Technical Note

A Publication of Technical Services

Date: **December 18, 1991**

Lit. No.: **TN013**

Title: **Immobilization of Protein to Millipore 96-Well Membrane-Bottom Plates**

Introduction

Nitrocellulose has long been shown to be an excellent material for adsorbing proteins.(1) The combination of a strongly adsorptive substance with the large surface area obtainable in a membrane
filter has proved to be a very useful tool in a variety of areas. One of the first areas to be explored was the potential for concentrating viruses through adsorption to nitrocellulose filters.(2) Clinical immunoassay systems have been developed, such as the FIAXtf) system from lOT.

Over the past five years, a variety of researchers have employed nitrocellulose membranes to perform different types of assays.(3,4) Towbin, et al.(5) employed nitrocellulose for adsorbing proteins from polyacrylamide gels using an electrophoretic transfer technique, the so-called Western blot, which has become standard practice.(6,7,8) Hawkes(9) developed a dot blot technique which utilized a direct spotting of the protein onto the membrane, rather than electrophoretic or diffusional transfer from a gel.
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This tech note explores the variables associated with the adsorption of protein to nitrocellulose, particularly as they relate to the structure of Millipore 96-well membrane-bottom plates, in order to provide the user with a set of guidelines which can be utilized to tailor the specific techniques required to perform a given assay.

Results and Conclusions

The immobilization of protein to nitrocellulose via adsorption occurs very rapidly. The graph shown in Figure 1 demonstrates the speed with which protein adsorbs to the membrane. The majority of the protein (>50%) will adsorb within 15 minutes. The graph, however, points out two other important considerations. With the 96-well membrane-bottom plate, the volume in which a given quantity of protein is applied has a significant effect upon both the amount and the reproducibility of the bound protein. This is due to the configuration of the Millipore plate. Since >99% of the surface area available for protein adsorption lies within the membrane at the bottom of the well, only that part of the solution which is in contact with the membrane will participate in the reaction. Therefore, a given quantity of protein will adsorb more consistently and more rapidly if applied in a smaller volume.

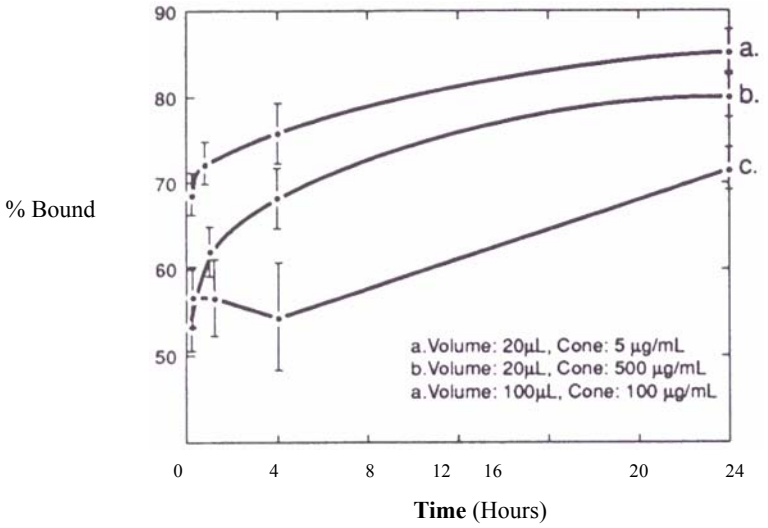

Figure 1: Immobilization vs time
Immobilization of IgG on the Millipore membrane-bottom plate as a function of time. The method used to generate the data is as follows: A solution of goat IgG in pH 7.2 pes at the indicated concentration was spiked with 1251-Goat IgG at a level such that the volume subsequently added to each well contained 25,000 cpm. This volume was then placed in each well and incubated for the indicated times. The solutions were drawn through the membranes under vacuum at the end of the incubation period. The membranes were then washed with pH 7.2 pes, punched, and counted. Results are shown as a percentage of the total counts added to each well.

Curve "b" in Figure 1 represents 1 0 ~g IgG applied in 20 ~L. Curve "c" represents 1 0 ~g applied in 1 00 ~L. This curve shows less consistency and less adsorption of protein. Although the volume of liquid which is required to completely fill the void volume of the membrane is only 5 ~L care should be taken in using less than 20 ~L for immobilization. Evaporation with subsequent loss of activity of the dried protein will occur.

In addition, the percentage and consistency of the protein which binds is also dependent on the quantity of protein applied. Curve "a" represents 0.2 ~g of IgG whereas curve "b" represents 1 0 ~g of IgG, both applied in the same volume. The capacity of the nitrocellulose membrane in the Millipore plate is 1 0-20 ~g per well, depending upon the specific protein and conditions. As this saturation capacity is approached, the percentage of protein bound begins to decline and the consistency also decreases. The lower quantity of IgG shows a more consistent and higher percentage bound at all points.

The effect of pH and salt concentration on protein adsorption was explored in the data presented in Figure 2.

Figure 2

Immobilization V5. pH and Salt Concentration

	Bovine Serum Albumin (% bound)	Goat IgG (% bound)
0.5M acetate buffer, pH 4.0	44	39
with 0.1 M NaCL	44	39
with 0.5M NaCL	45	45
0.5M phosphate buffer, pH 7.0	40	28
with 0.1 M NaCL	35	37
with 0.5M NaCL	45	35
0.5M carbonate buffer, pH 10.0	30	26
with 0.1 M NaCL	32	27
with 0.5M NaCL	42	34

Figure 2. Immobilization of proteins on nitrocellulose discs: 25 mm discs of nitrocellulose, 0.45 ~m pore size, were loaded into an appropriate filter holder and challenged with 30 mL of a protein solution containing 20 ~g/mL of either BSA or IgG in the indicated buffers. The solutions were spiked with either 1251-BSA or 1251-Goat IgG at approximately 3,000 cpm/mL. The solutions were drawn through the membrane with a peristaltic pump at 3 mUmin. The discs were then washed with the appropriate buffer, removed from the holder, and counted. Results are tabulated as a percentage of the applied counts.

It can be seen that there are some effects associated with changes in pH and salt concentration. The percentage of protein bound was lower than in Figure 1 because the challenge level represented roughly twice the saturation capacity of the membrane. The major point of this data is that, although some pH and salt effects can be seen, significant adsorption to nitrocellulose is seen under a wide variety of pH and salt conditions.

There is usually no need to alter the buffer containing the desired protein prior to immobilization. It should be noted, however, that although the quantity of immobilized protein may be the same under different immobilization conditions, the biological activity of the bound protein

may not be the same. In immunological systems using a bound antigen or a bound polyclonal antibody, this does not appear to be a concern. However, at least in microtiter plates, bound monoclonal antibodies have been

shown to retain varying amounts of activity based on the immobilization condition,(10) and the possibility that this is also true in the Millipore membrane-bottom plates bears consideration.

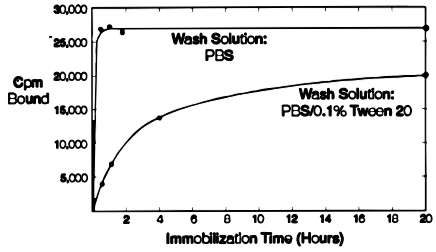

Figure 3. The effect of surfactants on the retention of immobilized protein as a function of immobilization time. Rabbit antiserum to human TSH was diluted with 0.1% BSA, and spiked with 1251- Rabbit IgG. 20 ~L was added to each well and incubated for the indicated times. Following the incubation, the solutions were drawn through the membranes under vacuum and the wells were washed with either pH 7.2 PBS or pH 7.2 PBS containing 0.1% Tween 20, as shown. The membranes were then punched and counted. Results show a dramatic effect with the use of surfactant at short immobilization times. This effect decreases with increasing immobilization times, indicating the immobilized protein is more tightly bound with longer immobilization periods.

Figure 3 demonstrates the effect of the surfactants on the binding of proteins to nitrocellulose. It has been reported that bound protein is eluted from nitrocellulose by detergents(11 ,12) and, as figure 3 demonstrates, surfactants do have a dramatic effect on the ultimate amount of protein which is retained on the membrane. It is somewhat surprising that this effect substantially decreases with time. As the length of time the protein remained in contact with the nitrocellulose prior to being exposed to Tween@ 20 increased, the reversibility of the adsorption process decreased. The adsorbed protein undergoes a stabilization process which results in tighter binding to the membrane over time. This data has an important effect on structuring the immunoassay. Although it is possible to adsorb protein virtually instantaneously to nitrocellulose, if Tween or a similar surfactant such as Triton@ X-100 or Nonidet P-40 are utilized in the assay, the bound protein will not remain on the membrane during the course of the assay. Since surfactants are typically included in buffer washes during immunoassays to reduce the level of non-specific binding, overnight immobilization is recommended. In those cases where detergents are not used, rapid immobilization will occur and, as Figure 3 indicates, the protein will remain bound throughout the assay.

The information presented in figure 3 raises another question: Does this "stabilization" process which bound protein undergoes result in a loss of biological activity? This question was addressed by the experiment shown in Figure 4.

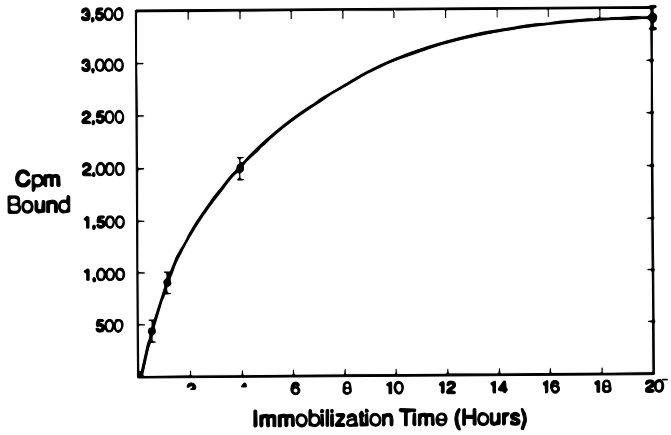

Figure 4. The quantity of TSH which can be specifically bound to an antibody-containing membrane as a function of immobilization time when surfactants are utilized. Rabbit antiserum to human TSH was diluted with 0.1% BSA exactly as in Figure 3, but without the addition of 1251-Rabbit IgG. 20 ~L was added to each well and incubated for the indicated times. Following the incubation, the solutions were drawn through the membranes under vacuum and the wells were washed with PBSfTween. 20 ~L 1251- TSH was added to each well and allowed to incubate 20 hours at 20.C. The wells were emptied and rinsed as before, with the membranes subsequently punched and counted. The results show a dramatic increase in the quantity of TSH which can be harvested as the antibody immobilization time increases. This increase parallels the increase in antibody present on the membrane seen in Figure 3.

The same antibody preparation which had been used in Figure 3, a rabbit antiserum directed against human thyroid-stimulating hormone (TSH), was utilized in Figure 4 to bind radiolabelled TSH. The immobilization and washing protocols were identical. The graph therefore represents the quantity of TSH which the bound antibody is able to harvest. As can be seen by comparing Figures 3 and 4, the shape of the curves are similar, indicating the bound antibody does not lose activity during the stabilization process. This is further indicated in Figure 5, which is a plot of the number of counts per minute of TSH harvested per unit quantity of immobilized antibody.

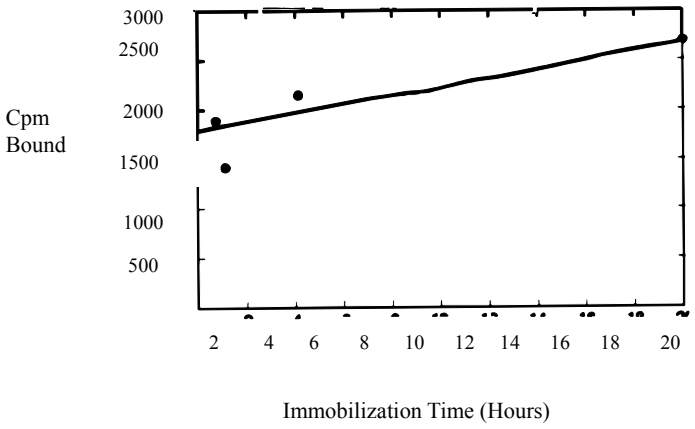

Figure 5. Biological activity of adsorbed antibody as a function of immobilization time. The data used in Figures 3 and 4 was utilized to generate a normalized plot of bound TSH per ng rabbit A'TSH. We would expect to see a loss of harvesting activity if longer immobilization times caused conformational changes in the adsorbed antibody. However, the amount of TSH harvested does not decrease as a function of immobilization time. Therefore, the stabilization process does not appear to adversely affect biological activity.

The data shows no reduction in activity with increasing immobilization time. Overnight immobilization therefore represents the preferred methodology when surfactants are used, both in terms of the total quantity of immobilized protein which remains on the membrane throughout the assay, as well as the activity of that immobilized protein.

Summary

The following guidelines should be utilized when immobilizing protein to Millipore 0.45 ~m nitrocellulose membrane-bottom plates.Utilize as small a volume as is feasible given the protein concentration of the immobilization solution.If surfactants are to be used in the wash solutions during the assay, immobilization should occur overnight «12 hours).When surfactant is not being used, shorter immobilization times are possible «4 hours).

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