Application Note

Blocking Agent and Detergent in ELISA

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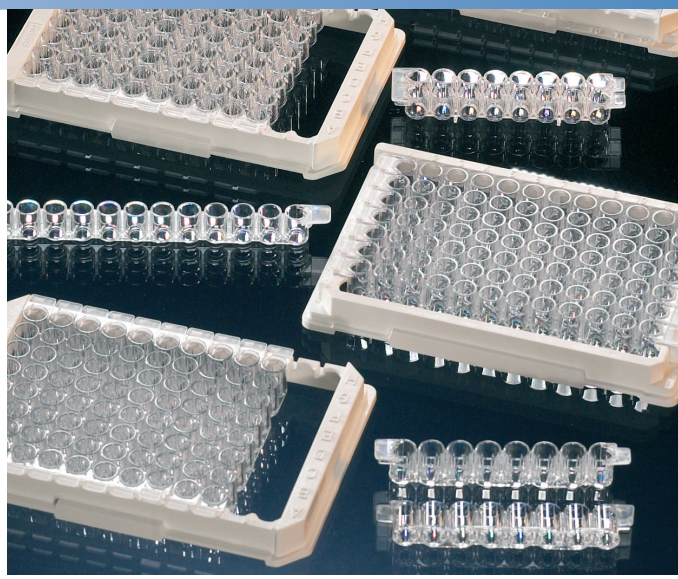
Key Words

Thermo Scientific™ Nunc™ Immuno MicroWell™ plate, Thermo Scientific™ Nunc™ PolySorp™ MicroWell™ plate, Thermo Scientific™ Nunc™ MaxiSorp™ MicroWell™ plate, Thermo Scientific™ Nunc™ MicroWell™ plate, ELISA, blocking, detergent, adsorbing surfaces, Tween™ 20, Triton™ X-100, CHAPS™, BSA, casein.

Goal

The goal of this application note is to describe the pinciples when a blocking agent is used with detergent in ELISA. Further to illustate the competition between the two molecules in respect to attachment to the plate. The results indicate the optimal assay is to use a blocking step after coating with presence of detergent during incubation with secondary reactant or extended to the wash just after coating depending on the detergent (Tween 20).



If a blocking agent is used together with detergent in ELISA, one must take into account that these reagents are competitors as far as blocking effect is concerned. Therefore they may counteract each other if not used with care.

In continuation of the investigation presented in Thermo Scientific Nunc Application Note No. 8 [1], each of the three neutral detergents, Tween 20, Triton X-100, and CHAPS, was examined with each of the two blocking agents, BSA and casein, using a two-layer antibody sequence in Thermo Scientific Nunc Immuno MicroWell plates with MaxiSorp and PolySorp surfaces.

Introduction

Agents may be used in ELISA for blocking possible excess solid surface after coating with one immuno-reactant to avoid unspecific immobilization of succeeding reactants. One reason for using a true blocking agent would be to substitute detergent for blocking: if detergent is present during incubation with secondary reactants, it might in some way interfere with the immunologic specificities or cause unspecific immobilization of the reactants [1]; if detergent is present during wash after secondary reactants, possible weak immunologic affinities might be broken by the washing activity of the detergent. Another reason for using a blocking agent would be to stabilize the immobilized reactant by sterical support [2]. This is relevant for storage of coated surfaces, especially for competitive assays needing unsaturated coatings.

A typical blocking agent would be a neutral macromolecule, large enough to establish a stable attachment to the surface, yet small enough to find its way between immuno-reactants, e.g. antibodies. Bovine serum albumin (BSA) of MW 67,000 is commonly used as a blocking agent. Also the more heterogeneous casein is often used and may be more effective than BSA [3, 4].

The problem of using both detergent and blocking agent occurs during the wash after 1st layer immobilization on the surface. If detergent is used in this wash, one may risk (depending on the detergent) unstable attachment of the succeeding blocking agent. On the other hand, if detergent is avoided in this wash, one may risk that the blocking agent will be hindered from reaching the surface by loosely attached 1st layer reactant [1]. This implies that by later wash with detergent, spaces may be exposed for unspecific immobilization of subsequent reactants (see Fig. 1).

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Step	Reagent	Time	0.05% Detergent added							
1st layer	SaR, 5 µg/mL in PBS or None	Overnight	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
1st wash	PBS + 0.2 M extra NaCl	3x	–	–	–	–	+	+	+	+
Blocking	BSA, 0.5% in PBS or Casein, 0.5% in PBS	0.25 hr	(–)	(–)	(–)	(–)	(–)	(–)	(–)	(–)
2nd wash	PBS + 0.2 M extra NaCl	3x	–	+	+	–	–	+	+	–
2nd layer	R:HRP, 1.3 µg/mL in PBS or S:HRP, 1.3 µg/mL in PBS	2 hr	–	–	+	+	–	–	+	+
3rd wash	PBS + 0.2 M extra NaCl	3x	–/+	–/+	–/+	–/+	–/+	–/+	–/+	–/+
Three first position detergent code used in Fig. 2			– – –	– + –	– + +	– – +	+ – –	+ + –	+ + +	+ – +

Table 1

Procedure with MaxiSorp or PolySorp MicroWell surfaces. Each of the two blocking agents was tested together with each of the three detergents used in sixteen alternative combinations [last eight columns], all in one experiment. The procedure was followed by HRP reaction using H_2O_2/OPD substrate. SaR = swine anti-rabbit antibody (Dako Z 196) = catching antibody; R:HRP = peroxidase conjugated rabbit antibody (Dako P 128) = target conjugate; S:HRP = peroxidase conjugated swine antibody (Dako P 217) = indifferent conjugate.

Therefore, blocking agent and detergent should primarily be regarded and administered as alternatives. It is the objective of this work to investigate, if and how the use of blocking agent and detergent in concert can be simplified to ensure minimal counteraction and still maintain the respective desired effects.

Materials and Method

Each of the three neutral detergents, Tween 20 (Merck 822184), Triton X-100 (Merck 8603), and CHAPS (Sigma C-3023), was tested together with each of the two blocking agents, BSA (Sigma A-4503) and casein (Sigma C-5890) in a catching antibody assay according to the procedure listed in Table 1. Thermo Scientific Nunc Immuno Modules MaxiSorp F8 (Cat. No. 468667) and PolySorp F8 (Cat. No. 469078) were used.

Results and Discussion

Preliminary experiments clearly demonstrated that addition of detergent in the blocking step destroys the effect of the blocking agent. Therefore this situation was left out of the experimental schedule.

From the results with Triton X-100 and BSA (Fig. 2 below), one makes the following observations:

1. If the detergent is used only in the 2nd wash (– + – –), or only in the 1st and the 2nd washes (+ + – –), significant unspecific signals occur. In the first situation (– + – –), probably the blocking agent has been hindered from reaching areas of the surface occupied by loosely attached 1st layer reactant. This has then been washed off by detergent in the 2nd wash, having opened spaces for unspecific attachment of later reactants (see Fig. 1 left).
   In the other situation (+ + – –), probably some of the blocking agent has been only loosely attached to the surface due to the preceding wash with detergent. This has resulted in removal of some blocking agent by detergent in the 2nd wash and thus opened spaces for unspecific attachment of later reactants (see Fig. 1 right).

2. The disadvantages stated above can be remedied by including detergent during conjugate incubation. Actually, all situations with detergent and conjugate present simultaneously (i.e. codes with "+" in the third position) exhibit specific signals. The unspecific signals can also be eliminated by detergent in the 3rd wash with PolySorp, but not with MaxiSorp. This can be explained by assuming that unspecific conjugate attachment is more loose (i.e. detergent sensitive) on PolySorp than on MaxiSorp.

3. Presence of detergent in the 1st wash only (+ – – –), or supplemented with detergent in the 3rd wash (+ – – +), implies specific signals. Probably the loosely attached blocking agent (cf. point 1) has been allowed to stay and exert its blocking effect on the surface due to the absence of detergent in the succeeding influential steps.

4. Complete absence of detergent (– – – –) also implies a specific signal, but on MaxiSorp it tends to be reduced. This is presumably due to sterical hindrance of 1st layer specificities by loosely attached antibody, which has not been washed off due to the absence of detergent. However, the reduction seems to vanish in the presence of detergent during the 3rd wash. Therefore the explanation may rather be that detergent remnants exert an amplifying effect on the substrate reaction; cf. the + – – – situation seems to give a somewhat reduced signal, compared to situations with later uses of detergent. This indicates an increasing amplification with decreasing detergent "distance" from the substrate reaction, thus supporting the amplification suggestion. However, this effect by Triton X-100 was not demonstrable in the previous work [1], and the complexity calls for a separate investigation, which will not be covered here.

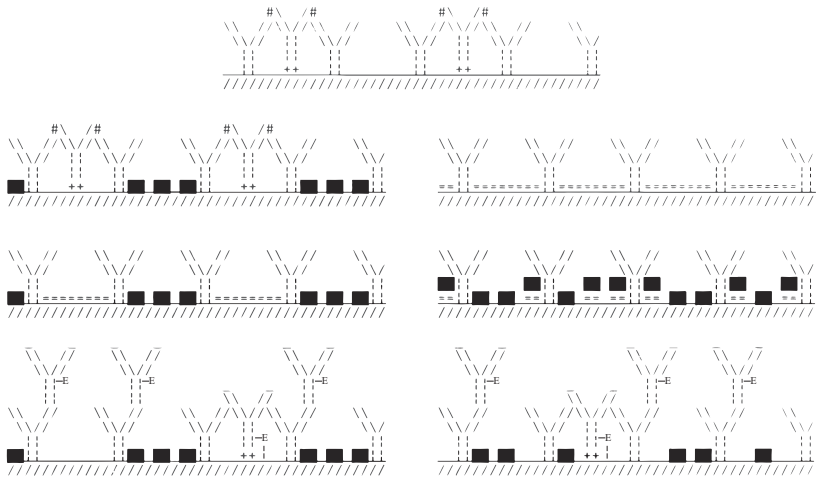


Fig. 1

Illustration of the presumed difficulties with use of detergent combined with blocking agent in a 2-layer sequence consisting of an immobilized capture antibody (Y-shapes) and a secondary target enzyme conjugate (Y-E). For simplicity, only the right arm antibody specificities are considered. The uninvolved specific sites of the conjugate are indicated by the small bars above the antibody arms.
Centre above: Surface coated with capture antibody including some loosely attached antibodies (++) which may cause sterical hindrance of some specific sites (#).
Left: Omission of detergent in wash after coating may let loosely attached antibody stay on the surface keeping the blocking agent (■) away from some areas [above], which by succeeding wash with detergent [middle] will be opened for unspecific attachment of target conjugate [below].
Right: Detergent (= =) in wash after coating removes loosely attached antibody [above], but may cause labile blocking by blocking agent [middle], resulting in some unspecific attachment of target conjugate [below]. Note that the unspecifically attached conjugate may be bound firmly, possibly through the enzyme (¦), i.e. it may not be washed off by detergent.

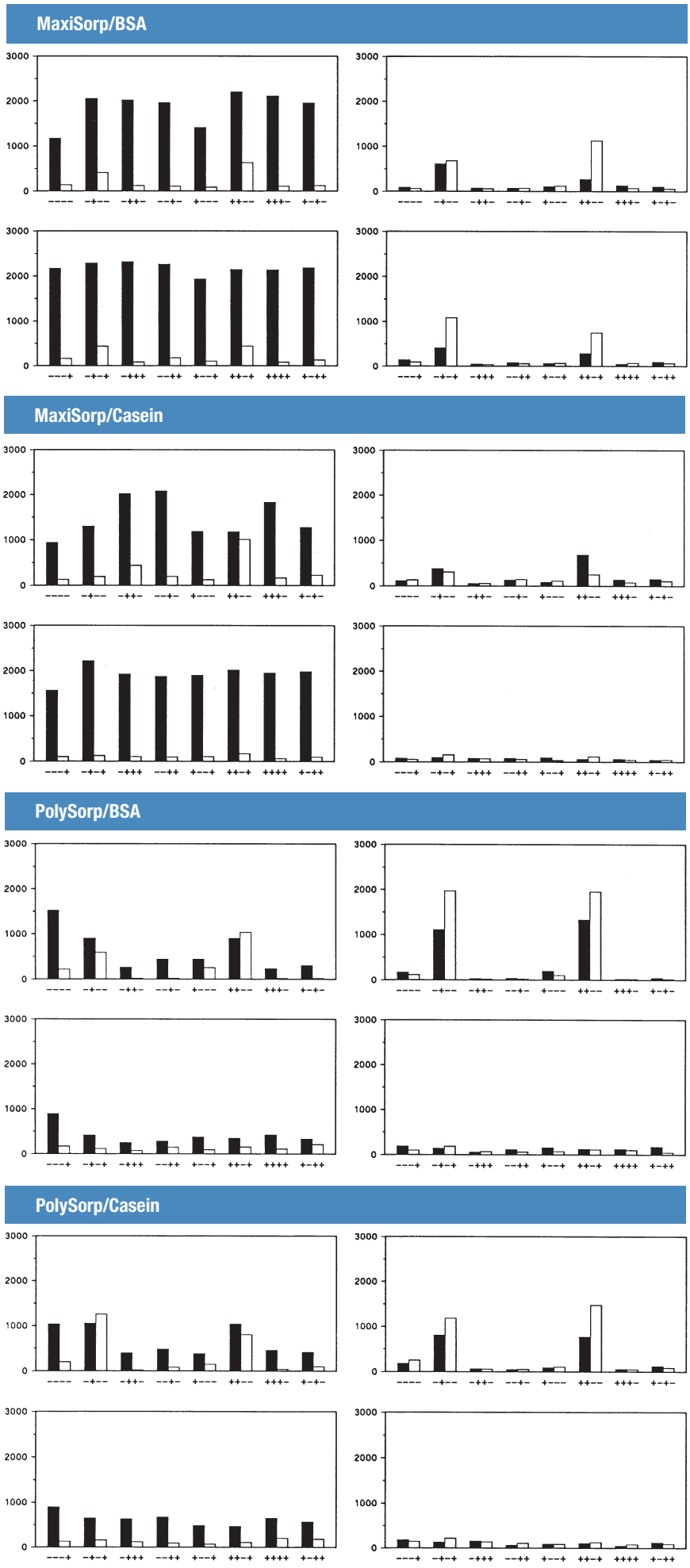
The results with casein (Fig. 2 below) very much resemble those with BSA, except that the unspecific signals can now be eliminated by detergent in the 3rd wash with both MaxiSorp and PolySorp. This indicates that casein, contrary to BSA, renders the unspecifically attached conjugate removable by detergent with both surfaces. Therefore casein seems to be a more effective blocking agent than BSA.

With Tween 20 (results not shown), regardless of the blocking agent used, no significant unspecific signals are observed in any situation. This is not surprising, since Tween 20 has been shown to exert a stable blocking effect if used just once [1]. In addition, the general backgrounds are reduced if the detergent is used in the 3rd wash.

With CHAPS (results not shown), like with Tween 20, no unspecific signals are observed. In this case the explanation is probably that CHAPS, contrary to the other detergents, is effectively replaced by either blocking agent (cf. Fig. 1 right). The backgrounds are somewhat higher than with the other detergents, even though they are reduced if the detergent is used in the 3rd wash. In addition, signal magnitudes are the same with both surfaces, indicating that the washing capability of CHAPS is less than that of the other detergents.

Fig. 2

Results with Triton X-100 for MaxiSorp [left diagram blocks] and PolySorp [right diagram blocks] in concert with blocking by BSA [above] or by casein [below]. Absence or presence of detergent in the 3rd wash is presented in separate diagrams indicated by minus or plus, respectively, in the fourth position of the detergent code. Left diagrams (in the MaxiSorp and PolySorp blocks respectively) show results with target conjugate; right diagrams show results with indifferent conjugate; ■ = 1st layer present: □ = 1st layer absent. Note the unspecific signals, clearly exceeding the general backgrounds, in some –+– ( ) and ++– ( ) situations.



Conclusion

The following general guidelines concerning the use of blocking agent and detergent in ELISA have been extracted from this investigation with special reference to the previous work [1]:

1. The optimal combination of blocking agent and detergent seems to be achieved simply by omission of detergent until wash after incubation with the last reactant. However, detergent wash may break specific couplings in systems having weak immunologic affinities. This may be merely a question of detergent selection. The gentle CHAPS might be a suitable choice in critical systems.

2. Unspecific adsorption can be avoided if detergent is included during incubation with secondary reactant. Actually, presence of detergent in this step is an efficient substitute for a blocking step [1], thus rendering a blocking step superfluous. However, the purpose of using a blocking agent may be (except to obtain coating storage stability) to avoid the co-presence of detergent and secondary reactant due to possible detergent interference with specific reactions. A simple solution to this might be just to wash after coating with Tween 20 and nothing else, since this detergent performs like a typical blocking agent [1].

3. The conclusion from the previous investigation [1], of using detergent in the wash succeeding coating (to wash off loosely adsorbed reactant), is obviously questionable. A desired use of detergent after coating should be limited to the wash between coating and blocking. But in this case, the blocking may not be stable, indicated by the observed blocking collapse if detergent (i.e. Triton X-100) is also used in the wash just after blocking. Again, CHAPS might fit the situation.

4. Casein is a more effective blocking agent than BSA. However, the blocking agent neutrality should always be questioned. Particularly with casein, and especially when assaying serum for auto-immune diseases, one must take into account the risk of cross-reaction with casein antibodies remaining in the patient's serum from ingestion of milk.

In summary, the optimal way of using blocking agent with detergent is to combine a blocking step (after coating) with presence of detergent only in wash after the last reactant. The use of detergent can possibly be extended to the wash just after coating, but this seems to be more risky and demands a careful detergent choice. In this context it should be noted that different detergents may be successfully used in different steps.

This investigation reveals that the combined use of blocking agent and detergent is a matter of considerable complexity. The above statements should be taken with the proviso that the investigation is based on a simple model system which may not be representative of all ELISA systems.

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