. Regardless of the storage medium, it is important to protect proteins from repeated freeze-thaw cycles that have a denaturing effect. Stock solutions can be aliquoted such that one aliquot is used per assay, thus eliminating the need to thaw and refreeze the entire stock. Even when using this precautionary approach, proteins stored in a frost-free freezer are susceptible to freeze-thaw damage caused by the temperature cycling required to maintain the frost-free condition of the freezer. If a non-frost-free freezer is not available to store bioreagents, we recommend that they be refrigerated, not frozen. The shelf life of an enzyme stored in a frost-free freezer can be literally reduced to weeks or days.

Effect of pH on Immobilization

Buffer composition in general can affect biomolecule immobilization to surfaces. For example, the use of phosphate buffered saline (PBS) as the buffer is recommended for procedures requiring the dry storage of pre-coated polystyrene surfaces. The phosphate in PBS has the ability to structure water around the molecules, making them less susceptible to denaturing effects.

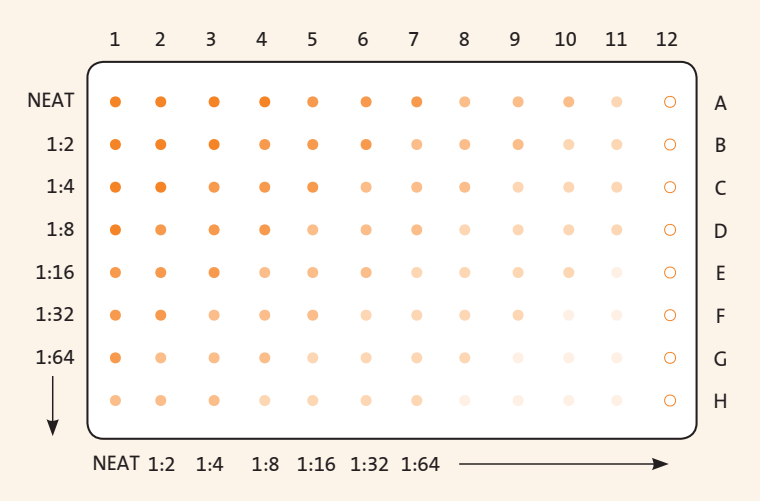

Figure 1. Checkerboard Titration Method. (A) Antigen or antibody is serially diluted across the plate (starting with a NEAT sample concentration of approximately 10 µg/mL), allowed to immobilize to the surface, and excess is washed away. (B) The surface is then blocked appropriately. (C) Primary antibody or antigen is serially diluted down the plate, allowed to react with the immobilized molecule, and excess is washed away. (D) The third reagent, usually the labeled detection agent, is used at a constant (and excess) concentration of approximately 10 µg/mL (such that it is not the limiting factor in the assay), allowed to react and excess is washed away. (E) Proceed with the remainder of the assay. (F) Following signal detection (colorimetric, fluorometric, etc.) in a plate reader, select the optimal reading range. This is usually chosen by observing the range of readings across the plate. Readings will increase from the bottom right corner to the upper left corner of the plate; the optimal reading lies just prior to the set of wells exhibiting the highest readings). (G) From the well corresponding to the optimal reading, derive the optimal concentrations of both titered reagents.

The literature on lyophilization of biomolecules can give additional methods of enhancing stability.

Passive immobilization is greatly affected by pH. Due to the hydrophobic character of most passive surfaces, biomolecules tend to bind best at a pH that causes the exposure of hydrophobic regions resulting from partial denaturation. (Hydrophobic regions of most proteins tend to be hidden within their tertiary structure, protected from their aqueous environment.). IgG, for example, tends to bind best when buffer pH is close to, but not equal to, its pI of 7 to 8. Precision is best at a slightly basic pH (9.6) due to exposed hydrophobic regions (Table 2).

The optimal pH for other proteins must be determined through experimentation. Suggestion: dilute the biomolecule in buffer (i.e., PBS) at three pH levels (5.2, 7.4, 9.6), allow 1 hour for immobilization to the surface to occur, compare immobilization efficiency (consider signal strength and precision [CV]).

Ionic immobilization is not only affected, but actually governed by pH. Ionic coupling of small, negatively charged biomolecules to an aminated surface can be accomplished by increasing the pH to 8 or above and maintaining an ionic strength of 0.01M or less. As with hydrophobic immobilization, optimal pH for immobilization should be determined experimentally. The optimal pH needs to be maintained throughout the entire assay to avoid ion exchange potential. (There is a risk of desorption if radical changes to the pH and ionic strength of buffers are encountered.)

The pH associated with covalent immobilization is governed by the reaction. Buffers in general are crucial to the reaction between the functional group on the surface and the functional group on the molecule. The N-oxysuccinimide (NOS) surface which couples to amine groups via the formation of a peptide bond is reactive at a basic pH of 8 to 9 in a buffer that is free of extraneous amines (Tris buffered saline is not suitable). However, at this pH, it is important to consider that the NOS group on the surface is readily hydrolyzed and should be coupled to an amine group as soon as possible. The Maleimide surface that couples to molecules via accessible sulfhydryl groups through a thiol linkage is most reactive at a slightly acidic pH of 6 to 6.5. An important addition to the buffer is EDTA, a chelator that (i) oxidizes metal contaminants that can interfere in the covalent reaction and (ii) prevents the formation of unwanted disulfide bonds between adjacent SH groups on the biomolecules. Hydrazide surfaces couple to periodate activated carbohydrate moieties at an acidic pH of 5 to 5.5 in an acetate buffer. This is convenient since the periodate activation itself requires these same buffer conditions. Since the Universal Covalent surface depends on passive adsorption as the initial attractant of the biomolecule to the surface, pH requirements match those discussed for a hydrophobic surface. Experiments to determine the proper pH must be conducted for each biomolecule to be immobilized.

Incubation Time and Temperature

Incubation time and temperature have a profound influence on protein immobilization to surfaces. Typically, as the incubation temperature is increased, the incubation time can be decreased and vice versa.

Table 2. Effect of pH on Passive Immobilization of IgG

pH of PBS	Average OD	CV (%)	Low well (%)	High well (%)
5.2	1.07	2.6	7.3	5.5
7.4	1.15	2.3	5.9	5.9
9.6	1.15	1.7	5.4	3.9

Table 3. Immobilization Time and Temperature

Temperature	Time (hr.)	CV (%)	Low well (%)	High well (%)
4˚C	16	1.4	3.6	4.1
Room Temperature	1	2.3	5.5	6.1
37˚C	2	2.2	6.4	4.6

The best incubation conditions for the initial surface coating step is 4°C overnight (16 hours) (Table 3). This environment is stable (constant temperature, low air-borne contamination levels, reduced air currents) and the incubation time allows for equilibrium between bound vs. free biomolecules to occur.

Caution: Even at 4°C, evaporation can be a problem. In most situations, evaporation primarily occurs in edge wells (entire perimeter or isolated edges). Evaporation affects precise immobilization by:

- Altering the surface area available to bind the biomolecule
- Increasing the effective concentration of the biomolecule in wells with the highest amount of evaporation
- Increasing the likelihood that proteins immobilized to areas that become “dried-out” will denature

To avoid evaporation, the use of plate covers or plate sealers for at least the top plate in a stack is recommended

For convenience and speed, assays tend to be performed at room temperature for short, typically one hour, incubations. This time and temperature allows adequate surface coating to occur in a precise manner in a reasonable time frame. When properly performed, an assay incubated at elevated temperatures, typically 37°C, can decrease the required incubation time and maintain precision. The data shown in Table 3 indicate that immobilization of the protein required a 2-hour incubation at 37°C, one hour longer than the room temperature incubation. Why? This 37°C incubation was performed in a forced hot air incubator. Due to polystyrene’s poor heat conductance, it takes approximately 30 minutes for a 96-well microplate to reach temperature equilibrium in this type of incubator. For precise well-to-well binding to occur, the incubation time needed to be increased to 2 hours (at 1 hour, an edge effect was evident).

To perform precise immunoassays at elevated temperatures, the method of heat transference to the plate must be controlled. Even heat distribution to all 96 wells simultaneously can be accomplished by using a heat block (we recommend aluminum for even conductivity of heat) that radiates heat through the bottom of the plate rather than across the plate. The heat block should be slightly smaller than the footprint of the plate so that the plate sits snugly around the block. There should be an air gap between the wells and the heat block to ensure even heat

distribution via radiation. The plates and blocks should be placed on a device such as a slide warmer. Placing plates on blocks inside a forced air incubator does not serve to decrease temperature gradients across the plate. As shown in Table 4 (which compares protein immobilization to plates incubated in an incubator or oven versus one on heat blocks), the edge effect caused by temperature gradients can be eliminated using a heat block device.

Table 4. Effect of Temperature Gradients at 37°C on Assay Results

Device	Average OD	CV (%)	Low well (%)	High well (%)
Incubator	1.20	5.5	7.1	28.0
Heat Block	1.30	3.0	6.3	7.3

Conclusion

Biomolecule immobilization to hard plastic surfaces, such as polystyrene 96-well microplates, is primarily governed by the chemistry of the surface. However, many factors can alter the surface to biomolecule interaction that can result in (i) complete inhibition of binding or (ii) well-to-well precision problems. To ensure precise binding, the assay environment must be controlled. This environment includes items such as the atmosphere surrounding the assay vessel (contaminants, temperature, etc.) and the aqueous solution containing the biomolecule of interest (ionic strength, pH, biomolecule concentration, reaction inhibitors). To expect precise assay results, one must control as many precision-affecting factors as possible. This application note has only touched the surface in terms of describing methods to increase the precision of biomolecule immobilization to surfaces; however, adhering to the techniques that we have discussed can only lead to improvement of assay precision.

There are bioassay steps subsequent to the immobilization of a biomolecule to a surface than can affect assay precision and accuracy. Many of these steps, such as effective blocking procedures (Application Note CLS-DD-AN-456) and optimizing the separation methods (Application Note CLS-DD-AN-457), are detailed in other editions of this application note series.

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