Effective Blocking Procedures

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Introduction

Solid phase immunoassays, such as ELISA, involve the immobilization of biomolecules, primarily proteins, to the surface via passive or covalent interactions. The ability of the surface to interact with proteins and other biomolecules is obviously an essential feature; however, non-specific binding (NSB) of other proteins or biomolecules to unoccupied spaces on the surface during subsequent steps of the assay can be detrimental to the specificity and sensitivity of the assay results. Non-specific binding to the surface can be minimized by saturating these unoccupied binding sites with a blocking reagent — a collective term for various substances that are used to reduce NSB without taking an active part in specific assay reactions. (Other factors can influence NSB, such as protein-protein interactions that are unique to each ELISA system, and must be considered during assay development and optimization.) Blocking reagents and methods are typically chosen in an empirical manner, since a single standardized procedure has not been determined suitable for all applications. However, for any given application or assay, a best method usually can be found quite

readily if one chooses a blocking reagent/method based on:

- the type of surface,
- the type of biomolecule immobilized to the surface, and
- the type of detection probe/system employed.

The two major classes of blocking reagents are:

- proteins, and
- detergents (typically non-ionic).

Both classes have advantages and disadvantages, which will be discussed in this bulletin and measured against the properties of an ideal blocking reagent. (Keeping in mind that a universal blocking reagent for all assays is idealistic, not realistic.) An ideal blocking reagent should:

- inhibit non-specific binding (passive and covalent) of assay components to the surface,
- inhibit non-specific protein-protein interactions,
- exhibit no cross-reactivity with subsequent assay components (i.e., antibodies, protein A),
- act as a stabilizer for (or assist in renaturing) biomolecules by minimizing the effects of denaturation caused by phase transitions associated with solid phase assays,
- exhibit low enzyme activity (or other activity that may interfere with the detection method),
- not disrupt the bonds that immobilize the specific protein or biomolecule to the surface, and
- exhibit consistent, reproducible performance with every lot.

Blocking a surface to reduce non-specific binding is a compromise between low background and high sensitivity and specificity. The best blocking reagent and method for any particular assay will be an optimized, but not absolute, choice.

Typical Problems Associated with Blocking Reagents

Since no blocking reagent or method is ideal for all assays, one must consider the advantages and disadvantages of each type and assess how these features will affect the assay. Some of the major problems associated with blocking reagents in general are:

- lot-to-lot inconsistencies (certain sources of bovine serum albumin, fish gelatin, and normal mammalian serum vary in quality from lot-to-lot),
- masking of surface bound proteins by interfering with specific protein-protein interactions (fish gelatin tends to block protein-protein interactions more tenaciously than protein-surface interactions, thus reducing specific binding more so than non-specific binding),
- lack of molecular diversity (many single molecule blocking reagents lack the diversity to block surfaces comprised of hydrophobic, ionic and covalent regions),
- cross-reactivity with assay components (i.e., Protein A will cross-react with the non-specific IgG molecules of normal mammalian serum),
- disruption of non-covalent bonds between specific biomolecules and the surface (i.e., non-ionic detergents may displace hydrophobically attached proteins and biomolecules),
- interference with detection due to endogenous enzyme activity, intrinsic fluorescence, etc.

Detergent Blockers

One of the major classes of blocking reagents is detergents — non-ionic and ionic. For solid phase immunoassays on polystyrene (or other hard plastic), ionic detergents are seldom used as the sole blocking mechanism due to:

- their propensity to disrupt ionic and hydrophobic biomolecule-surface bonds,
- their ability to solubilize proteins, and
- their tendency to inhibit (or terminate) enzyme-substrate reactions.

Zwitterionic detergents are simply poor blockers so are not even considered as blocking reagents. Typically, detergents used as blocking reagents are non-ionic;

the most common being Tween 20. Detergents are considered temporary blockers; they do not provide a permanent barrier to biomolecule attachment to the surface because their blocking ability can be removed by washing with water or aqueous buffer. To be useful as the sole blocking reagent in an assay, detergents must be present in all the diluents/buffers subsequent to coating the surface with a capture molecule. However; when used in conjunction with a protein blocker, detergents provide added convenient and inexpensive blocking ability during wash steps, etc. by blocking areas on the surface that may become exposed due to protein/biomolecule desorption.

Non-ionic detergents are advantageous for the following reasons: They are:

- inexpensive, even though they must be used at a concentration equal to or greater than their Critical Micelle Concentration (CMC) value (typical concentrations for Tween 20 are 0.01% to 0.10%),
- extremely stable and can be stored in diluted form (i.e., wash buffers) at room temperature for extended periods of time without experiencing any loss of blocking activity,
- useful in washing solutions because their presence blocks areas on the surface that may be physically stripped of specifically bound biomolecules during the wash step and helps dislodge loosely bound biomolecules that are physically trapped in corners.

Major disadvantages associated with non-ionic detergents are:

- they may disrupt non-covalent biomolecule-surface bonds,
- they block hydrophobic interactions only,
- residual detergent left in wells following the immobilization of a peroxidase conjugate can interfere with its enzymatic activity,
- they are not permanent blockers, and
- they cannot be used with lipopolysaccharides due to their ability to successfully compete against these biomolecules for surface space.

Our recommendation for using a non-ionic detergent as a blocking reagent for hard plastic assays (i.e., 96 well plates or strips) is to include it in the wash buffer and not use it as the sole blocking reagent for the assay. The preferred non-ionic detergent for this purpose is Tween 20, which is also the most commonly used at concentrations ranging from 0.01 to 0.1%. Some non-ionic detergents, such as Triton X-100, although excellent blockers of non-specific binding to the surface, can cause a high loss of specific binding, resulting in false negative results. By using non-ionic detergents at low concentrations in wash buffers, the negative aspects can be avoided, while the benefit of added blocking ability can still be exploited.

Protein Blockers

Protein blockers can serve two purposes:

- block non-occupied sites on the surface and
- space out and stabilize biomolecules bound to the surface to reduce steric hindrance and denaturation problems associated with solid phase assays.

Unlike non-ionic detergents, proteins are permanent blockers and only need to be added once after the surface is coated with the capture molecule. However, it is common practice to add protein blockers to diluents used for subsequent assay reactants to further reduce background and stabilize surface bound biomolecules. Some of the most commonly used protein blockers are:

- bovine serum albumin,
- non-fat dry milk or casein,
- whole normal serum, and
- fish gelatin.

Each of these blockers has its own advantages and disadvantages.

Bovine Serum Albumin

Bovine serum albumin (BSA) is typically used at a 1 to 3% concentration. BSA is inexpensive and can be stored dry or as a sterile solution at 4°C. The use of BSA as a blocking reagent is well documented and has been proven to be a good blocker of non-specific protein-surface binding

on medium and high binding surfaces, as well as many of the pre-activated covalent surfaces. An advantage associated with using BSA is its compatibility with Protein A. Disadvantages associated with BSA include:

- lot-to-lot variability — primarily related to the fatty acid content (BSA used as a blocking reagent should be fatty acid free),
- presence of phosphotyrosine in Fraction V preparations that cross-reacts with anti-phosphotyrosine antibodies,
- cross-reactions with antibodies prepared against BSA-hapten conjugates (BSA is typically linked to small haptens that lack the ability to elicit an immune response as individual molecules), and
- lack of diversity required to block some covalent surfaces (surfaces that feature hydrophobic, ionic and covalent characteristics).

Despite its disadvantages, BSA is probably the most widely used blocking reagent for solid phase immunoassays.

Non-Fat Dry Milk

Non-fat dry milk (NFDM) is typically used at 0.1 to 0.5% concentrations and is relatively inexpensive; however, preparations vary in quality. We have found only one source of NFDM (a 2% solution) that exhibits acceptable lot-to-lot consistency and stability. NFDM, either homemade or commercial, has a tendency to deteriorate rapidly if not properly prepared and stored. Although casein, a non-fat dry milk component, can be used as a stable blocking reagent (primarily for DNA blots), NFDM tends to be more dispersible in aqueous buffers than pure casein. This may explain why it is the better blocker of the two on hard plastic surfaces. Although NFDM is compatible with Protein A and exhibits little cross-reactivity with typical immunoassay components, it does express the following reactivity related problems:

- milk contains phosphotyrosine which reacts with anti-phosphotyrosine antibodies,
- some preparations of NFDM may contain histones that interfere with anti-DNA determinations, and
- alkaline phosphatase activity can be inhibited by some preparations of NFDM.

Overall, these are minor issues. NFDM is an excellent blocking reagent. Due to its molecular diversity and amphipathic characteristics, NFDM is the preferred blocking reagent for many covalent surfaces.

Fish Gelatin

Although fairly popular as a blocking reagent, fish gelatin has some major disadvantages. Typically, gelatin is not an adequate blocker when used alone and is actually the least effective biomolecule-surface blocker discussed in this bulletin. It blocks mainly protein-protein interactions, sometimes masking specific surface bound proteins and interfering with immunoreactivity. The inferior surface blocking ability and the protein-masking characteristic of gelatin results in higher background and decreased sensitivity. Gelatin also tends to vary in quality from lot-to-lot. The greatest advantage associated with fish gelatin is its lack of cross-reactivity with mammalian antibodies and Protein A.

Whole Sera

For extremely difficult blocking problems, the use of normal whole sera at a 10% concentration is recommended. Due to its molecular diversity, whole sera effectively blocks non-specific:

- biomolecule-surface (passive adsorption) interactions,
- biomolecule-covalent surface interactions, and
- protein-protein interactions, while acting as a protein stabilizer as well.

The disadvantages of using normal whole sera as a blocking reagent center around its cross-reactivity with Protein A and anti-IgG antibodies. Since many immunoassays rely on a system that utilizes a labeled (enzyme, radiolabel, etc.) secondary anti-IgG antibody, blocking with nor-

mal whole sera can lead to false positive reactions and high non-specific binding due to this cross-reactivity issue. Alternatives to normal mammalian sera are fish or chicken sera. Both lack the cross-reactivity problems associated with their mammalian equivalents, yet retain the positive aspects of being molecularly diverse in order to block surfaces with mixed characteristics (hydrophobic, hydrophilic and covalent functional groups).

Miscellaneous Blockers

As assays become more sensitive and surfaces become more diverse, there is a need for alternative blocking reagents that perform a variety of functions beyond reducing non-specific background. Examples of alternative blockers include polymers such as polyethylene glycol (PEG), polyvinyl alcohol (PVA), and polyvinylpyrrolidone (PVP). These blocking reagents are known for their ability to coat hydrophobic surfaces and render them both non-binding as well as hydrophilic. This hydrophilicity-producing characteristic has been exploited for assays designed as one-step on lateral flow matrices (i.e. over-the-counter pregnancy tests).

Matching the Blocker to the Surface

Passive Surfaces

Hydrophobic surfaces consist of those typically referred to as medium binding. These surfaces can be effectively blocked with either non-ionic detergents or protein blockers. In our experience, the combined use of 0.02% Tween 20 and 1% BSA has been ideal for most assays on medium binding surfaces.

Surfaces that are comprised of *hydrophobic and ionic* binding sites are typically termed high binding. Due to the ability of IgG and its conjugates to displace detergents, high binding surfaces are slightly more difficult to block than medium binding surfaces. The combined use of a non-ionic detergent (0.02% Tween 20) and a protein blocker (1% BSA, 0.2% NFDM, 10% normal sera, etc.) is suggested to effectively minimize non-specific binding. The choice of protein blocker is more dependent on the assay's reactive biomolecules than on the surface itself.

Surfaces that are highly charged and exhibit little to no hydrophobic character must be blocked with a protein blocker. Since an ionic surface is typically only used for the immobilization of small, ionic molecules, the chosen blocker must be both relatively small to prevent the eclipsing of the specific capture molecule and express the appropriate ionic species in order to interact with the surface charge. BSA (1 to 3%) or non-fat dry milk (0.2 to 2%) can be used for most assays; however, a smaller molecule such as ethanolamine (10%) may be necessary when very small biomolecules are specifically bound. Non-ionic detergents are useless in terms of blocking an ionic surface.

Covalent Surfaces

(See the *Corning Surface Selection Guide* on the Corning web site for additional information on ELISA Plates with Covalent Surfaces.)

An *amine* surface used with bifunctional crosslinkers must be blocked with a protein blocker capable of interacting with unreacted hydrophobic sites, ionic sites and covalent sites. We suggest using non-fat dry milk (02 to 2%) if possible. Another option is to use 10% normal serum as a primary blocking reagent or as a constituent of the post-coating assay buffer(s). Non-ionic detergents are inefficient as blockers for this surface, but including Tween 20 in the wash buffer can enhance the removal of non-bound, physically trapped biomolecules.

Pre-activated covalent surfaces (N-oxysuccinimide, Maleimide, Hydrazide, Universal) most always consist of hydrophobic and covalent regions. Amphipathic proteins tend to be the most efficient blockers of covalent surfaces. Non-ionic detergents will not block covalent inter-

actions, but their presence in wash buffers is recommended regardless of the surface used. The following is a recommended method for blocking the four covalent surfaces listed above:

1. After covalently immobilizing a specific biomolecule to the surface, block the plate with 2% BSA for approximately 30 minutes. The BSA diluent should be compatible with the surface and pH adjusted to allow the covalent interaction between the blocker and the surface to occur. If a protein blocker other than BSA is used, it must possess an appropriate functional group that can interact with the covalent sites on the surface.
2. Due to the complexity of the surface chemistry, the addition of 10% normal sera (such as fetal bovine, goat, fish or chicken sera) to all reactant diluents is recommended and necessary for most assays. Normal sera have the molecular diversity necessary to block non-specific binding due to hydrophobic, ionic, and covalent interactions.

Conclusion

In summary, the selection of an appropriate blocking system is essential to the development of a specific and sensitive assay. Most often the choice is based on convenience, literature and "what has traditionally worked." In reality, empirical testing is required to both choose the best blocker(s) and optimize the blocking procedure. This testing is heavily influenced by the surface chemistry as well as interactions unique to the specific assay reactants, primarily cross-reactivity. A blocker can totally inhibit non-specific reactions with the surface and not reduce signal-to-noise due to cross-reactivity issues.

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It is advisable that during the development of a blocking procedure, each of the proposed blockers and blocking conditions (buffers, incubation times, etc.) be evaluated for cross-reactivity with all other assay reactants. The ideal blocker and blocking procedure will effectively and reproducibly eliminate non-specific surface attachment and improve assay sensitivity and specificity — resulting in a high signal/low noise assay.

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 Separation Step on 96 Well Plates

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Introduction

Enzyme Linked Immunosorbent Assays (ELISA), regardless of whether the antigen or antibody is bound to the surface, require a physical separation step to remove free from bound analyte. This is typically referred to as a "wash step". For multiple well plates, washing is accomplished by consecutive filling of the wells with wash solution followed by emptying them via decanting or aspirating. In order to maximize assay precision and sensitivity, complete separation of free from bound fractions is required. We have found that the wash step can be a major factor affecting assay precision and optimizing this step is crucial to obtaining consistent and reliable results. Some of the factors associated with the wash step that should be optimized are:

- the composition of the wash solution,
- the dispensing mechanism used to fill the wells with wash solution, and
- the fluid aspiration conditions, including vacuum strength.

The use of a well-maintained automated microplate washer is a positive step in assuring precise, accurate washing for every assay.

Wash Solution Composition

The wash solution should be comprised of a physiological (enzyme friendly) buffer, such as phosphate buffered saline, Tris buffered saline or imidazole buffered saline. We have experienced favorable results using imidazole buffered saline, which is compatible with all the enzymes typically used for enzyme immunoassays. When alkaline phosphatase is used, phosphate buffers should be avoided due to the effect of phosphates on the enzyme's activity. Inorganic phosphate can act as a pseudosubstrate for alkaline phosphatase and effectively reduce its specific activity with the substrate. If peroxidase is used, sodium azide must not be added to the wash solution. Sodium azide is an inhibitor of peroxidase activity. Water is a poor wash buffer due to its variable pH and lack of protein buffering capability (surface bound proteins need to be protected from denaturation).

The addition of a detergent such as Tween 20 is beneficial. Detergents aid in the removal of loosely bound protein and act as a hydrophobic blocking reagent to block sites on the surface that may become available due to protein desorption during the wash step. A concentration of 0.01 to 0.03% is recommended. The goal is to remove loosely bound protein without stripping off specifically bound protein or inactivating enzymes, which could occur if detergent concentrations are too high, when greater than their critical micelle concentration (CMC) value.

Effect of Fluid Force on Immobilized Biomolecules

To achieve precise washing, an equal volume of wash solution should be dispensed into each well of a microplate with equal force. The gentle addition of wash solution that occurs with an automated washer is ideal. A gentle flow of fluid into the well removes free protein without stripping off bound protein. When the addition of wash solution is too vigorous, protein can be stripped off and enzyme activity inactivated by the sheer force of the fluid as it enters the well. An example of vigorous and inconsistent wash solution addition is the use of a squirt bottle to dispense the wash solution. The force of fluid from well to well cannot be controlled and is usually too vigorous to allow precise washing.

The volume of the wash solution dispensed per well should be high enough to cover the entire surface area coated with antigen or antibody. We recommend that the entire well be filled (approximately 300 µL per well for a 96 well plate).

It is often tempting to increase the vigorousness of the wash buffer dispensing step whenever background is higher than desired in an effort to remove unbound protein that could be causing the higher than expected values. However, this is not recommended for the above stated reasons. Instead of increasing the fluid force into the wells, increasing the number of wash cycles will help eliminate background problems caused by residual unbound protein left in the wells.

The optimal number of wash cycles can be determined through experimentation; however, our results indicate that less than three wash cycles leaves residual unbound protein in the wells and more than five results in unwanted protein desorption. Since washing is actually a dilution process (some of the original solution remains after each aspiration step as a film of fluid on the surface), the goal is to dilute the original solution as much as possible without stripping off bound protein. (Note: total aspiration is undesirable due to the denaturation effect of drying out the surface bound protein.) This optimal dilution scheme occurs between 3 and 5 cycles. The addition of a five minute soak step following the last wash cycle is extremely beneficial in terms of removing the remaining unbound protein that may be trapped in the well corners.

Optimizing the Aspiration Step

The major cause of precision problems that are associated with the wash step occurs during aspiration. Although the best method of removing liquid from the wells (in relation to Coefficient of

Variation; CV) is hand decanting, aspiration can be used, if optimized, to reduce the adverse effects to the surface bound protein. The items requiring optimization are (i) needle position, (ii) aspiration direction (top-down), and (iii) vacuum strength.

For flat bottom wells, the aspiration needles should be positioned midway between the center and edge of the well. (The center position should be occupied by the dispense needles.) The needles should be distanced from the bottom of the wells such that they do not touch the surface. Ideally, the surface should never be completely dried out during the wash step, so the position of the needles should allow a small amount of liquid to be left in the wells after aspiration is complete. This small liquid volume accumulates at the edge of the well because of the gravitational forces on the film of liquid left on the sidewalls. After the final wash cycle, this small volume can be hand decanted by rapping the plate upside down on an absorbent paper towel. It is important to remove this residual liquid prior to adding substrate, since wash solutions containing detergents can suppress product (colorimetric, fluorometric, or luminometric) development by a substantial degree. This will create precision problems if the volume of the residual liquid is not consistent from well to well.

The best automated plate washers use top-down aspiration; the needles begin aspirating as soon as they enter the liquid such that aspiration occurs as the needles descend to the bottom of the well. This type of aspiration reduces shear action and prevents air currents from drying out the surface bound protein. It is very crucial that the needles stop aspirating as soon as the liquid is removed or else they will draw air over the protein coated surface and cause unnecessary drying. Top-down aspirators tend to alleviate this problem because the needles aspirate on their way down to the bottom of the well and stop when their destination is reached. Needles that go to their lowest position prior to the start of suction, aspirate for a given amount of time (sufficient to remove approximately a full well) regardless of the amount of liquid in the well. This can result in "dry aspiration", with unnecessary drying, once the liquid is removed.

Drying out the surface is an assay's worst enemy. Even minimal drying can result in a loss of protein activity, especially enzyme

Table 1. Edge Effect from a 20 Minute Time Delay

MIN	0.224											
MAX	0.759											
RANGE	0.535											
AVG	0.669											
STD	0.103											
CV	15.365											
LOW W	66.5%											
HIGH W	13.5%											
	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%	0	0	0	0	0	0	0	0	0.759	0	0	0
of the Average	0	0	0	0	0	0.774	0	0	0.751	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	0.233	0.229	0.224	0	0.330	0594	0.326	0.589	0.452	0.595	0
	0	0	0	0	0	0	0	0	0	0	0	0
Wells <10%	0	0	0	0	0	0	0	0	0	0	0	0
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	0.604	0	0	0	0	0	0	0

Table 2. Effect of Aspiration Vacuum Strength on Assay Precision

Vacuum (mmHg)	No. of Plates Run	No. of Plates Out-of-Spec	CV	High Well	Low Well
400	112	0	2.0	4.8	5.2
550	10	2	3.3	7.3	8.4
250	10	3	2.4	7.2	7.1

activity. Table 1 depicts the effect of drying on enzymatic activity after a 20 minute delay from aspiration after the last wash cycle to the addition of substrate. The expected optical density (OD) reading for this assay when performed correctly is 1.200. As the data show, the color development is severely suppressed after 20 minutes without fluid in the wells. Edge wells are affected more than inner wells as shown by their lower OD's. (Wells represented by a "0" are within 10% of the average OD for the entire plate. Only wells that lie outside this 10% range show up as high or low wells).

Optimization of vacuum strength is crucial to maintaining low CV's in an assay. If vacuum strength (measured in mm Hg or PSI) is too high, shear forces and air currents will denature bound protein and inactivate enzymes. If vacuum strength is too low, excessive residual wash solution will remain in the wells and suppress enzymatic activity. Each type of well shape (flat or round) and type of plate (solid plate, strip plate, different manufacturers' plates) have their own optimal vacuum strength. However, we have found that 400mm Hg is optimal for most products (see Table 2 for results).

Conclusion

Crucial to assay precision is optimization of the separation step. Virtually all unbound proteins must be removed for maximum sensitivity, specificity, and precision to be realized. What would appear to be a simple and foolproof manipulation for multiple well plates — washing — is actually a step in an immunoassay that can cause the most troublesome precision problems. A balance needs to be struck between leaving too much unbound protein behind and stripping off or denaturing specifically bound protein. This balance can be achieved by adhering to the following recommendations:

- The wash solution should be a physiological buffer that will not interfere with immunological or enzymatic activity and should contain a low concentration of detergent to aid in the removal of unbound protein.
- An equal volume of wash solution should be dispensed into each well with equal (gentle) force to avoid stripping off bound protein.
- Wells should be washed a minimum of three times and a maximum of five times for best results. The addition of a 5 minute soak period following the final wash cycle will aid in the removal of unbound protein trapped in the well corners.
- Aspiration should be controlled so that the well surface does not dry out. Optimizing the vacuum strength is crucial to good assay precision.
- Finally, the well surface should be kept moist at all times. When running multiple plates, keep the wells filled with wash solution until ready to proceed with the next step or leave the plates inverted on a wet paper towel to keep the surface hydrated during delays.

These are the essential ingredients in optimizing the separation step of an ELISA.

Technical Assistance

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