

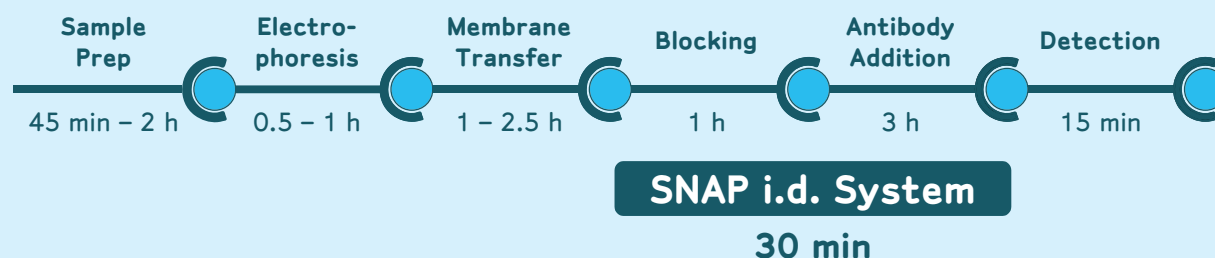
# Key Steps for Successful Immunodetection Using the SNAP i.d.<sup>™</sup> Protein Detection System

## INTRODUCTION

The western blot is a powerful tool used extensively in protein research to detect and compare the relative levels of proteins without the need for their prior purification. Its widespread appeal is based on its overall simplicity, coupled with the high resolution of proteins separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) prior to membrane transfer. This versatile technique for the detection and characterization of proteins continues to develop with approaches that now enable more rapid electrophoresis, faster protein transfer, increased miniaturization, and higher specificity. Additionally, recent enhancements in chemiluminescent detection have increased significantly both the sensitivity and flexibility of western blots. While much attention has focused on improving these aspects of western blotting, the immunodetection step, up until now, has been a lengthy process typically requiring four hours or more of elapsed time to complete.

The SNAP i.d. Protein Detection System has been developed to shorten the time required for immunodetection (i.e., blocking, washing and antibody incubations) to approximately 30 minutes. This is achieved without any additional reagent consumption (e.g., antigen, antibody or detection reagents) or any compromise in the results; sensitivity, background, and signal-to-noise ratios are the same or better than that achieved with traditional immunodetection techniques. The SNAP i.d. system's unique design enables the use of small volumes of antibody for incubations and either polyvinylidene difluoride (PVDF) or nitrocellulose blotting membranes. Because it is vacuum-driven, extremely rapid blocking and washing of blots are achieved. The SNAP i.d. system is compatible with fluorescent, chemiluminescent, or chromogenic detection methods. Moreover, the sequence of steps required to process a western blot with the SNAP i.d. system is identical to those used in traditional immunodetection (Table 1).

The SNAP i.d. system replaces time-consuming steps in the preparation of western blots.



As with any new technology, the successful implementation of the SNAP i.d. Protein Detection System requires users to optimize their protocol in order to obtain maximal performance. In most cases, neither the steps upstream of immunodetection (e.g., sample preparation, sample load, and transfer), nor those downstream of immunodetection (e.g., detection reagent choice and/or instrumentation) will need to be altered with the SNAP i.d. system. However, the design of the SNAP i.d. system requires that users optimize two key steps in their immunodetection protocol: blocking and antibody concentrations.

**Table 1. Comparison of Standard and SNAP i.d. Immunodetection Protocols**

| Step                          | Standard Method | SNAP i.d. Method   |
|-------------------------------|-----------------|--------------------|
| Blocking                      | 1 h             | 20 s               |
| Primary Antibody Incubation   | 1 h             | 10 min             |
| Washing                       | 3 x 10 min      | 3 x 20 s           |
| Secondary Antibody Incubation | 1 h             | 10 min             |
| Washing                       | 3 x 10 min      | 3 x 20 s           |
| <b>Total Time</b>             | <b>4 h</b>      | <b>&lt; 30 min</b> |

## BLOCKING

In traditional immunodetection protocols, blocking of the unoccupied sites of the blotting membrane prior to antibody incubations is typically achieved via a one-hour incubation step and gentle agitation. This is done in order to ensure sufficient time for the blocking agent to penetrate into the pores of the blotting membrane. With the SNAP i.d. system, sufficient blocking is accomplished in only few seconds and without an incubation step. This is achieved as a result of the application of vacuum, which drives the blocking of all sites within the blotting membrane nearly instantaneously.

Over the years, researchers have successfully employed a variety of agents, including bovine serum albumin (BSA), casein, gelatin, or even serum to achieve membrane blocking. By far, the most common blocking agent used today is non-fat/low fat dry milk (NFDM). NFDM is highly efficient in blocking, and because it is readily available and inexpensive, it can be used at relatively high concentrations (i.e., up to 5%). However, the high blocking efficiency of NFDM can actually compromise or reduce chemiluminescent signals when used at high concentrations (Figure 1). The SNAP i.d. system is compatible with most commonly used blocking agents as shown in Table 2. Since NFDM is the most widely used blocker, the results presented herein will focus on the optimization of blocking with NFDM. Researchers interested in optimizing other blocking reagents should follow a similar approach.

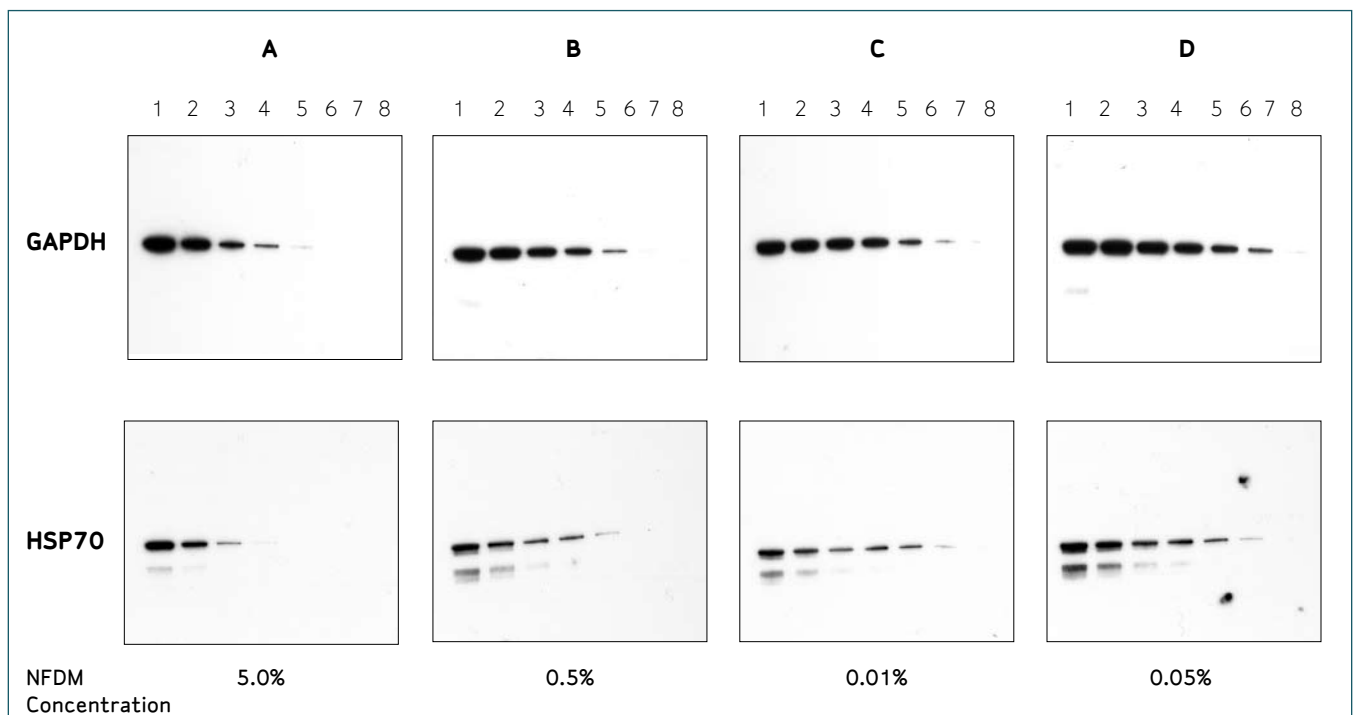
**Table 2. Compatibility with Common Blocking Agents**

| Blocker                              | Compatible     | Recommended Concentration |
|--------------------------------------|----------------|---------------------------|
| Non-fat/low fat dry milk             | yes, ≤ 0.5%    | 0.5%                      |
| Casein, N-Z-Amine AS (Sigma)         | yes, ≤ 5%      | 1%                        |
| Bovine Serum Albumin (BSA)           | yes, ≤ 5%      | 1%                        |
| PVP-40 (Polyvinylpyrrolidone)        | yes, ≤ 1%      | 1%                        |
| Immunoblot Blocking Reagent          | yes, ≤ 0.5%    | 0.5%                      |
| BLOT-QuickBlocker™ Reagent           | yes, ≤ 0.5%    | 0.5%, pre-filter          |
| ChemiBLOCKER™ Reagent                | yes            | ≤ 50%                     |
| SEA BLOCK Blocking Buffer (Pierce)   | yes            | undiluted                 |
| SuperBlock® Blocking Buffer (Pierce) | yes            | undiluted                 |
| Li-Cor® Odyssey® Blocking Buffer     | yes            | undiluted                 |
| Gelatin                              | not compatible | N/A                       |

## USE OF NON-FAT DRY MILK WITH THE SNAP i.d. SYSTEM

One of the biggest adjustments that new users of the SNAP i.d. system will need to make is to use much lower concentrations of NFDM than usual for blocking and/or antibody incubations. As shown in Figure 1, a concentration as low as 0.05% is possible with the SNAP i.d. system. At all concentrations tested (panels B – D), the SNAP i.d. blots displayed blocking that was at least as good as that obtained using 5% NFDM and the standard immunodetection method (compare background in panels B – D to panel A).

In addition, a 2- to 4-fold increase in immunodetection sensitivity was obtained when  $\leq 0.5\%$  NFDM was used with the SNAP i.d. system, as compared to standard immunodetection and 5% NFDM (Figure 1, compare panels B – D to panel A). While an increase in sensitivity may not be achievable with all antibodies, the application of vacuum to blocking with the SNAP i.d. system enables sufficient blocking with 0.5% NFDM. With proper optimization, the use of even lower concentrations of NFDM is possible as shown below. *Caution: the use of NFDM at concentrations higher than 0.5% is not recommended, as clogging of the SNAP i.d. blot holders will likely occur.*



**Figure 1. Highly efficient blocking using low concentrations of non-fat dry milk and the SNAP i.d. system.**

A 2-fold dilution series of rat liver lysate (from 12  $\mu$ g in lane 1, to 0.09  $\mu$ g in lane 8) were subjected to SDS-PAGE and transferred to Immobilon<sup>®</sup>-P blotting membranes as described under Materials and Methods. The blots in panel A were processed according to the standard immunodetection protocol while those in panels B – D were processed using the SNAP i.d. protocol (see Materials and Methods for details). The blots were processed using the indicated concentration of NFDM for both blocking and antibody incubations. Parallel blots were probed with either a mouse monoclonal antibody specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or heat shock protein 70 (HSP70) as indicated above. Primary antibodies were diluted 1:40,000 for standard immunodetection (panel A) or 1:13,000 for SNAP i.d. immunodetection (panels B – D). The goat anti-mouse horseradish peroxidase conjugated secondary antibody was diluted 1:50,000 for standard immunodetection or 1:10,000 SNAP i.d. immunodetection. The results shown are from a 1 minute exposure to x-ray film.

## ANTIBODY CONCENTRATION

Optimization of both primary and secondary antibody concentrations is another key requirement for successful immunodetection. This is true for both standard immunodetection methodologies as well as the SNAP i.d. system. Because of the wide range of antibody avidities/affinities, optimization is required whenever a new antibody is first used in an application. Only after performing multiple immunodetections can conditions be developed that will enable the routine production of publication quality western blots and ensure that the antibody response is linear over a defined, experimental range. If a blot is incubated with sub-optimal concentrations of antibody, it will result in poor/no detection of the protein(s) of interest, or non-linear signals. If, on the other hand, a blot is incubated with a too high a concentration of primary antibody, the resulting background could make it impossible to discern specific from nonspecific signals. The same challenges apply to immunodetections using the SNAP i.d. Protein Detection System; however antibody optimizations can be accomplished in a fraction of the time by virtue of the system's 30-minute processing time.

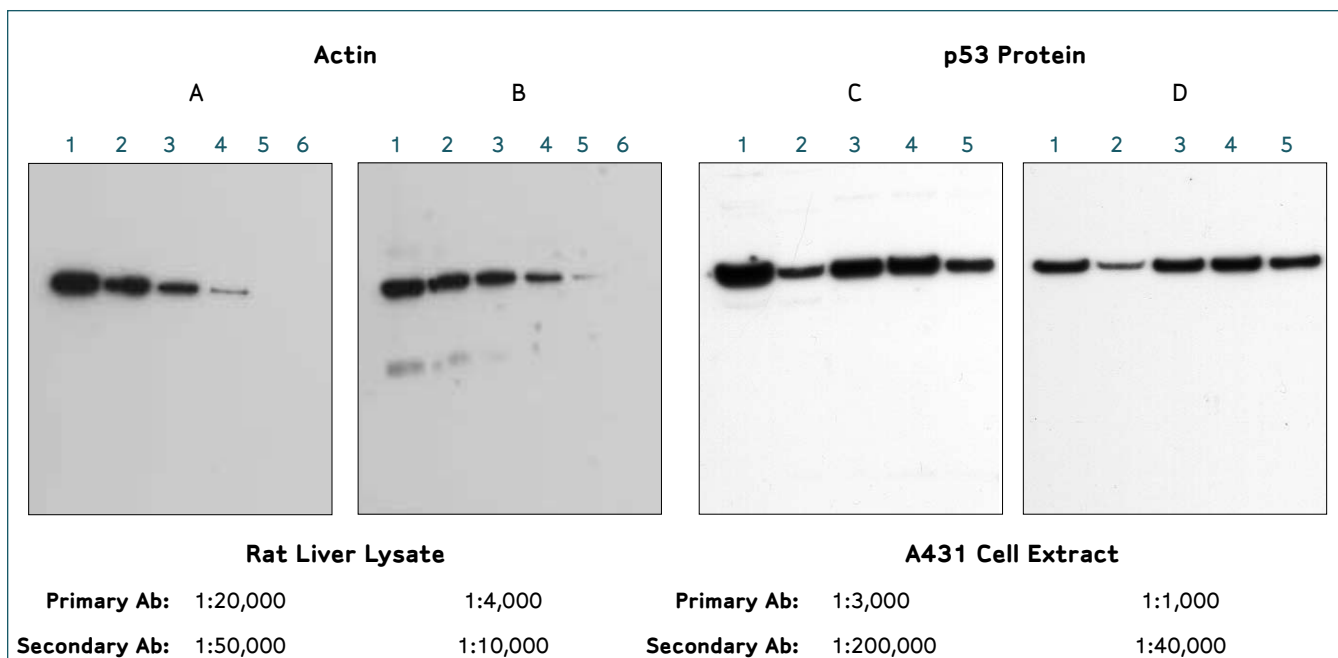
**Table 3. Volumetric Requirements of the SNAP i.d. Protein Detection System.**

|                   | Single Well | Double Well | Triple Well |
|-------------------|-------------|-------------|-------------|
| Blocking Solution | 30 mL       | 15 mL       | 10 mL       |
| Antibody          | 3.0 mL      | 1.5 mL      | 1.0 mL      |
| Wash Buffer       | 30 mL       | 15 mL       | 10 mL       |

It is recommended that all blocking and antibody solutions be prepared in standard phosphate- or Tris-buffered saline solutions supplemented with 0.1% Tween® 20 surfactant. In addition to improving blocking efficiency, the Tween 20 surfactant serves to lower the surface tension of the solutions, thereby allowing their even distribution over the surface of the blot holder.

In order to achieve this rapid immunodetection, the SNAP i.d. system has been designed so that the antibody incubations are performed in extremely low volumes (Table 3). These low volumes, coupled with the application of higher concentrations of antibodies, serve to drive the kinetics of antibody-antigen complex formation such that sufficient antibody binding is achieved in 10 minutes. For most antibodies, the antibody concentration will need to be about three to five times higher than that used in standard immunodetection protocols. However, since the volume required for antibody incubations will be reduced by one-third to one-fifth, there is no net increase in the amount of antibody required with the SNAP i.d. system.

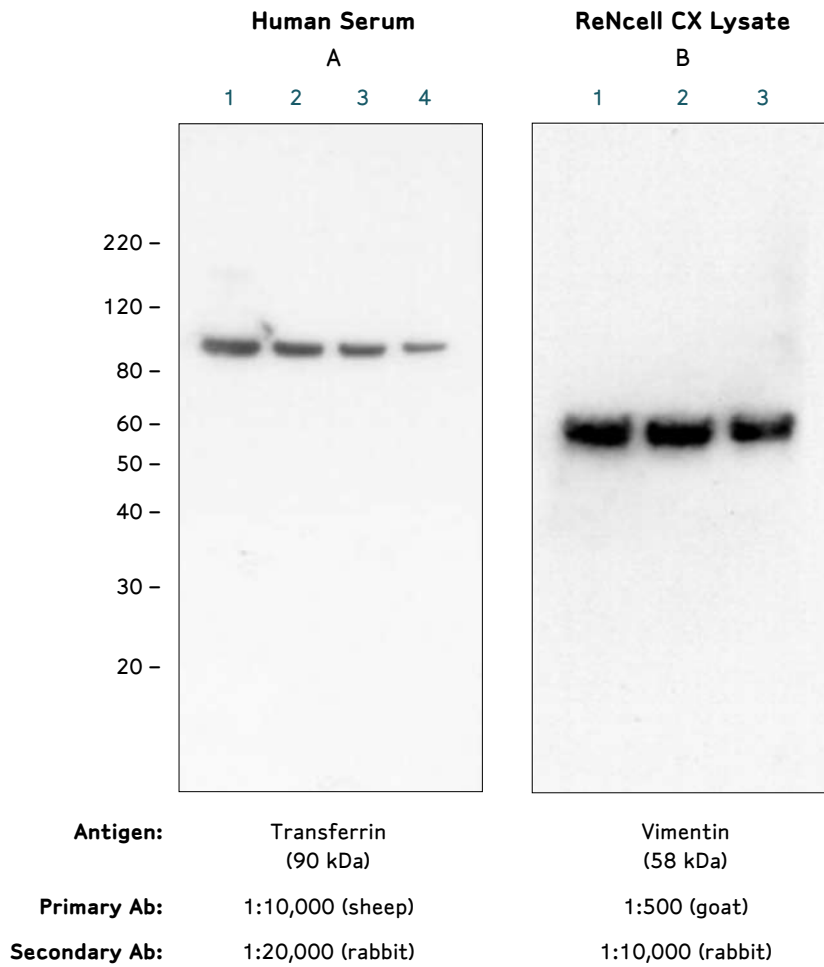
When adapting the SNAP i.d. system, both the primary and secondary antibody concentrations must be optimized. It is recommended that the starting point be an antibody concentration that is at least 3-fold higher than that used with the standard method. While some antibodies may require slightly higher concentrations of primary antibody (Figure 2, compare panel A to panel B), others have been observed to require lower concentrations in order to achieve similar immunodetection. An a priori prediction of the optimal antibody concentration for use with the SNAP i.d. system is not possible because of the variability in avidity/affinity of polyclonal and monoclonal antibodies, the variability in antigen loads and antibody concentrations currently used, and the amount of blocking NFDM (Figure 2). However, once the primary and secondary antibodies have been optimized, 30-minute immunodetections with low background and high signal-to-noise ratios can routinely be achieved (Figure 3). Any slight differences in immunodetection signal intensity can be compensated for by using longer or shorter exposure times to x-ray film.



**Figure 2. Optimization of antibodies with the SNAP i.d. Protein Detection System.**

**Panels A and B** - Blots of a 2-fold dilution series of rat liver lysates (12 – 0.38 µg protein) were prepared as described under Materials and Methods and processed for immunodetection using either the standard protocol (panel A) or the SNAP i.d. system protocol (panel B). After blocking with 0.5% NFDM (both blots), the blots were probed with a monoclonal antibody specific for the housekeeping gene, actin. In this experiment, both the primary monoclonal and secondary HRP-conjugated antibodies were used at 5-fold higher concentrations for immunodetection with the SNAP i.d. system as compared to the standard method.

**Panels C and D** - A whole-cell lysate (300 µg of protein in 500 µL) prepared from human epidermoid carcinoma (A431) cells was evaluated for binding to an immunoaffinity column specific for the tumor suppressor protein/transcription factor p53. Aliquots (16 µL) from the load (lane 1), flow through (lane 2) and 500 µL washes (lanes 3 – 5) were collected and subjected to gel electrophoresis and blotting as described under Materials and Methods. The blots were processed for immunodetection using either the standard protocol (panel C) or the SNAP i.d. system protocol (panel D). Both blots were blocked using 0.5% NFDM and then probed with an antiserum specific for p53. As a result of prior optimization experiments with this antibody, the p53 antibody was used at a 3-fold higher concentration for immunodetection with the SNAP i.d. system (as compared to the standard protocol), whereas the HRP-conjugated secondary antibody was used at a 5-fold higher concentration. The blots shown represent a 5-minute exposure to x-ray film.



**Figure 3. Immunodetection after optimization of primary and secondary antibody concentrations.**

Western blots of a 2-fold dilution series from either human serum (1:50 to 1:400, panel A) or ReNcell™ CX cell lysates (14 to 3.5 µg, panel B) were prepared as described under Materials and Methods. The blots were loaded into 2-well SNAP i.d. blot holders and subjected to immunodetection according to the SNAP i.d. protocol. The blots were blocked with 0.5% non-fat dry milk and probed with the indicated polyclonal antiserum (species shown in parentheses) diluted in TBST buffer as indicated. The human serum samples were probed with a polyclonal antiserum specific for human transferrin, while the ReNcell CX lysates were probed with a polyclonal antiserum specific for vimentin. The washed blots were then probed with the appropriate HRP-conjugated secondary antibodies. All blots were visualized with using the Immobilon Western HRP Substrate prior to exposure to x-ray film.

## CONCLUSIONS

As with any new technology, optimization is required for maximal performance. For the SNAP i.d. system, optimization of both antibody concentrations (primary and secondary antibodies) and blocking buffers is essential. The data shown in Figure 1 demonstrate that the application of a vacuum dramatically increases the efficiency of blocking, such that it can be achieved nearly instantaneously with as little as 0.05% non-fat dry milk. Furthermore, the use of higher antibody concentrations in a proportionally reduced incubation volume allows users to decrease antibody incubation times without using any more antibody. Once the SNAP i.d. system is optimized, users can expect results similar to those obtained with standard immunodetection but in a fraction of the processing time.

## MATERIALS AND METHODS

### Electrophoresis and Blotting

Solubilized protein samples were subjected to SDS-PAGE (4-12% gradient gels) using commercially prepared, 10-well, 1 mm thick minigels. After electrophoresis (typically 45 minutes at 200 V), the gels were removed from their cassette and equilibrated for 10 minutes at room temperature in transfer buffer (25 mM Tris, 192 mM glycine) supplemented with 10% methanol. The resolved proteins were transferred to Immobilon-P polyvinylidene difluoride (0.45  $\mu$ m PVDF) blotting membranes using a semi-dry transfer apparatus (BioRad) for 35 minutes at 10 V. After transfer, the blots were rinsed briefly in Milli-Q<sup>®</sup> water and allowed to either air dry for future use or assembled directly into the SNAP i.d. blot holder for immediate immunodetection.

### Immunodetection – Standard Protocol

After transfer, the blots were incubated in a small plastic tray with 10 mL of blocking solution (5.0% NFDM in TBST) for 1 hour at room temperature with gentle agitation. All antibodies were diluted in blocking solution and incubated for 1 hour each at room temperature, again with gentle agitation. Washes were performed with TBST. See Table 1 for wash times.

### Immunodetection – SNAP i.d. Protocol

Blots processed with the SNAP i.d. Protein Detection System were processed as described in the system User Guide. Briefly, after the blot holders containing the blots were placed in the SNAP i.d. system, blocking buffer (containing 0.5% NFDM unless otherwise indicated) was added and the vacuum immediately activated. Primary antibodies diluted in blocking buffer were added to the blot holders and incubated for 10 minutes at room temperature (see Table 3 for volumes). The vacuum was initiated and the blots were washed three times with TBST. After the vacuum was turned off, the blots were incubated with a horseradish peroxidase (HRP) conjugated secondary antibody diluted in blocking buffer for an additional 10 minutes at room temperature. The vacuum was activated once again and the blots washed three additional times with TBST prior to visualization of the immunoreactive protein.

### Visualization of Immunoreactive Proteins

Probed blots were incubated with 5.0 mL of Immobilon<sup>®</sup> HRP Western Substrate for 5 minutes at room temperature. After patting the blots dry, they were exposed to x-ray film for one minute and developed.

### Buffers

**TBST** – Tris-buffered saline solution (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% Tween 20 surfactant).

**Blocking Buffer** – TBST supplemented with non-fat dry milk as specified.



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