



Application Note

Fluorescent Immunodetection Using the SNAP i.d.[™] Protein Detection System

INTRODUCTION

Recent advances in fluorescent dye chemistry and blotting membranes coupled with improvements in instrumentation have accelerated the application of fluorescence detection methodologies to western blotting. Immunodetection via fluorescently-labeled primary or secondary antibodies offers researchers several advantages over conventional, enzymatically-catalyzed chemiluminescent detection. These advantages include enhanced dynamic range, a greater degree of multiplexing, and the potential for more accurate quantitation of the immunoreactive species. Despite these advances, the overall work flow for immunodetection has remained largely unchanged and has, up until now, required 4 hours or more to complete.

The SNAP i.d. Protein Detection System has been developed to substantially shorten the time required for fluorescent immunodetection through the use of vacuum. All of the immunodetection steps, starting with blocking and proceeding through antibody incubations and washing, can be completed within 30 minutes. This is achieved without any additional reagent (e.g., fluorescent antibody) consumption. Sensitivity, background, and signal-to-noise ratios are the same or better than that of traditional immunodetection techniques. The SNAP i.d. system uses small volumes for antibody incubations and is compatible with either nitrocellulose or low fluorescence background Immobilon[®]-FL polyvinylidene difluoride (PVDF) blotting membranes. The SNAP i.d. Protein Detection System works with most blocking reagents and fluorescently-labeled secondary antibodies.

OPTIMIZATION

In order to obtain maximal performance with the SNAP i.d. Protein Detection System, users must optimize their protocols. Optimization of the blocking buffer and the concentrations of antibodies used are all required.

When using non-fat dry milk (NFDM) for blocking with the SNAP i.d. system, the NFDM concentration must be adjusted to 0.5%. The use of NFDM at concentrations employed in standard immunodetection ($\geq 0.5\%$) is not recommended as clogging of the SNAP i.d. blot holders will likely occur. Although this is a much lower concentration than usual for blocking and/or antibody incubations, successful immunodetections can be achieved using NFDM at concentrations as low as 0.05%. (See SNAP i.d. Protein Detection System User Guide for a complete list of compatible blocking buffers and their recommended concentrations).

Antibodies that have been used previously with standard immunodetection will generally need to be used at higher concentrations with the SNAP i.d. system. A suggested starting point is to use a three- to five-fold higher concentration of antibody, but in one-third to one-fifth the volume used for standard immunodetection (see SNAP i.d. Protein Detection System User Guide for details). This keeps the total amount of antibody used the same as that used with most standard immunodetection protocols.

RESULTS

Figure 1 shows a comparison of standard immunodetection versus immunodetection using the SNAP i.d. system. Blots of human brain lysates were probed with a monoclonal antibody specific for the integral axonal membrane protein (i.e., growth associated protein 43) termed, GAP43 and visualized with a Cy3[®] labeled secondary antibody. The standard immunodetection protocol employed one hour incubations each for blocking, primary (1:500 dilution) and secondary (1:1,000 dilution) antibodies (panel A). The blot shown in panel B was processed according to the protocol provided with the SNAP i.d. system. Blocking was complete within 20 seconds and antibody incubations were performed for 10 minutes each. The antibodies were optimized previously for use with the SNAP i.d. system and were used at 1:100 and 1:200 dilutions for the primary and secondary antibodies, respectively. Panels C and D show quantification of GAP43 protein bands. Fluorescent band intensity (y-axis) is plotted against the lysate amount (x-axis) from the

corresponding blots in the upper panel. The blot images (panels A and B) demonstrate the sensitivity of the SNAP i.d. protocol, and the lower panels (C and D) show that the response was linear over the range evaluated, providing the basis for accurate quantification.

Fluorescence detection in a more complex model system is shown in Figure 2. Receptor-mediated signaling in response to growth factors, hormones and cytokines often result in complex patterns of tyrosine phosphorylation. The 4G10[®] anti-phosphotyrosine antibody is an important tool that is used to study tyrosine kinase activity via western blotting. The experiment shown demonstrates the applicability of the SNAP i.d. Protein Detection System to the study of tyrosine phosphorylation using the 4G10 monoclonal antibody. All blots shown were blocked with the Li-Cor[®] Odyssey[®] blocking buffer and processed using either standard immunodetection (1:2,000 dilution of primary antibody in 7.5 mL of TBST) or the SNAP i.d. system (1:400 final dilution of primary antibody in 1.5 mL of TBST). Note that although there was an increase in the

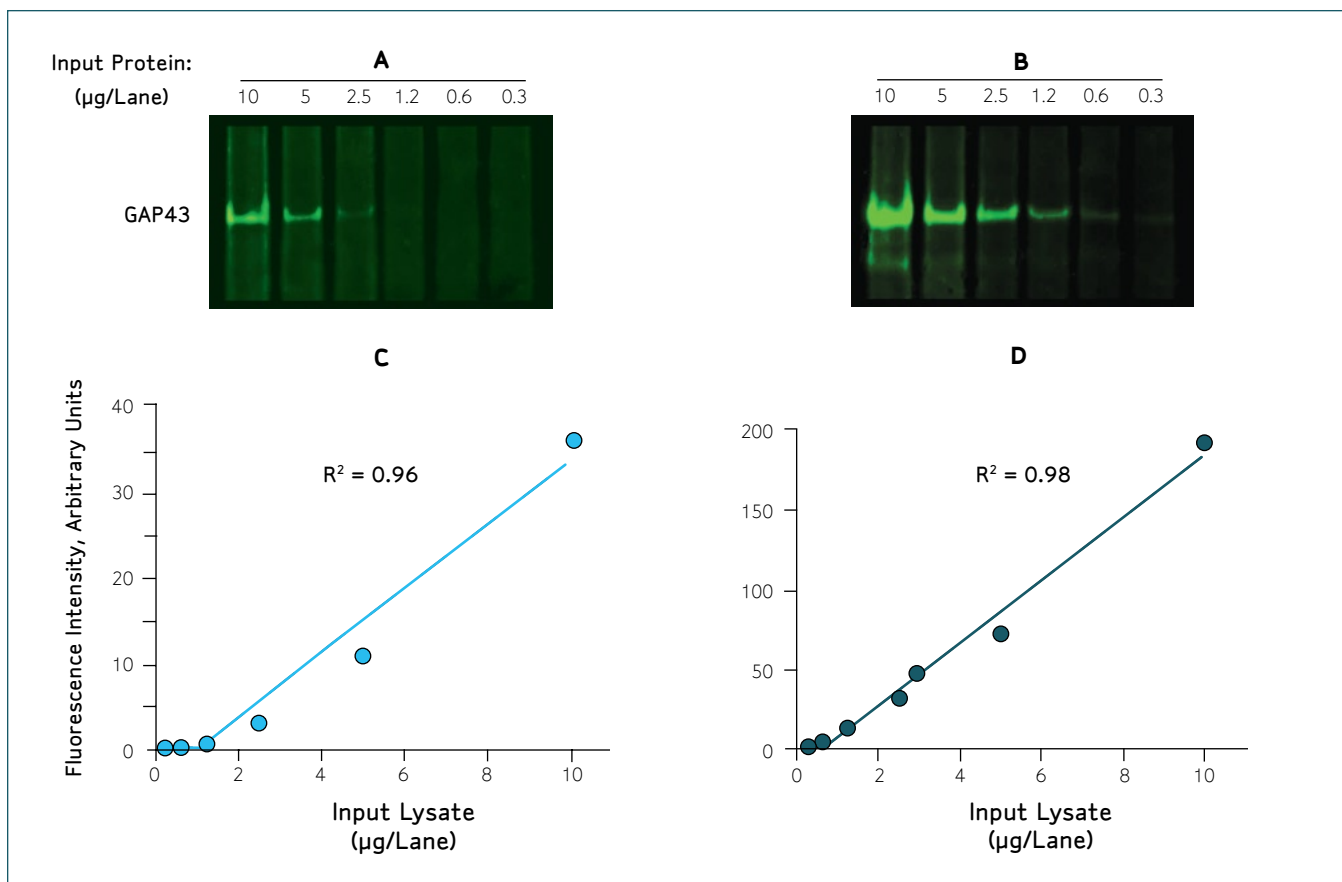


Figure 1. Cy3 fluorescent immunodetection of GAP43 in human brain lysates.

Blots of a 2-fold dilution series of human brain lysates were prepared as described under Materials and Methods. The blots were visualized simultaneously using a Fujifilm FLA-5100 imaging system. Representative images from multiple scanned blots are shown. Panel (A) shows the standard blot and panel (B) displays the SNAP i.d. blot. The graphs in panels (C) and (D) show the quantification of the immunoreactive bands from panels (A) and (B), respectively.

concentration of the primary antibody used with the SNAP i.d. system, the total amount of antibody used is the same as that for standard immunodetection (i.e., 3.75 μ g). Optimization of the concentration of the goat anti-mouse secondary antibody with the SNAP i.d. system is shown. Three different dilutions (1:250, 1:500 and 1:1,000) of the IRDye® 680 antibody were evaluated with the SNAP i.d. system and compared to standard immunodetection (1:1,250 secondary antibody dilution). This experiment shows that the SNAP i.d. system can produce fluorescent western blotting results similar to that achieved with standard immunodetection.

CONCLUSIONS

The availability of fluorescently-labeled secondary antibodies, coupled with improved instrumentation, has resulted in increased use of fluorescence for immunodetection. With the SNAP i.d. Protein Detection System, researchers interested in fluorescence can significantly decrease the

time required to process their blots without loss of signal or additional reagents. Once optimized, the SNAP i.d. system produces results similar to those obtained with standard immunodetection – but in a fraction of the 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-FL polyvinylidene difluoride (0.45 μ m PVDF) blotting membranes using a semi-dry transfer apparatus (BioRad) for 35 minutes at 10 V. After transfer, the blots were cut into three pieces and rinsed briefly in Milli-Q® water prior to assembly into the SNAP i.d. triple well blot holder.

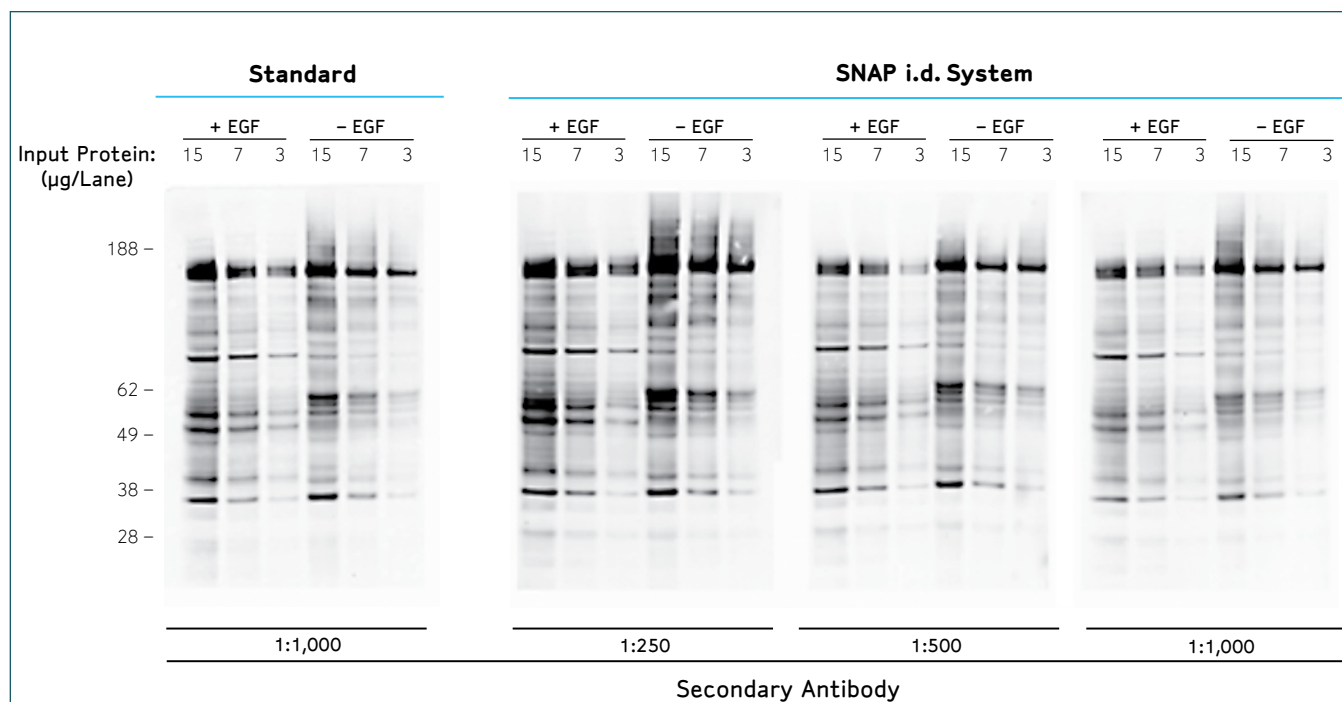


Figure 2. Immunodetection of phosphorylated proteins.

Whole-cell lysates from either control or epidermal growth factor (EGF)-stimulated A431 cells were subjected to SDS-PAGE and transferred to Immobilon-FL blotting membrane as described under Materials and Methods. The blots were processed via standard immunodetection or immunodetection with the SNAP i.d. system as shown. All blots were first probed with the 4G10 monoclonal antibody followed by an IRDye secondary antibody (IRDye 680). The blots were visualized using a Li-Cor Odyssey Infrared Imaging System. Grayscale images of the scanned blots are shown.

Immunodetection – Standard Protocol

After transfer, the blots were incubated in a small plastic tray with 7.5 mL of blocking solution (1% casein in Tris-buffered saline solution containing 0.1% Tween® 20 surfactant unless otherwise indicated) for 1 hour at room temperature with gentle agitation. Antibodies were diluted in TBST and incubated for 1 hour each at room temperature, again with gentle agitation. Washes were performed with TBST.

Immunodetection – SNAP i.d. Protocol

Blots were processed as described in the SNAP i.d. Protein Detection System User Guide. Briefly, after the blot holders containing the blots were placed in the SNAP i.d. system, blocking buffer was added and the vacuum was activated. Primary antibodies diluted in TBST were added to the blot holders and incubated for 10 minutes at room temperature. 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 fluorescently-labeled secondary antibody diluted in TBST for an additional 10 minutes at room temperature. The vacuum was activated once again and the blots washed three additional times with TBST buffer prior to visualization of the immunoreactive proteins.

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 1% casein or Li-Cor Odyssey blocking buffer.



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