Application Note

Amicon® Pro Purification System enables fast, efficient antibody biotinylation for cleaner immunodetection assays

Introduction

Many protein detection methods, including Western blotting, flow cytometry, ELISA, and immunohistochemistry, rely upon the use of target-specific probes, such as antibodies, that are detected via two-step indirect detection using chemical tags or fluorescent labels. For example, detection of unlabeled immunoprecipitates via Western blotting is routinely accomplished using a primary antibody specific to the protein of interest followed by an anti-species secondary antibody conjugated to horseradish peroxidase (HRP). However, interpretation of results from such detection schemes can be confounded by the presence of additional Western blot signals resulting from cross-reactivity of the secondary antibody with the heavy and light chains of either the endogenous immunoglobulin in total cell lysates or the immunoprecipitating immunoglobulin. Ambiguous results due to cross-reactivity can be reduced through careful experimental design and use of pre-adsorbed antibodies.

An alternative approach to indirect protein detection that circumvents the need for secondary anti-species antibodies is to use a biotin/streptavidin (SA) detection pair. Figure 1 shows how using biotin/SA pairs can improve detection of target proteins. Multiple characteristics of biotin/SA pairs are advantageous for purification or detection. First, biotin is very small (244 Daltons); its covalent attachment to proteins rarely interferes with function. The small size also allows for conjugation of multiple biotin molecules on a single antibody, which amplifies signals and increases detection sensitivity. Further, the binding between biotin and streptavidin is highly specific; it is the strongest known non-covalent interaction ($K_d = 10^{-15}$ M) between a protein and its ligand. Lastly, the complex between these two molecules forms very rapidly and is unaffected by extremes of pH, temperature, organic solvents and other denaturing agents.

The commercial availability of protein labeling kits, including kits for biotinylation, provides researchers with the flexibility to customize their own detection panels. However, because many protocols require buffer exchange prior to and following antibody labeling, the current workflow is time-consuming and subject to significant protein loss at multiple points of sample transfer. The Amicon® Pro purification system, depicted in Figure 2, is an adaptable centrifugal device coupling affinity purification with downstream sample concentration and buffer exchange.

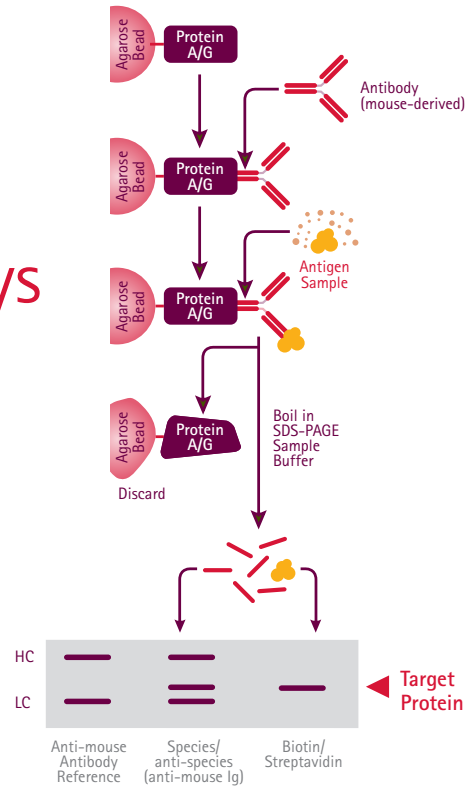
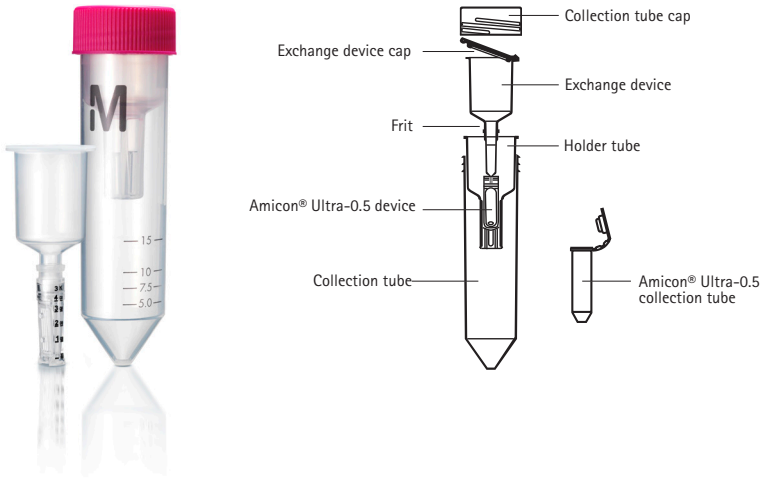


Figure 1.
Using biotin/streptavidin pairs can improve detection of target proteins by eliminating the cross-reactivity of secondary detection antibodies with the heavy and light chain bands present following traditional immunoprecipitation. The cross-reactive interference arises due to binding of the secondary detection antibody (an anti-mouse Ig, in this case) to the mouse-derived immunoprecipitating antibody.

Two attributes of the Amicon® Pro device make it a convenient device for preparing pure, labeled antibody. First, the device enables highly efficient buffer exchange via diafiltration with simultaneous sample concentration in a single 15 minute spin. Second, the entire workflow can be performed within a single device, reducing the potential for sample loss. In this report, we describe the successful use of the Amicon® Pro device for small-scale biotinylation of a target antibody. The biotinylated antibody was used for detection of protein immunoprecipitated from cell lysate.

Figure 2.

The two main components of the Amicon® Pro purification system are the large capacity exchange device and the Amicon® Ultra 0.5 mL filter. Notable features of the exchange device include its device cap, which can be labeled and used in the centrifuge instead of the collection tube cap. The unique tip design of the exchange device connects with the Amicon® Ultra 0.5 mL filter for continuous flow/buffer exchange.



Materials and Methods

Antibody biotinylation – standard protocol. Biotinylation of mouse anti-tubulin β (clone KMX-1, # MAB3408, Merck Millipore) was performed using Innolink™ Biotin 354S (Cat. No. 203119), according to manufacturer's instructions. Briefly, initial buffer exchange was performed on 50 µg mouse anti-β tubulin antibody (Cat. No. MAB3408) using a centrifugal diafiltration device. Three 10-minute spins were required. The sample was transferred to a new 1.5 mL tube, 0.2 mg/mL reconstituted Innolink™ Biotin 354S was added, and the mixture was incubated at room temperature for 2 hours. The reaction was transferred to a fresh diafiltration apparatus and the buffer was exchanged using three spins with 0.5 mL phosphate-buffered saline (PBS) plus sodium azide for each spin. Antibody recovery was determined by measuring absorbance at 280 nm.

Antibody biotinylation using the Amicon® Pro purification system. The protocol for biotinylation of mouse anti-β tubulin using Innolink™ Biotin 354S was modified for application to the Amicon® Pro purification system. (Assay parameters can vary greatly and should be optimized for each reaction.) Briefly, the Amicon® Pro device was assembled with the Amicon® Ultra 0.5 mL filter (10K NMWL) attached. 50 µg anti-β tubulin antibody was mixed with 1.5 mL 0.1 M PBS containing 0.2 mg/mL Innolink™ Biotin 354S, applied to the Amicon® Pro system and centrifuged at 4000 g for 15 min. The labeling reaction, now inside the Amicon® Ultra 0.5 mL device, was incubated at room temperature for an additional 30 min. Next, 1.5 mL PBS containing sodium azide was added to the exchange tube of the Amicon® Pro system. The unbound biotin was cleared and biotinylated antibody buffer exchanged by centrifugation at 4000 g for 15 min. The concentrated, labeled antibody was recovered from the Amicon® Ultra 0.5 mL device by reverse spin. Antibody recovery was determined by measuring absorbance at 280 nm.

Note: To ensure chemical compatibility with the Amicon® Ultra 0.5 mL device, which tolerates less than 10% (v/v) dimethylformamide (DMF), Biotin 354S was reconstituted at 5 mg/mL in DMF (this is 10X normal concentration). For the labeling reaction, Biotin 354S was added to 4% (v/v).

Immunoprecipitation of native tubulin β. For each reaction, 0.5 mg EGF-stimulated A431 cell lysate (Cat. No. 12-110) was combined with 10 µg anti-β tubulin antibody and incubated at 4 °C overnight with end-over-end mixing. Next, half of this reaction was transferred to the exchange tube of an Amicon® Pro device containing 50 µL packed volume of pre-washed Protein A agarose (Cat. No. 16-125) and incubated for 1 hour with gentle agitation. 1.5 mL of PBS was added and the filtrate was cleared by centrifugation at 1000 g for 1 min. 200 µL of sodium dodecyl sulfate polyacrylamide gel electorphoresis (SDS-PAGE) loading buffer was added to the resin, resuspended by pipetting and then transferred to a microfuge tube. Following incubation at 95 °C for 5 min, the beads were returned to the Amicon® Pro device and centrifuged at 1000 g for 1 min to collect the filtrate.

Electrophoresis and Blotting. Immunoprecipitated samples (10 µL of the filtrate above) and control lysate (4 µg in 1X SDS-PAGE loading buffer) were denatured at 70 °C for 10 min and loaded onto 1 mm thick 4-12% gradient NuPAGE® Bis-Tris gels (Life Technologies). Gels were run at 200 V for 40 min. Gels were removed from the cassette and equilibrated for 10 min in transfer buffer (25 mM Tris, 192 mM glycine) supplemented with 10% methanol. After equilibration, the proteins were transferred to Immobilon®-P PVDF membrane (0.45 µm PVDF, Cat. No. IPFL00010) using a semidry transfer system (Bio-Rad) for 35 min at 10 V. Blots were briefly rinsed in Milli-Q® water and assembled directly into the SNAP i.d.® 2.0 blot holder for immunodetection.

Immunodetection using the SNAP i.d.® 2.0 system. Each blot processed in the SNAP i.d.® 2.0 system, (Cat. No. SNAP2MM) was assembled according to the user guide. Briefly, once blot holders were placed in the SNAP i.d.® 2.0 system, Bløk™-CH blocking buffer (Cat. No. WBAVDCH01) was added and the vacuum immediately activated. Primary antibody (mouse anti-β tubulin or biotinylated anti-β tubulin, both diluted to 1 µg/mL in blocking buffer) was added to the blot holder and incubated for 10 min at room temperature. The vacuum was initiated and blots washed three times with tris-buffered saline with Tween® 20 (TBST) surfactant. After the vacuum was turned off, the blots were incubated with HRP-conjugated secondary antibody (1 µg/mL goat anti-mouse IgG-HRP (Cat. No. AP124) or 0.1 µg/mL SA-HRP (Cat. No. 18-152)) diluted in blocking buffer for an additional 10 min. The vacuum was activated and blots washed three times with TBST. Probed blots were visualized with 5 mL Luminata™ Classico Western HRP Substrate (Cat. No. WBLUC0500) for 5 minutes. Blots were patted dry, exposed to X-ray film for 1 min, and developed.

	Traditional 0.5 mL centrifugal diafiltration device	Amicon® Pro Purification System
Sample clean-up	45 min	15 min
Biotinylation reaction	120 min	30 min
Remove free biotin, buffer exchange, concentrate	45 min	15 min
Total protocol time	**3.5 hours**	**1 hour**
Antibody recovery	**40%**	**72%**

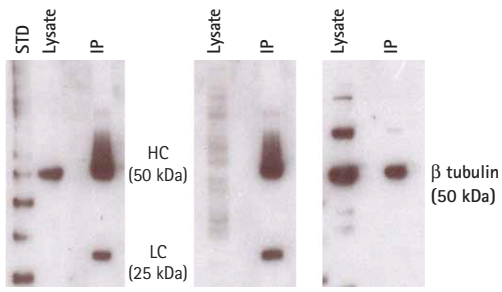
Table 1.
Comparison of biotinylation workflows with respect to time and antibody recovery.

Results

The biotin-streptavidin interaction is commonly exploited for detection because of the highly specific binding between these two molecules. However, in cases where an antibody must first be biotinylated before being used, the tedious and inefficient methods currently available for small-scale labeling (≤ 50 µg) present a major obstacle. Out of the four steps typically involved in antibody labeling (initial antibody cleanup, labeling, removal of unbound label/buffer exchange, and concentration of labeled protein), the two buffer exchange steps are the most time-consuming and risk the greatest sample loss.

We compared the overall processing time and recovery efficiency of the Amicon® Pro system to that of a standard protocol provided with the Innolink™ Biotin 354S reagent (Table 1). Biotinylation using the Amicon® Pro system reduced processing time by 70%. This difference would be even more significant if traditional tube or cassette-based dialysis methods were employed, which typically involve overnight incubations.

The improved recovery is most likely a reflection of sample containment within a single device for the entire process. Higher recovery using the Amicon® Pro system may also be a result of the gentler method of buffer exchange. The smaller diafiltration device (0.5 mL) used with the traditional biotinylation protocol required multiple concentration/dilution cycles to achieve complete buffer exchange. Such cycles may cause either physical protein loss due to aggregation and precipitation, or loss of protein function due to destabilization of the tertiary structure.



Used for Western blotting:			
Primary antibody	Unlabeled anti-β tubulin	No antibody	Biotinylated anti-β tubulin
Secondary antibody or molecule	Anti-mouse-HRP	Anti-mouse-HRP	SA-HRP

Figure 4.
Biotin/SA detection pairs permit visualization of the β tubulin signal in Western blots of immunoprecipitated (IP) fractions. Total A431 cell lysate and IP fractions were resolved by SDS-PAGE, transferred to membrane, and probed for the presence of β tubulin. Given that the same antibody was used for both IP and primary detection in immunoblotting, cross-reactivity of the secondary antibody resulted in appearance of bands at 25 and 50 kDa. Of greater concern was that the signal from the heavy chain masked signal expected from β tubulin, a protein of similar molecular weight.

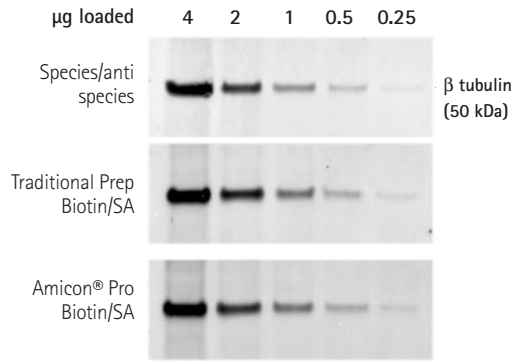


Figure 3.
Both preparations of biotinylated anti-β tubulin successfully detect β tubulin in Western blots. Aliquots of EGF-stimulated A431 cell lysate (0.25–4 µg) were resolved by SDS-PAGE, transferred onto Immobilon®-P membrane, and probed for β tubulin in the SNAP i.d.® 2.0 system using the various indirect detection pairs. In each case, the same quantity of primary (β tubulin-specific) antibody was used.

To assess the functional performance of the newly biotinylated antibodies obtained using the two protocols described, we compared their detection ability with that of a species/anti-species β tubulin detection pair (Figure 3). Using similar amounts of primary antibody for each blot, both biotin/SA pairs demonstrated the same, if not slightly better sensitivity, than the species/anti-species detection pair.

When species/anti-species antibody pairs are used, interpretation of Western blotting results is often confounded by cross-reactivity of the secondary antibody with immunoglobulin heavy (50 kDa) and light (25 kDa) chains present in either the total lysate or immunoprecipitated fractions. The signals that arise from this cross-reactivity are particularly problematic when the protein(s) of interest are similar in molecular weight to the immunoglobulin chains. To demonstrate the advantage of employing a biotin/SA pair (compared to a species/anti-species pair), we assessed the ability to detect β tubulin following immunoprecipitation (Figure 4). In blots probed with the species/anti-species pair, two prominent bands were present in the IP fraction (leftmost blot); these correspond to binding of the secondary antibody to the light and heavy chains of the anti-tubulin antibody originally used to capture tubulin from the lysate. This result was confirmed when an identical blot was probed with secondary antibody alone (middle blot). In each case, the 50 kDa heavy chain masked the signal expected from the similarly sized β tubulin protein. By contrast, a single, prominent band was detected in the immunoprecipitated fractions probed with the biotin/SA pair.

Conclusion

We have demonstrated the distinct advantages of using the Amicon® Pro purification system for small-scale antibody labeling. Specifically, this centrifugal device offers a comparatively streamlined workflow, providing significant time-savings when compared to functionally equivalent platforms. In addition to time saved, using the Amicon® Pro purification system also led to reduced sample loss, a potentially important consideration given the cost of commercial antibodies. Moreover, we confirmed the importance of using biotin/SA pairs whenever possible to eliminate immunoglobulin cross-reactivity. With the flexibility of the Amicon® Pro purification system, small amounts of antibody can be labeled quickly and easily with reliable performance and superior yield.

Featured Products

Amicon® Pro Ordering Information

To choose the appropriate Amicon® Pro device, determine the molecular weight cut-off (MWCO) of your protein of interest and your desired affinity purification scheme.

Amicon® Pro Purification Kits, 12/pk Includes reagent kit (resin and buffers)	Reagent Kit Only	MWCO				
		3,000	10,000	30,000	50,000	100,000
Amicon® Pro Affinity Concentration Kit **Ni-NTA**	ACR5000NT	ACK5003NT	ACK5010NT	ACK5030NT	ACK5050NT	ACK5100NT
Amicon® Pro Affinity Concentration Kit **Protein A**	ACR5000PA	ACK5003PA	ACK5010PA	ACK5030PA	ACK5050PA	ACK5100PA
Amicon® Pro Affinity Concentration Kit **Protein G**	ACR5000PG	ACK5003PG	ACK5010PG	ACK5030PG	ACK5050PG	ACK5100PG
Amicon® Pro Affinity Concentration Kit **GST**	ACR5000GS	ACK5003GS	ACK5010GS	ACK5030GS	ACK5050GS	ACK5100GS

Amicon® Pro Purification System – No Reagents Included	MWCO				
	3,000	10,000	30,000	50,000	100,000
Amicon® Pro Purification System Trial Pack, 2/pk	ACS500302	ACS501002	ACS503002	ACS505002	ACS510002
Amicon® Pro Purification System, 12/pk	ACS500312	ACS501012	ACS503012	ACS505012	ACS510012
Amicon® Pro Purification System, 24/pk	ACS500324	ACS501024	ACS503024	ACS505024	ACS510024

Other Featured Products

Description	Catalogue No.
Innolink™ Biotin 354S	203119
Mouse anti-β tubulin antibody	MAB3408
EGF-stimulated A431 cell lysate	12-110
Protein A agarose	16-125
Immobilon®-P PVDF membrane	IPFL00010
SNAP i.d.® 2.0 System	SNAP2MM
Bløk™-CH Noise-canceling Reagent	WBAVDCH01
Goat anti-mouse IgG-HRP	AP124
SA-HRP	18-152
Luminata™ Classico Western HRP Substrate	WBLUC0500

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