Rapid purification and characterization of recombinant proteins and antibodies: Capturem high-capacity membranes

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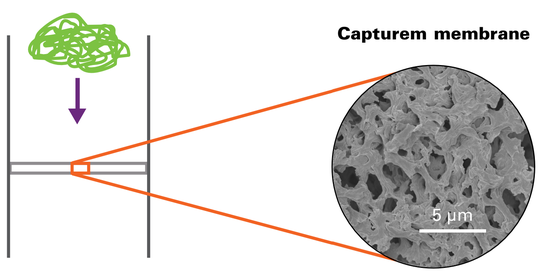
Abstract

Recombinant protein production is immensely important in many research settings, including academic research institutions, biopharmaceutical organizations, and enzyme and agricultural industries. Fusion tags are widely used to improve yields and enable purification and characterization of protein structure and function. Polyhistidine tags, which incorporate 6–10 histidines at either terminus of the target proteins, are the most popular tag used for purification. The affinity of the histidines to immobilized metal ions such as Co^{2+} and Ni^{2+} is utilized to selectively bind the tagged construct to the matrix, while washing away unwanted materials, before eluting the target with low-pH or imidazole-containing buffers. Typical purification methods using immobilized metal affinity chromatography (IMAC) columns take several hours to complete due to long column equilibration/binding times and slow diffusion of large macromolecules through the resin bed. The long times increase the risk of proteolytic degradation and activity loss due to unfolding or denaturation. Membrane-based affinity systems have rapid flow-induced mass transport and short residence times; however, they have been plagued with low capacity due to small internal surface areas. Here, we describe a novel, nylon-membrane-based IMAC system with chemically enhanced surface areas of the pores that allow protein binding capacities comparable to, or better than, resins at 75 mg or more per cm^3 of membrane. Unlike traditional resin-based systems, the entire purification process—from loading the lysate to eluting pure protein—can be completed at room temperature in less than five minutes. We have assembled these membranes into spin columns and filtration devices and demonstrated their ability to purify his-tagged proteins produced in bacterial and mammalian cells. The millisecond residence time of the proteins on the membrane during binding minimizes the possibility of degradation. These membranes function perfectly in the presence of additives such as ethylenediaminetetraacetic acid (EDTA), reducing agents such as dithiothreitol (DTT), and under denaturing conditions (in the presence of urea and guanidium hydrochloride). We have extended the high-capacity membrane technology to immobilize Protein A and G, enabling extremely fast purification of antibodies from various matrices based on the affinity of these proteins for the fragment crystallizable region (Fc) region of antibodies. Antibody purification can be accomplished in less than 15 minutes, with capacities of up to 40 mg/ml or more, far exceeding the capacity of resin-based columns. More recently, we have immobilized trypsin and pepsin enzymes on these membranes to carry out proteolysis of proteins for their characterization, identification, and quantitation through mass spectrometry analysis. In contrast to the long incubation periods (6–24 hours) of conventional in-solution digestions, the proteolytic membranes generate peptides suitable for downstream analysis, with equivalent or improved sequence coverage, in less than a minute. Additionally, we expanded the membrane technology with immobilized streptavidin suitable for enrichment of target proteins, antibodies, and oligos. These novel membrane-based spinnable affinity columns and filtration devices will be useful for purifying a variety of recombinant proteins and antibodies and their proteomics characterization in academic and industrial settings.

Capturem™ membrane technology overview



- Resin-free
- High capacity due to large internal surface
- Rapid flow-induced mass transfer
- No incubation time
- Room-temperature workflow

Materials and Methods

Experimental conditions for Capturem his-tagged minipreps: Spin columns were equilibrated by the addition of 400 µl xTractor™ Buffer to the column followed by centrifugation at 11,000*g* for 1 min. Equilibrated spin columns were then loaded with 400–800 µl clarified lysate from cells expressing a 6xhis-tagged fusion protein (GFPuv) and centrifuged at 11,000*g* for 1 min at room temperature. The bound protein was then washed with 300 µl wash buffer, followed by centrifugation. Elution was performed by the addition of 300 µl elution buffer containing 500 mM imidazole, followed by centrifugation. The eluted fraction was analyzed by electrophoresis on a 4–20% SDS polyacrylamide gel to verify the purity of the protein. Gels were stained with Coomassie blue and destained according to standard protocol. An expected band was observed at a molecular weight of ~29 kilodaltons (kDa), corresponding to 6xhis-tagged GFPuv (unless otherwise specified in the figure caption).

Experimental conditions for the antibody purification experiment are described in Figure 3. Details for pepsin digestion experiments are provided in Figure 5. Details for trypsin digestion experiments are provided in Figure 6. Antibody enrichment using Capturem Streptavidin for successive antibody capture is described in Figure 7.

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1 High capacity with novel, membrane-based, spinnable, metal ion affinity columns

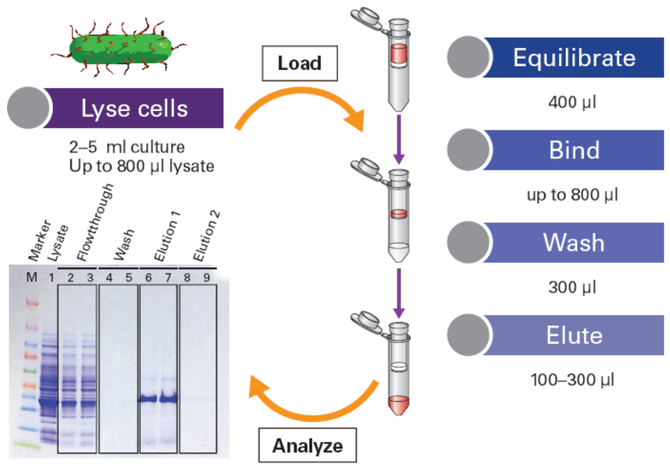


Figure 1. Capturem miniprep workflow for protein purification. The Capturem miniprep protocol was followed as described in Materials and Methods. Briefly, the his-tagged protein is first bound to the membrane, then washed and eluted with the appropriate buffers. Over 90% of the bound protein can be eluted with as little as 100 µl of elution buffer. Each step is followed by spinning the tube for 1 min at 11,000*g*. The working bed volume of the membrane is ~3 µl. This entire purification is complete in <5 min.

2 Volume and yields of Capturem purification formats

	Bed volume	Sample volume	Time	Approximate yield
Nano	0.24 ± 0.01 µl	400 µl	5 min	5–25 µg
Mini	3.0 ± 0.2 µl	800 µl	5 min	40–100 µg
96 wells	3.0 ± 0.2 µl	1 ml	15 min	40–100 µg
24 wells	30 ± 2 µl	4.5 ml	15 min	0.4–1 mg
Maxi	64 ± 2 µl	25 ml	15 min	1–2.5 mg
Large volume	480 ± 20 µl	500 ml	30 min	10–25 mg

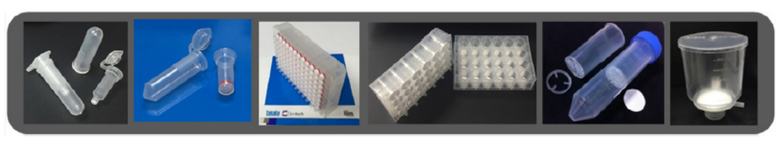
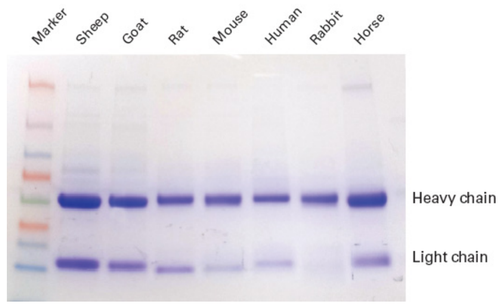


Figure 2. Loading volumes, protocol times, and approximate yields for different column formats. The loading volumes, protocol times, and approximate yields for the different column formats are listed (top). Yields are based on representative samples and will vary based on loading concentration and sample details (e.g., sample type, species, antibody isotype, protein size, etc.). Pictured from left to right: nanoprep, miniprep, 96-well, 24-well, maxiprep, and large-volume Capturem formats (bottom).

3 Animal sera antibody binding test



Animal sera	Purified antibody
Sheep	256
Goat	184
Rat	170
Mouse	140
Human	129
Rabbit	102
Horse	215

Figure 3. Capturem Protein A columns were tested for their ability to purify antibodies from animal sera: sheep, goat, rat, mouse, human, rabbit, and horse. Columns were equilibrated with 800 µl of Protein A binding buffer (0.5 M phosphate, pH 8.0, containing 2 M NaCl), then centrifuged at 1,000*g* for 1 min. Serum samples (250 µl) were diluted in 1 ml of binding buffer, and 600 µl of each sample was loaded onto the column, followed by centrifugation at 1,000*g* for 1 min. The loading process was repeated with the remaining 600 µl. The columns were then washed with 800 µl of binding buffer and centrifuged at 1,000*g* for 1 min. The bound antibody was eluted with 300 µl of elution buffer (0.1 M glycine, pH 2.5) into 30 µl of neutralization buffer (1 M Tris, pH 8.5) to neutralize the eluted antibody. The eluates from each sample were resolved by gel electrophoresis and stained with Coomassie blue. Absorbance at 280 nm was measured with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and used to quantify the amount of antibody in eluted fractions.

4 Schematic of the Capturem spin digest workflow

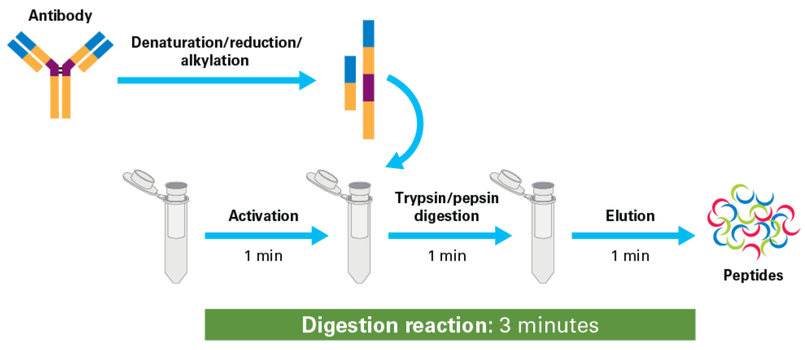


Figure 4. Digestion workflow using Capturem Pepsin or Capturem Trypsin spin columns. Digestion of prepared sample is complete in just 3 min.

5 Capturem Pepsin spin digest of anti-Her2 mAb

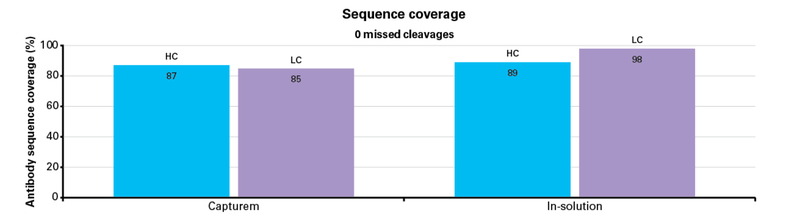


Figure 5. Peptic peptides from spin digest cover antibody sequences in MS analysis similar to in-solution digest. 50 µg of anti-HER2 mAb (Trastuzumab, Herceptin, human IgG1, kappa chain) was digested using two methods: Capturem Pepsin and in-solution pepsin incubated for 4 hr. In-solution digests were performed at 37°C with 50 µg of antibody in a reaction volume of 500 µl (weight ratio of 1:20 pepsin:antibody). In-solution digests are stopped by heating at 95°C for 10 min, followed by the addition of NaOH. The Capturem Pepsin method was performed as per the standard protocol in the user manual. MS analysis was performed following digestion. 1D LC-MS/MS QTOF analysis was performed by JadeBio, Inc.

6 Complete digestion of apomyoglobin with Capturem Trypsin

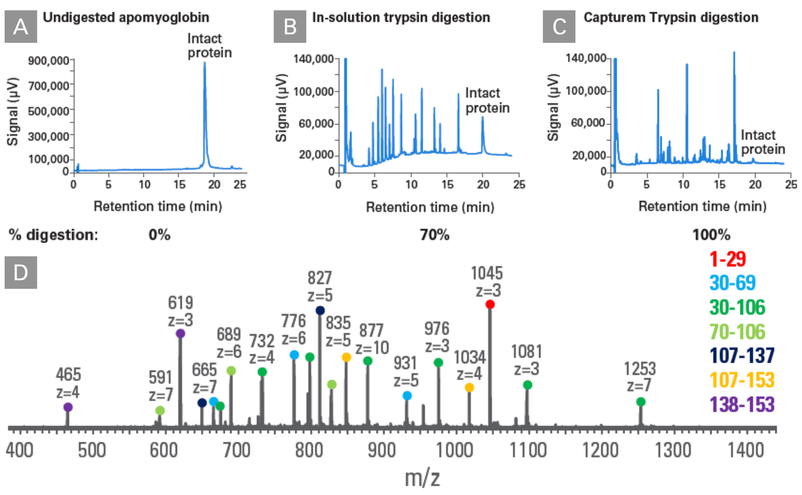


Figure 6. Rapid and complete digestion of apomyoglobin (Apo) using Capturem Trypsin results in high sequence coverage in MS analysis. Reverse phase HPLC (RP-HPLC) analysis was used to compare undigested Apo (Panel A) with Apo digested using in-solution trypsin for 16 hr at room temperature (Panel B), and Apo digested on a Capturem Trypsin column for <1 min (Panel C). For both digestions, Apo (80 µg) was dissolved in 200 µl of 10 mM ammonium bicarbonate before digestion. For Capturem Trypsin digestion, the columns were activated by loading 200 µl of the included activation buffer onto the columns, followed by centrifugation at 500*g* for 1 min. The digestion reaction solution was then loaded onto an activated column, followed by a single centrifugation at 500*g* for 1 min. For in-solution digestion, 4 µg of trypsin was used at an enzyme:substrate ratio of 1:20 (w/w). The reaction mixture was incubated at room temperature for approximately 16 hr. RP-HPLC analysis was performed using a Rainin Dynamax HPLC system. Mass spectrometry analysis of Apo peptides generated by Capturem Trypsin (Panel D). A deconvoluted ESI-Orbitrap mass spectrum was generated for Apo digested on a Capturem Trypsin column, using Xtract software. Data kindly provided by: Dr. Merlin Bruening, University of Notre Dame. For details about the mass spectrometry analysis, please visit takarabio.com/capturem-trypsin.

7 Capturem Streptavidin pulldown

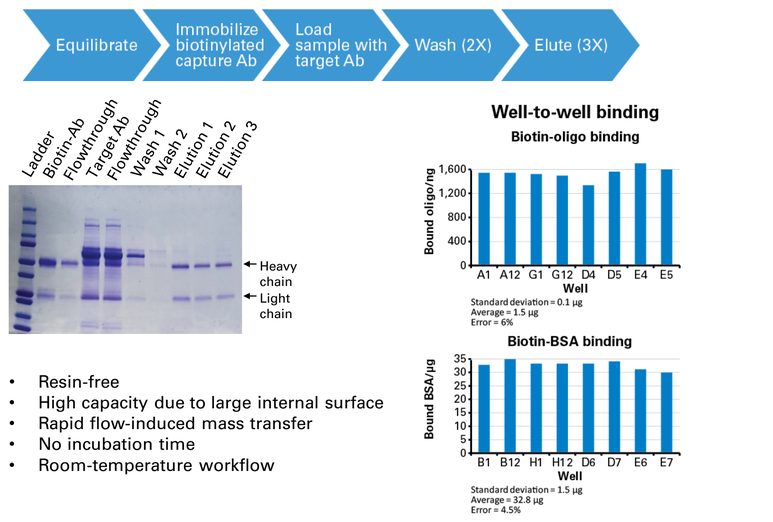


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Figure 7. Binding capacity and reproducibility of Capturem Streptavidin pulldown experiments. 48 µg of biotinylated rabbit IgG in 200 µl Binding Buffer was passed through an equilibrated Capturem Streptavidin spin column and 32.0 ± 1.4 µg were immobilized. After a single wash step, a sample containing the spiked-in target antibody (~100 µg of anti-rabbit IgG from goat) in hybridoma medium with 20% mouse serum was diluted with Binding Buffer and applied to the column. After two washing steps with Binding Buffer and then PBS, the target antibody was eluted with 1.0 M glycine in three steps to yield 42 ± 5 µg of highly pure target antibody. Technical replicates of biotinylated oligo or biotinylated BSA were loaded into a 96-well plate to test reproducibility. Absorbance measurements were used to determine the amount of biomolecules bound to the membranes. For protein binding experiments, 100 µg biotinylated BSA was diluted in 200 µl Binding Buffer and applied to each well. For oligonucleotide binding, 3.8 µg of oligo in 200 µl Binding Buffer was used.

Conclusions

- **No-waiting purification workflows**—Antibody and his-tagged purification protocols are complete in <5 min for the miniprep and 15 min for the maxiprep, protecting the protein from possible degradation or loss of activity.
- **High yield**—His-tagged miniprep columns yield up to ~0.3–1 mg/ml of protein and his-tagged maxiprep columns yield up to 0.5–4.5 mg/ml of protein. Protein A miniprep columns yield up to ~0.1–1 mg/ml of antibody and Protein A maxiprep columns yield up to 0.1–2 mg/ml of antibody.
- **Appropriate for different samples and conditions**—The purification membranes can be used for proteins expressed in mammalian cells or bacteria. Results are consistent regardless of denaturing conditions or the presence of additives (e.g., EDTA, DTT, BME, and TCEP).
- **Improved characterization**—Both trypsin and pepsin columns completely digest protein samples in less than 1 min, with protein coverage comparable to or better than in-solution digestion reactions.
- **Rapid enrichment**—Streptavidin-functionalized membranes enable a variety of enrichment protocols (e.g., oligo capture, successive antibody capture, etc.) for the capture of biotinylated products or pull-down of binding partners for a captured biotinylated ligand.