High-capacity system for rapid purification of antibodies using Protein A and Protein G membranes

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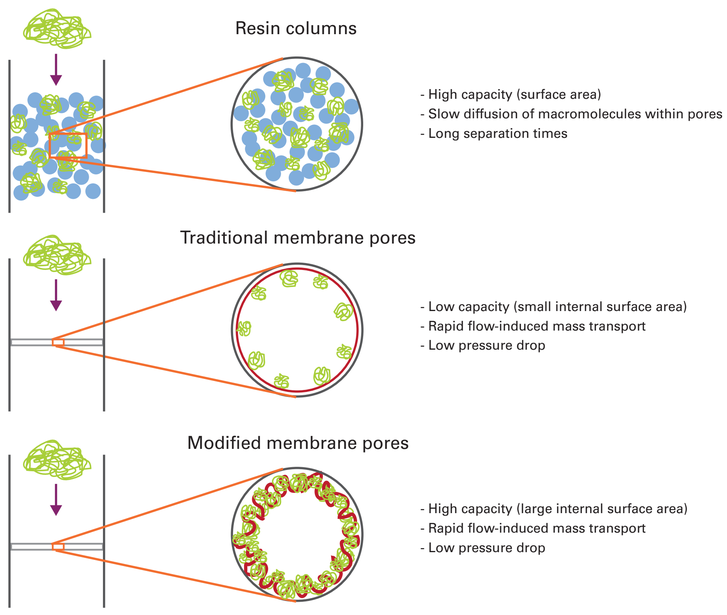
Abstract

Antibody engineering, production, and purification are critical in a wide range of research settings such as academic research institutions and biopharmaceutical organizations. There is a constant need for better, faster, and more efficient processes for antibody purification at various scales. Protein A has historically been one of the most widely used methods for affinity purification of immunoglobulins (IgG), and allows the opportunity for several-fold enrichment in fewer steps along with high recovery rates. Agarose resins with immobilized Protein A are typically used for this process, with capacities ranging from 18 to 35 mg/ml. Resin-based purification requires a significant amount of work and may take up to a few hours to complete due to long column equilibration/binding times and slow diffusion of large macromolecules through the resin bed. These longer times, in turn, increase the possibility of antibody aggregation or degradation, or loss of activity due to unfolding or denaturation. Membrane-based affinity systems have rapid flow-induced mass transport, and short residence times; however, traditionally they have been plagued with low capacity due to small internal surface areas. Here we describe a novel, membrane-based system with Protein A or Protein G affinity chemistry in which the pore surface area has been chemically enhanced, leading to a protein binding capacity better than that of resins at 75 mg or more per cm^3 of membrane. However, unlike traditional resin-based systems, the entire purification process—from loading the sample to eluting pure antibody—can be completed at room temperature in less than five minutes. We have assembled these high-capacity membranes into spin columns and filtration devices such as 96-well plates, and demonstrate that they can purify antibodies from a variety of samples such as animal sera, cell culture supernatants, etc. We further characterize the binding properties of these Protein A membranes, and demonstrate their utility in immunoprecipitation (IP) and co-immunoprecipitation (Co-IP) experiments. We have compared our Protein A and Protein G membranes with commercially available resins, and show that Capturem membranes result in more concentrated antibodies in significantly less time. These novel membrane-based affinity columns are extremely useful for purification and characterization of various antibody isotypes for a variety of applications.

Capturem™ membrane technology overview



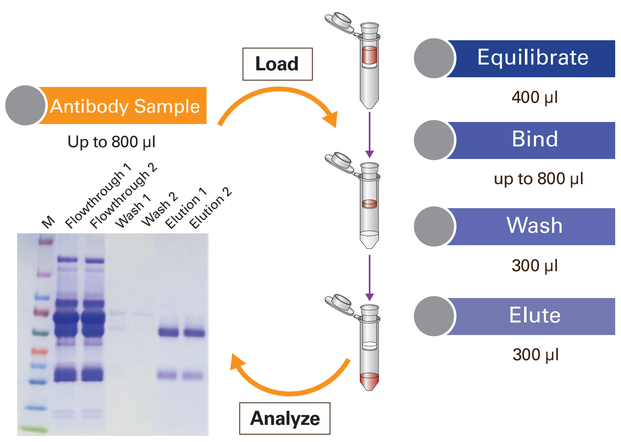
Differences between column-based separation and membranes. Traditional membrane pores can only hold so much protein due to available surface area. However, our proprietary modified membranes have much higher surface area, leading to much higher protein binding capacity compared to unmodified membranes.

Materials and methods

Miniprep spin columns were first equilibrated by addition of 400 µl of binding buffer to the column, followed by centrifugation at 1,000*g* for 1 min. Equilibrated spin columns were then loaded with 800 µl of serum diluted in binding buffer, and centrifuged at 1,000*g* for 1 minute at room temperature. The flowthrough was reloaded on the column and centrifuged at 1,000*g* for 1 min. The bound antibodies were then washed with 300 µl of binding buffer, followed by centrifugation. Elution was performed by addition of 300 µl of elution buffer, followed by centrifugation. The eluate was neutralized by adding neutralization buffer in the collection vessel. Experimental conditions for maxiprep columns are described in the corresponding figure caption (Figure 5).

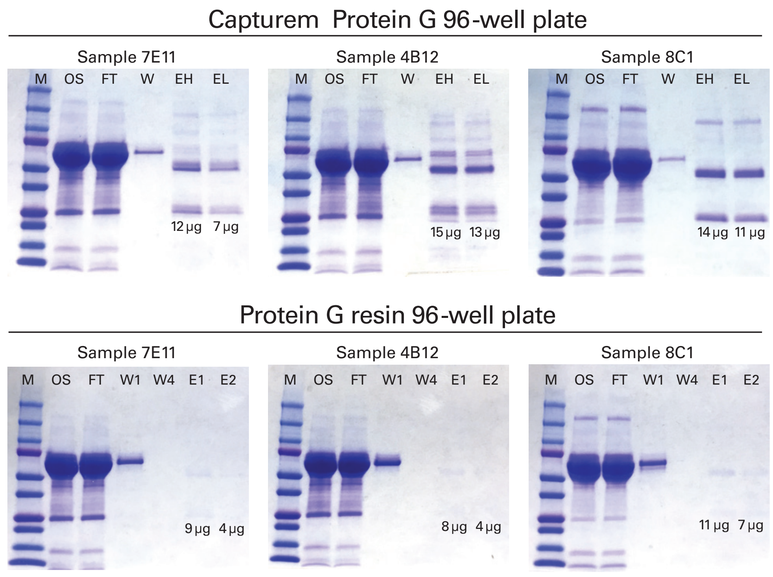
The eluted fraction was analyzed by electrophoresis on a 4–20% SDS polyacrylamide gel to verify the purity of the immunoglobulins. The absorbance of the sample was measured at 280 nm and used to calculate the concentration and yield.

1 Fast, high-capacity membrane-based antibody purification



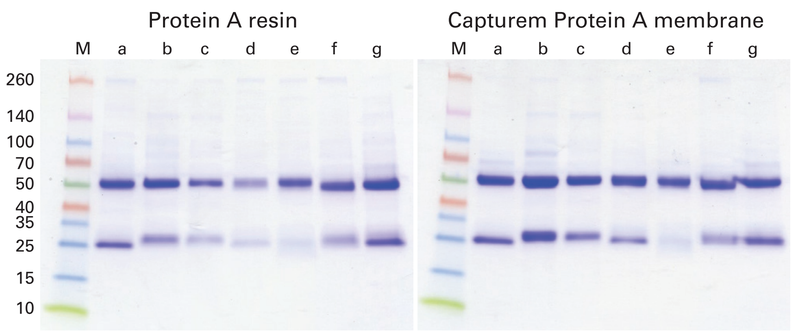
Capturem Protein A Miniprep workflow for antibody purification. Each mini spin column can be loaded with up to 800 µl of a diluted sample (antibody sample diluted from 1:1 to 1:20 with buffer). The same diluted sample was purified in duplicate. Antibodies are first bound to the membrane, followed by washing with 300 µl of wash buffer, and elution with 300 µl of elution buffer. Over 80% of the antibody can be eluted with as little as 100 µl of elution buffer. The gel shows upper and lower bands, which are the heavy and light chains, respectively. Each step is followed by spinning the tube for 1 min at 1,000*g*. The working bed volume of the membrane is <2 µl. This entire purification is complete in ~5 min.

2 Animal sera antibody binding test



Gels demonstrating the purification of monoclonal antibody from dilute hybridoma supernatants using either Capturem Protein G 96-well plates (top) or a commercially available Protein G resin 96-well plate (bottom). The Capturem plate had a significantly faster workflow (15 min vs. 2 hr) and a more concentrated eluate due to the low elution volume (50 µl vs. 200 µl for each resin elution). OS = original sample; FT = flowthrough; W = wash; EH = elution, well with the highest yield; EL = elution, well with the lowest yield; W1 = wash 1; W4 = wash 4; E1 = elution 1; E2 = elution 2.

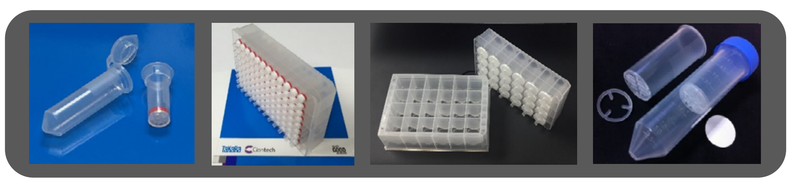
3 Comparison with resins



Amount in elution samples (µg)		
Sample	**Protein A resin**	**Capturem Protein A membrane**
a) Mouse	90	122
b) Sheep	94	207
c) Goat	55	104
d) Rat	42	191
e) Rabbit	70	94
f) Horse	80	251
g) Human	114	180

Capturem Protein A mini spin columns were compared against a Protein A resin. Sera from the following species were tested: mouse, sheep, goat, rat, rabbit, horse, and human. For the resin (Thermo Fisher Scientific), 20 µl of slurry was used for each purification, following the vendor's protocol for antibody purification. Storage buffer was first removed, followed by equilibration of the beads with Protein A binding buffer (Thermo Fisher Scientific; 600 µl x 2). The equilibration buffer was then removed and the beads were incubated with diluted serum samples (1 ml of serum in 3 ml of buffer, 1.2 ml of diluted sample) for 4 min with end-over-end rotation. Following loading, the resin was washed twice with 600 µl of Protein A binding buffer, drained, and eluted with 600 µl of elution buffer plus 60 µl of neutralization buffer in a collection tube. Purification steps for the Capturem samples were as listed in the Materials and methods section. Serum samples were the same concentration and dilution as those used for the Protein A resins. Purities of the final elution samples from both sets were analyzed by gel electrophoresis and stained with Coomassie blue. Upper and lower bands are heavy and light chains, respectively. The amount of antibody eluted was determined by measuring absorbance at 280 nm.

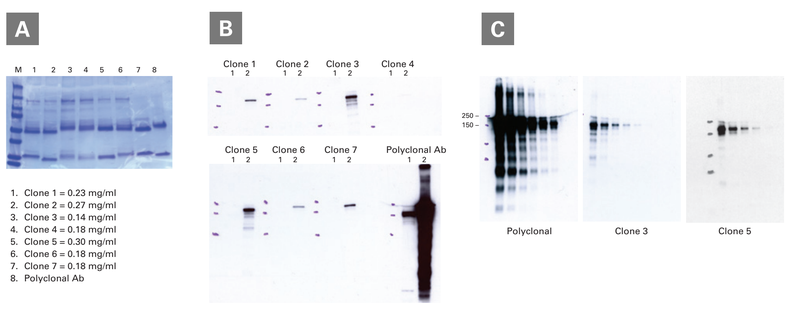
4 Volume and capacity for different Capturem column formats



	Miniprep	96-well plate	24-well plate	Maxiprep
Membrane bed volume	~3.4 ± 0.2 µl	~3.4 ± 0.2 µl	~34 ± 1 µl	~68 ± 2 µl
Sample volume	~800 µl	~1 ml	~4.5 ml	~25 ml
Purification time	5 min	15 min	15 min	15 min
Typical yield for Protein A and Protein G membranes	~50–100 µg	~50–100 µg	~0.5–1 mg	~1–2 mg

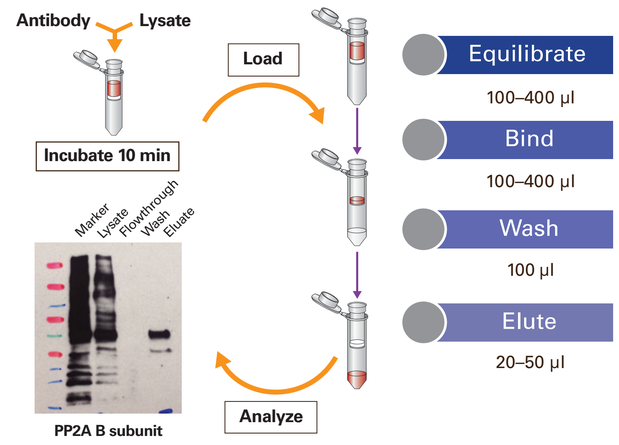
Sample volume and capacity (dependent on antibody isotype) for different column formats.

5 Cas9 antibody screening



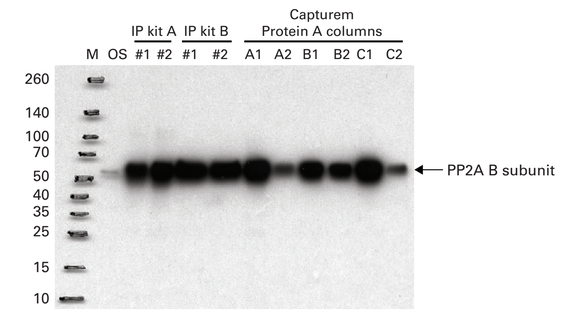
Screening for the best monoclonal antibody against Cas9 from hybridoma supernatants. All clones were the mouse IgG1 isotype, and thus purified using the high-salt method as detailed below. Capturem Protein A maxi columns were equilibrated with 6 ml of buffer (10 mM sodium borate, pH 8.9, 3 M NaCl), and centrifuged at 2,000*g* for 3 min. The NaCl concentration of the crude antibody supernatant was adjusted to 3.3 M, and to this, 1/10 of its volume of 1.0 M sodium borate (pH 8.9) was added. The supernatant (20 ml) was then loaded on a Capturem Protein A maxi column, followed by centrifugation at 2,000*g* for 3 min. The loading process was repeated with the flowthrough to maximize antibody binding. The column was then washed with 10 ml of wash buffer (3 M NaCl, 10 mM sodium borate, pH 8.9), centrifuged, and eluted with 0.5 ml of elution buffer (100 mM glycine, pH 3.0). The eluate was neutralized by adding 50 µl of 1 M tris, pH 8.5, into the collection tube. Elution was done in a low volume in order to yield a concentrated antibody. **Panel A.** Antibodies purified from cell culture supernatants using Capturem columns were resolved on an SDS-PAGE gel. Upper and lower bands are heavy and light chains, respectively. **Panel B.** Cell lysates expressing low (lane #1) or high (lane #2) amounts of Cas9 were resolved by gel electrophoresis and transferred to nitrocellulose membranes. The membranes were blotted using the antibodies purified with the maxi columns. **Panel C.** Cas9 protein dilutions (from left to right: 20, 10, 5, 2.5, 1.25, and 0.625 ng), blotted with the selected antibodies.

6 Protein A column IP



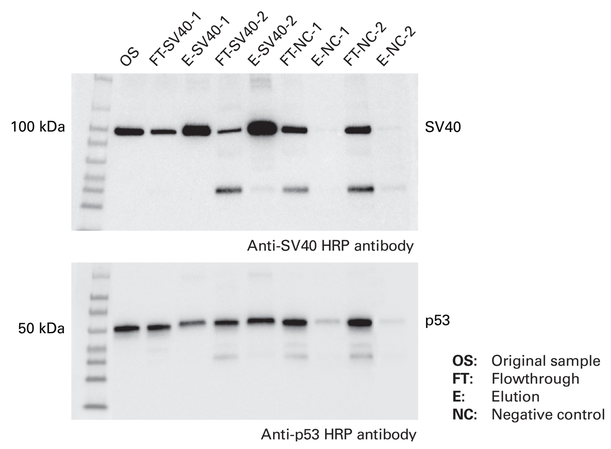
IP performed with Capturem Protein A columns. NIH3T3 cell lysates were incubated with 1 µg of protein phosphatase type 2A (PP2A) B subunit antibody for 10 min at room temperature with end-over-end rotation. Mini spin columns containing membranes with immobilized Protein A were equilibrated with 400 µl of equilibration/loading buffer (1.0 M glycine, 2 M NaCl, pH 9.0), and centrifuged at 1,000*g* for 1 min. The antibody-lysate complex was then diluted to 400 µl in lysis buffer, and applied to the spin columns, followed by centrifugation at 30*g* for 4 min. The mini spin columns were then washed with 100 µl of wash buffer (PBS) and centrifuged at 1,000*g* for 1 min. Elution was carried out in 100 µl of elution buffer (0.1 M glycine, pH 2.5, with 10 µl 1 M tris, pH 8.5) in the collection tube, centrifuged at 1,000*g* for 1 min. The various fractions were then resolved by gel electrophoresis, transferred onto PVDF membranes, and probed with an anti-PP2A antibody. The protein band in the eluate fraction corresponds to the PP2A B subunit.

7 IP compatibility



Capturem Protein A columns are compatible with a variety of buffers. NIH3T3 cell lysates (100 µl, containing 130 µg of total protein) were incubated with 0.6 µg of protein phosphatase type 2A (PP2A) B subunit antibody in the IP buffers provided with either Active Motif (A) or Thermo Fisher Scientific (B) IP kits, or the Promega lysis buffer (C), bringing the total volume up to 400 µl. The antibody-lysate mixture was allowed to incubate at 4°C for 1 hr, and was then loaded on Capturem Protein A columns which had been equilibrated with 100 µl of the corresponding IP buffer. The columns were centrifuged at 1,000*g* for 1 min, washed with 100 µl of wash buffer, and centrifuged at 1,000*g* for 1 min. The bound immunocomplexes were then eluted with 30 µl of either low pH buffer (0.1 M glycine, pH 2.5) (A and C) or Thermo Fisher Scientific low pH elution buffer (B). The elution process was repeated a second time to ensure complete elution of antibody complexes. IP experiments were also done using the Active Motif and Thermo Fisher Scientific IP kits following their respective manufacturers' protocols, starting with the same amount of lysate and antibody as used for Capturem columns. All elution samples were then resolved on a gel, transferred to PVDF membranes, and probed for the PP2A B subunit using an appropriate antibody. The gel shows the presence of the PP2A B subunit in the original sample (OS), which is greatly enriched in the immunoprecipitated samples. Elution samples #1 and #2 are shown for those performed entirely with the IP Active Motif kit (IP Kit A) and the IP Thermo Fisher Scientific kit (IP Kit B). Elution samples #1 and #2 for Capturem Protein A columns are shown for those performed with buffers from the Active Motif kit (A1, A2), the Thermo Fisher Scientific kit (B1, B2), and the Promega lysis buffer (C1, C2). Elution from Capturem columns with the low pH glycine buffer allows elution of most of the immunocomplex in the first elution.

8 Fast Co-IP of p53 and SV40 T antigen with Capturem Protein A



Co-IP of p53 and SV40 T antigen from 293T cells. p53 is a 53-kDa nuclear phosphoprotein that functions as a tumor suppressor and is involved in inhibiting cell proliferation upon DNA damage. Wild-type p53 is known to form specific complexes with several viral oncogenes such as SV40 T antigen (SV40 T). Using 293T cells expressing both p53 and SV40 T, we demonstrate the ability to co-immunoprecipitate the p53 and SV40 T at basal levels. To 293 T cell lysates (100 µg), 1 µg of anti-SV40 T antibody (rabbit polyclonal, V-300, SCBT) was added, and the mixture was incubated at room temperature for 20 min with end-over-end rotation. As a negative control, the lysate was incubated without the anti-SV40 T antibody. The IP was then carried out in duplicate, using the procedure detailed in Figure 6. The eluted sample was resolved on an Any kD Mini-PROTEAN TGX Precast Gel (Bio-Rad) and transferred to a PVDF membrane. The blots were then probed with mouse monoclonal antibodies against SV40 T (sc-147, SCBT), stripped, and then also probed with mouse monoclonal antibodies against p53 (sc-126 HRP, SCBT). IP of SV40 T shows the presence of bands for both SV40 T (97 kD) and p53 (53 kD).

Conclusions

Capturem Protein A and Protein G membranes are unique solutions for antibody purification, consisting of spinnable affinity columns and filtration devices containing novel, modified nylon membranes. The benefits of these systems make it a powerful tool for a wide range of research settings.

- **No-waiting workflow**—The protocol is complete in <5 min for the miniprep and 15 min for the maxiprep, from loading the antibody to elution, thus saving the antibody from possible degradation and/or loss of activity
- **High purity and yield**—Typical elutions of 50–100 µg antibody/column, with a concentration of ~0.1–1 mg/ml for the mini spin columns
- **Appropriate for different samples**—The purification protocol works for antibodies expressed in mammalian cells and whole serum
- **Fast immunoprecipitation**—Capturem Protein A enables IP in less than 5 minutes of hands-on time starting from antibody-antigen complex

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