Immobilization Principles – Selecting the Surface

ELISA Technical Bulletin - No. 1

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Life Sciences

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Table of Contents:

Introduction 1

Interfaces 2

Structure of Polystyrene 2

Immobilization Mechanisms 2

Chemical Properties (Surface Flavors) 3

Physical Properties 5

Conclusion 7

Technical Assistance 8

References 8

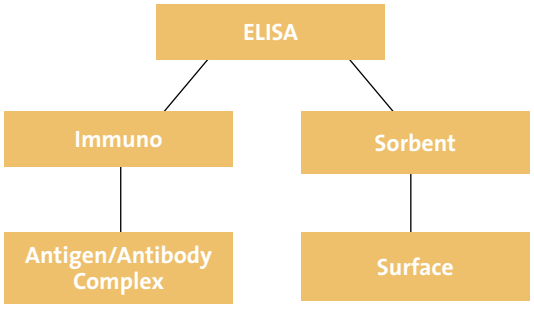

Figure 1.

Introduction

An Enzyme Linked Immunosorbent Assay (ELISA) has two major components (see Figure 1). The first is the immunological reaction that occurs between an antigen and antibody. This reaction is crucial and must be optimized in order for the assay to be reliable. The second component is the surface to which antigens and antibodies are immobilized. The surface is often taken for granted; however, its function is as crucial as the antigen-antibody complex with which it interacts. We consider the surface an integral component of any assay due to its effect on biomolecules as they attach or do not attach to the matrix. Biomole-

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cules attach to surfaces via a variety of mechanisms. This attachment phenomenon is controlled by the chemical properties of the surface, but can be influenced by (i) the physical properties of the containment vessel (96 well plate) and (ii) other factors such as pH and temperature. In this, the first in a series of five ELISA technical bulletins, we will be primarily concerned with the surface and its interactions with biomolecules.

Interfaces

Proteins and other biomolecules denature at interfaces. During the course of an immunoassay, several interfaces are formed that affect the structure and function of the biological components. The initial interfaces to exist during a typical assay are formed between (i) the solid surface and the aqueous biological solution (SOLID-LIQUID) and (ii) the aqueous biological solution and air (LIQUID-GAS). Biomolecules located at either interface can and do denature. As the biomolecules attach to the solid surface, their conformation is altered depending on the chemical properties of the surface. In many instances, the molecules unfold to expose hydrophobic regions that interact with the surface in what is considered passive adsorption.

During the course of an ELISA, after protein or biomolecule immobilization to the surface is realized, non-bound reagents are removed from the surface, leaving a liquid film behind. The same SOLID-LIQUID-GAS interfaces exist; however, the denaturing effect on the immobilized biomolecules is intensified by the removal of the liquid phase.

Many assays destined for storage for future use require that the immobilized biomolecules be dried to the surface. This drying step introduces a new set of interfaces — SOLID-DRY FILM-GAS — and increases the denaturing effect on biomolecules at the surface. At this point, biomolecules that were immobilized and active in a liquid environment may be so severely denatured that activity, immunological, enzymatic, etc., may be totally lost due to conformational (sometimes irreversible) changes.

Severe denaturing can be alleviated by adhering to principles used with lyophilization procedures. Proteins attached to surfaces need to be protected and stabilized during any drying process. This protection can be accomplished by the addition of stabilizing reagents such as inert proteins, sucrose, glycerol and other compounds that structure water around the bound biomolecule. The most practical stabilizing reagent for proteins bound to surfaces and destined for further immunological reactions is an inert protein — usually the same inert protein used as the blocking reagent.

For assays that require dry storage, the use of PBS (phosphate buffered saline) as the biomolecule diluent is recommended due to its ability to structure water around the surface-bound molecules. Other common diluents, such as carbonate buffers, do not have the same stabilizing effect as PBS. A more detailed discussion of recommended storage conditions that help reduce the negative effects of drying is presented in *ELISA Technical Bulletin #2*. In terms of surface selection, it is important to note that surfaces and their associated mechanisms of immobilization can and do play a role in the severity of denaturation problems that occur at SOLID-LIQUID interfaces.

Structure of Polystyrene

Polystyrene is a long carbon chain with pendant benzene rings on every other carbon (see Figure 2). Its structure makes it a very hydrophobic compound. This hydrophobicity is retained when polystyrene is molded into devices, such as 96 well plates. A non-treated polystyrene surface is by nature hydrophobic; however, polystyrene can be easily modified by radiation and other techniques that alter the chemistry of the surface. Essentially, the benzene ring, present at the surface, lends itself to chemical modification. Carboxyl and amine groups can readily be grafted to the surface. The polystyrene can also be modified through chemical reactions to allow the covalent attachment of a variety of reactive groups that can be used for the subsequent covalent immobilization of biomolecules.

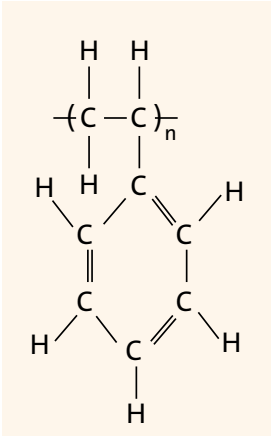

Figure 2. The Structure of Polystyrene

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Immobilization Mechanisms

Proteins and other biomolecules attach to surfaces by several different mechanisms. (Please refer to the Corning web-based *Surface Selection Guide* for additional information.) Passive adsorption consists of primarily hydrophobic interactions or hydrophobic/ionic interactions between the biomolecules and the surface. Typical nomenclature for passive binding surfaces includes Medium Binding for hydrophobic surfaces and High Binding for surfaces that are modified to have a small number of ionic carboxyl groups resulting in a slightly ionic, hydrophobic surface. Covalent immobilization to polystyrene can be accomplished through several means. On surfaces that are aminated or carboxylated, covalent coupling is achieved using bifunctional crosslinkers that couple the amine or carboxyl group on the surface to a functional group, such as an amine or sulfhydryl, on the biomolecule. Selection of the crosslinker determines the type of covalent bond that will be formed. Functional and covalently reactive groups, such as N-oxysuccinimide, maleimide and hydrazide groups, can also be grafted onto a polystyrene surface. These reactive groups are coupled to the polystyrene via a photolinkable spacer arm resulting in a stable, yet reactive surface.

Surfaces that are hydrophilic and neutrally charged are considered low binding. Since proteins and other biomolecules passively adsorb to surfaces through hydrophobic and ionic interactions, a surface lacking these characteristics naturally inhibits nonspecific immobilization via these forces.

Chemical Properties (Surface Flavors)

Medium Binding Polystyrene

As mentioned earlier, non-modified polystyrene surfaces are hydrophobic in nature and can only bind biomolecules through passive interactions. This type of surface is referred to as Medium Binding and is primarily suitable for the immobilization of large molecules, such as antibodies, that have large hydrophobic regions that can interact with the surface. Due to the large surface area needed to immobilize biomolecules in this manner, binding capacities are typically low (100 to 200 ng IgG/cm^2). Due to the single mechanism of attachment, Medium Binding surfaces are considered easy to block using either inert proteins or non-ionic detergents. Many assays employing the immobilization of non-purified antibodies or antigens (>20 kD) use this surface due to its ability to bind only the large, hydrophobic components in the mixture. This reduces the potential of cross-reactivity problems with smaller contaminants in the mixture. One drawback of hydrophobic immobilization is the denaturing effect it has on biomolecules as they unfold to expose hydrophobic regions that can interact with the surface.

High Binding Polystyrene

Polystyrene is easily modified to be what is considered High Binding via the use of radiation. The radiation effectively incorporates carboxylic acid on the accessible carbons of the "broken" benzene ring. The resulting surface is primarily hydrophobic with intermittent carboxyl groups capable of ionic interactions with positively charged groups on biomolecules. The mechanism of immobilization is passive adsorption through hydrophobic and ionic interactions. This is considered a general purpose surface capable of binding medium (>10 kD) and large biomolecules that possess ionic groups and/or hydrophobic regions. Binding capacity is increased as compared to the Medium Binding surface to approximately 400 to 500 ng IgG/cm^2 because ionic interactions require that a smaller portion of the molecule be in contact with the surface to obtain stable immobilization. The negative effects of denaturation are still evident on this surface. There is also a concern that the antigenic (or active) site is part of, or adjacent to, the positively charged region of the molecule that interacts with the surface creating a possibility of a steric interference. This surface cannot be effectively blocked using a non-ionic detergent alone; the addition of a protein blocking step is required.

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Aminated Polystyrene

Polystyrene can also be modified to possess positively charged amine groups that replace the benzene ring at the surface. This type of surface lacks hydrophobic character and is strictly ionic in nature. Using the appropriate buffers and pH, this surface can be used to ionically couple to small negatively charged biomolecules. The lack of hydrophobic areas on the surface precludes the immobilization of large, relatively hydrophobic molecules. This surface was specifically designed to be used with bifunctional crosslinkers (i.e., glutaraldehyde, carbodiimide) to covalently couple to functional groups (primary amines, thiols, carboxyls) on biomolecules. Due to its hydrophilic nature and capability of covalent immobilization through the use of additional crosslinkers, this surface can be used to immobilize molecules solubilized in detergents, such as Triton X-100 and Tween 20 (see Table 1 and Figure 3). A negative aspect of the amine surface is the stringency needed to effectively block the surface. Due to its hydrophilic/covalent surface characteristics, blocking reagent used with this surface must be able to interact with non-reacted amine groups, as well as with any functional groups (succinimide, aldehyde, maleimide) exhibited by the chosen crosslinker.

Tissue Culture Treated Polystyrene

Polystyrene surfaces that are primarily hydrophilic and negatively charged (carboxyl groups) are designed for cell attachment. Under typical assay conditions, this type of surface will act as an ion exchange matrix, allowing molecules with greater ionic potential (surface affinity) to replace previously immobilized molecules with lesser affinity for the surface. Non-specific background tends to be difficult to control. We advise that surfaces specified as Tissue Culture Treated not be used for ELISA or similar procedures.

Preactivated Covalent Surfaces

Polystyrene can be modified via a variety of methods to possess functional groups

Table 1. Covalent vs. Adsorbed

	Adsorbed/ Medium	Adsorbed/ High	Covalent/ Amine
OD No Triton X-100	1.403	1.400	1.693
OD with Triton X-100	0.000	0.074	1.378
% Bound	0.0%	5.0%	81.0%

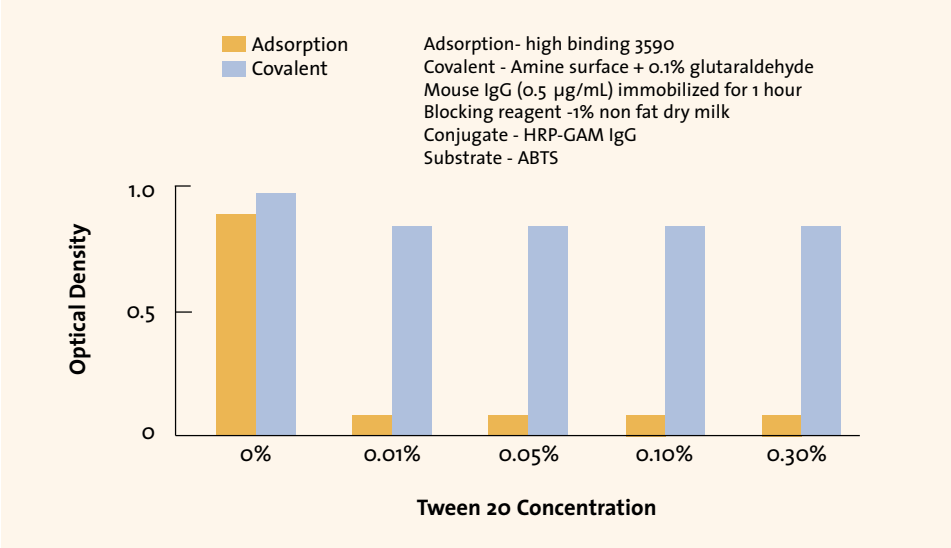

Figure 3. Effect of Detergent Concentration On Immobilization of IgG

designed to covalently immobilize biomolecules. Using a proprietary process, Corning has developed four such surfaces. All four surface "flavors" consist of functional groups that are covalently attached to the polystyrene via a photolinkable spacer arm. The four surfaces that are currently available are:

- N-oxysuccinimide (DNA-BIND™) — to couple to amine groups
- Maleimide (Sulfhydryl-BIND™) — to couple to free sulfhydryl groups
- Hydrazide (Carbo-BIND™) — to couple to periodate activated carbohydrate moieties
- Universal-BIND™ — to non-specifically couple through abstractable hydrogen via UV activation

Since the first three of these surfaces are highly specific for their coupling partner (amine, sulfhydryl, or activated carbohydrate), they are used to immobilize biomolecules in a site-directed manner. The Universal-BIND surface is specific for abstractable hydrogen, but this is not in a site-directed manner. The size of the biomolecule is irrelevant; single amino acids can be immobilized to the correct surface "flavor." Since the immobilized biomolecules are attached via a single point, denaturation effects are minimized, but not eliminated. The effects caused by excessive drying and the lack of stabilizing reagents are still evident.

Choosing the appropriate surface requires that the structure of the molecule to be immobilized be known, such that functional groups available on the molecule are matched with the correct surface. Of particular concern is that the functional group on the biomolecule that will be interacting with the surface must be positioned in such a manner that immobilization does not interfere with the activity (immunological or enzymatic). Blocking of these surfaces requires that the blocking solution contain a reagent that possesses the appropriate functional group as well as components capable of blocking hydrophobic sites along the spacer arm.

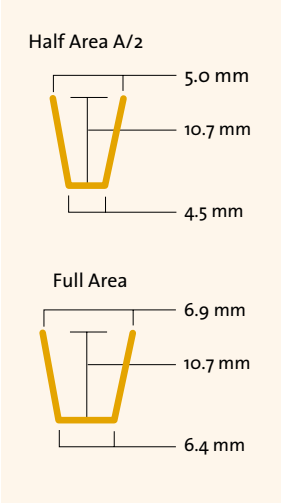

Figure 4. Well Size

Non-Binding Surfaces

Certain procedures require a surface that is non binding. Many proteins, enzymes in particular, may become activated or inactivated upon attachment to a surface. A non-binding surface can be ideal for assays requiring this feature.

Physical Properties

Assays can be performed in a variety of vessels from tubes to plates. One of the most versatile formats is the 96 well plate, which is offered with the selection of surface "flavors" discussed in the previous section. As the surfaces of these plates can vary, so can their physical properties. And, as the surface plays an important and crucial role in biomolecule immobilization and assay design, the physical properties can also affect assay performance. The physical properties that affect assay performance the most are (i) well size, (ii) well shape, and (iii) plate pigment.

Well Size

The 96 well plates are available with two well sizes — full size and half area (half volume). Full size wells are typically used for immunoassays. Total well volume is approximately 300 µL. The surface area covered by 100 µL of solution is approximately 1.0cm^2. This plate well size is compatible with all commercially available 96 well plate devices, such as automated washers and readers. However, Corning offers the half area well plate, having a total volume of approximately 150 µL with a working volume of 50 µL, as an alternative.

Most assays can be and are performed in full size (standard) 96 well plates. However, there are instances when a smaller well can be beneficial:

- limited supply of reagent and
- high cost of reagents.

Because of the surface-to-volume ratio and the diffusion distance associated with a smaller size well, assay volumes can be reduced by 1/2 without affecting the sensitivity of the assay. Since the half area well is approximately half the diameter

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Surface Selection Chart

Surface	Binding Interaction	Sample Properties
Medium Binding	Hydrophobic	Large biomolecules >20kD with large or abundant hydrophobic regions
High Binding	Hydrophobic and ionic	Medium-large biomolecules >10kD that are positively charged +/- hydrophobic regions
Aminated-Polystyrene	Ionic or covalent via bifunctional crosslinkers	Small negatively charged biomolecules OR biomolecules possessing an appropriate functional group, such as amine, carboxyl or thiol
N-oxysuccinimide (DNA-BIND™)	Covalent	Biomolecules possessing an available amine group
Maleimide (Sulfylhydryl-BIND™)	Covalent	Biomolecules possessing an accessible sulfhydryl group or reducible disulfide bond
Hydrazide (Carbo-BIND™)	Covalent	Biomolecules possessing carbohydrate moieties available for periodate activation
Universal-BIND™	Covalent via UV crosslinking	Biomolecules with abstractable hydrogen
Non-Binding	None - inhibits hydrophobic and ionic interactions	Non-specific

Figure 5. Well Shapes

of a full size well, the diffusion time for molecules in solution (center) to reach the surface is reduced by 1/3. This decreased distance leads to an increased adsorption rate on the surface as depleted molecules are replaced faster. This means that assay time and/or assay volume can, in many cases, be reduced by 1/2 to 1/3 that required for assays performed in standard size wells. Using a volume of 50 µL in a half area well allows for a more favorable surface-to-volume ratio (1:75) as compared to the ratio (1:100) obtained when 100 µL is used in a standard size well. This favorable ratio, combined with the reduced diameter of the well (travel distance), allows for reduced volume without compromising sensitivity.

These half area plates are unique and can be valuable for assays requiring smaller volume due to reagent availability or cost. However, because of the reduced diameter of the well, they are not compatible with all plate washers on the market and may need to be washed manually.

Well Shape

Plates and strips designed for assays are available with several well shapes including traditional flat bottom, round bottom and "eased" edge bottom (referred to as Easy Wash™) wells. Immobilization of biomolecules in general is not affected by well shape; however, assays may be designed to better accommodate one well shape versus another. Flat bottom wells are the most traditional well shape. They offer the advantage of being compatible with all microplate devices, such as washers and readers. The flatness feature allows for excellent optical transmission and low background adsorbance. The fact that this well shape retains a slight amount of fluid (approximately 5 to 10 µL) can be beneficial when the bound molecule is extremely susceptible to the negative effect of excessive drying. However, this retained volume of fluid can be easily tapped out of the well if required.

Round bottom wells have been traditionally used as substitutes for test tubes. This shape allows for:

- the elimination of surface related steric hindrance associated with large molecules bound in corners, and
- efficient removal of fluid, which is an advantage for assays employing the use of reagents that are negatively affected by residual wash solution.

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This shape, however, does not have the excellent optical characteristics of a flat bottom well. It is also mandatory that plate washers be adjusted to accommodate the round shape. This well shape is not recommended for use with biomolecules that are sensitive to the denaturing effects of excessive drying.

The third well shape is a hybrid of the first two (flat and round). The bottom edges of this hybrid are tapered such that the sharp corner associated with a flat bottom well is eliminated and fluid hang up is minimized. Testing has shown that this design does allow for a minimal retention of fluid such that the negative effects of excessive drying are not as prominent as with the round bottom design. Since the actual optical surface of the well is flat, transmission and background are comparable to that obtained with flat bottom wells. Again, the shape of the well does not affect biomolecule immobilization, but can affect one's assay technique.

Pigment: Clear versus Opaque Plates

The choice of pigment is a very straightforward decision. Clear plates made from pure polystyrene offer the most well defined binding characteristics and are ideal for colorimetric assays. However, for fluorescent and chemiluminescent assays, an opaque plate offers several advantages.

Solid black plates and strips are typically used when fluorometry is the detection method. Opaque black plates reduce background due to autofluorescence, reduce well-to-well crosstalk and enhance sensitivity by reducing "light scatter."

For assays designed to quantify light emission from bioluminescent or chemiluminescent assays, a solid opaque white plate is advantageous. The white material chosen by Corning has a low luminescent background and no well-to-well crosstalk. Opaque white plates and strips can actually enhance assay sensitivity by reflecting light emitted from flash and glow chemiluminescent reactions.

For procedures requiring optically clear wells — cell based assays, in particular — black and/or white plates with clear well bottoms are ideal. These plates combine the opaque qualities of the solid black and white plates while allowing:

- cell observation through clear wells,
- top and/or bottom options for signal detection, and
- dual labeling options utilizing colorimetric and fluorescent/chemiluminescent detection in a single plate.

Conclusion

Selecting the right surface for immobilizing biomolecules is crucial to the development of accurate, precise, and sensitive bioassays. Since the surface plays an indispensable role in the reactions that take place at its interface with the biological components of the assay, one must choose a surface that:

- forms a stable linkage with the bound molecules,
- immobilizes the molecules in such a manner that orientation and conformation result in optimal biomolecular activity, and
- does not interfere with biological reactions subsequent to the immobilization step.

In choosing a surface and assay format, one must consider the chemical properties of the biomolecule being immobilized and match them to the chemical properties of the surface. A surface that is attractive to a large hydrophobic biomolecule may not be appropriate for a small negatively charged one. Many non-proteinaceous molecules cannot passively adsorb to a surface and require a covalent method of immobilization.

Choosing the correct surface does not have to be a trial-and-error event if the binding interactions that can possibly take place between the biomolecule and the surface are carefully analyzed. Once an appropriate surface is selected, the next decision relates to the physical properties of the assay vessel.

Care must be taken to choose a well size and shape that fits the assay technique being employed. Most automated assays require full size wells that can be utilized with standard assay equipment; whereas a

manual assay designed around the use of rare, expensive reagents can be performed on half area wells to conserve valuable compounds. Plate formats, such as 384 well and 1536 well, further expand the choice of well size and shape. The choice of well shape is often a personal preference. However, if the molecule being immobilized to the surface is highly sensitive to residual wash solutions, a round bottom well should be used; whereas a flat bottom well should be used for biomolecules that cannot survive the denaturing effect of drying.

Choosing clear or pigmented plates is fairly simple — clear for colorimetric detection, black for most (but not all) fluorometric detection and white for luminescent detection. Solid opaque plates can be used with solution based assays; however, fluorescent or luminescent cell-based assays require clear bottom black or white plates.

The choices (surfaces and formats) for bioassays are many, but with careful consideration, a 96 well plate that possesses all the necessary characteristics for an optimized assay can be found. In future bulletins, we will be presenting information on methods that have been proven to improve assay results.

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Technical Assistance

For additional ELISA technical support and bulletins, or product information, please visit the Corning Life Sciences website at **www.corning.com/lifesciences** or call 1-800-492-1110.

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Optimizing the Immobilization of Protein and Other Biomolecules

ELISA Technical Bulletin - No. 2

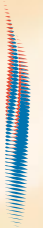

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Table of Contents

Introduction 1

Assay Environment. 2

Pipetting Optimization. 2

Biomolecule Concentration and Purity 4

Incubation Time and Temperature. 6

Conclusion 7

References 8

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 first technical bulletin of this series (*Immobilization Principles — Selecting the Surface*), 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 plate formats. In designing a Certification Test for our 96 well plate 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 will focus on the immobilization step of an ELISA and discuss methods to (i) improve biomolecule-surface interactions and (ii) improve well-to-well precision.

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Assay Environment

Particulate Contamination

The environment surrounding an assay plate 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. Preactivated 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 air-borne 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 1000 mL of reagent grade water, add 12.1 g Trizma base (Sigma Chemical Company #T6791) and 20 mg phenol red. Mix well.
2. Using the pipettes or pipetting apparatus set at the volumes that are normally used for assays, pipette the phenol red solution into each well of a 96 well plate or 8 well strip plate. Immediately (to reduce the effect of evaporation) read the optical density in each well at 540 nm.

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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 multichannel pipettor whenever possible to reduce the number of pipetting steps (less steps lead to reduced chance for error).
2. Be sure that the pipette tip is properly positioned and sealed to each channel of a multichannel pipettor. A loose tip will lead to inaccurate volume pick up and delivery (see 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 blow out step, a volume setting of 100 µL will deliver a consistent 98 µL of volume each time. (This can be verified using a calibrated pipette 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 pipette below the fluid line or against the sidewall of the well to remove the last drop that adheres to the pipette tip. The volume in this last drop can vary from tip to tip and with each pipetting maneuver.
5. Always pre-wet pipette tips with the solution being pipetted. Pipette tips are typically hydrophobic and will immobilize biomolecules. By pre-wetting, the inner and outer surfaces of the pipette 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 pipette 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

Table 1. Example of Pipetting Error

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

Loose pipet tip on the second channel of a 12-channel pipettor resulting in lower than average OD's. Zeros (0) represent OD's lying within 10% from the mean of all 96 wells.

[011] GATOR – EXHIBIT 1021

incubated at elevated temperatures. Room temperature is sufficient and the easiest to maintain during the pipetting process.

7. Finally, use high quality pipette 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

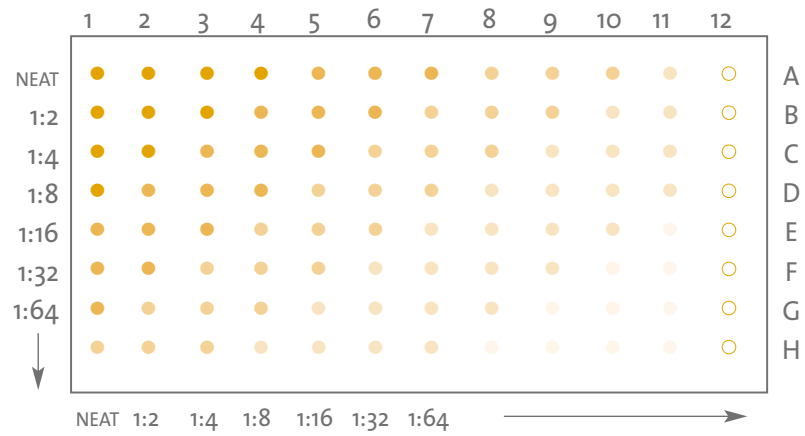
Concentration

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 (see 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 surface as a monolayer to ensure the greatest precision. A monolayer coverage reduces void areas on the surface (concentration too low) or unstable multilayer 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

Figure 1. Checkerboard Titration Method



A. Antigen or antibody is serially diluted across the plate (starting 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.

[012]

GATOR – EXHIBIT 1021

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 1000 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 stored 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. 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 (pH 9.6) due to exposed hydrophobic regions (see Table 2).

[013] GATOR – EXHIBIT 1021

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.

The best incubation conditions for the initial surface coating step is 4°C overnight (16 hours). (See Table 3). This

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	CV	Low well	High well
4°C	16 hrs	1.4%	3.6%	4.1%
RT	1 hr	2.3%	5.5%	6.1%
37°C	2 hrs	2.2%	6.4%	4.6%

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, and
- 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 plate 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 one 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. (LabLine markets a slide warmer that fits 8 plates.) 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.

Conclusion

Biomolecule immobilization to hard plastic surfaces, such as polystyrene 96 well plates, 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

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%

[015]

GATOR – EXHIBIT 1021

inhibitors). To expect precise assay results, one must control as many precision-affecting factors as possible. This bulletin 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 (ELISA Technical Bulletin #3) and optimizing the separation methods (ELISA Technical Bulletin #4), are detailed in other editions of this bulletin series.

Technical Assistance

For additional ELISA technical support and bulletins or product information, please visit the Corning Life Sciences website at **www.corning.com/lifesciences** or call 1-800-492-1110.

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