Optimizing the Separation Step on 96-well Microplates for ELISA Assays

Application Note

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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:

- Composition of the wash solution
- Dispensing mechanism used to fill the wells with wash solution
- Fuid 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 pseudo-substrate 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 microplate).

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 5-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 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 ODs. (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 values in an assay. If vacuum strength (measured in mmHg 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 400 mmHg is optimal for most products (see Table 2 for results).

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

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%											
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	0.759	0	0	0
	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
Wells <10% of the Average	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
	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	0.604	0	0	0	0	0	0	0

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.

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