



Efficient Multi-Well Protein Purification Strategies

Introduction

Many tools and techniques are available today for protein purification. Development of a purification process requires an analysis of various strategies to isolate a protein of interest. Protein purification from a complex sample, such as cell culture or serum, requires more than one chromatographic step. Typically the first step is ion exchange (IEX) or mixed-mode chromatography to fractionate the sample and yield a sub-population of proteins containing the target molecule. This is followed by subsequent chromatographic steps to further fractionate the sub-population until a desired purity level is reached. These steps can include affinity purification, such as Protein A for immunoglobulins, IMAC for histidine-tagged proteins, and size exclusion chromatography, as well as IEX or mixed-mode chromatography to isolate the target protein. Each step in the purification process requires optimization in order to maximize yield and purity of the target protein. Thus, small-scale purification experiments are used during the development and optimization phase to preserve precious sample while providing crucial information.

The methods in this Application Note demonstrate the use of AcroPrep™ Advance 96-well filter plates combined with Pall chromatography media as an efficient tool for fractionation of small-volume protein samples. The format can be used to develop protein purification strategies and/or as a platform for moderate to high throughput protein isolation. In either case, purified sample can be used for further analysis and/

or downstream applications. Development methods for protein purification strategies using small quantities of protein mixtures are presented. One method uses AcroPrep Advance filter plates with Supor® membrane combined with Pall's mixed-mode chromatography resin, HEA HyperCel™, to yield complete separation of three proteins in a protein sample. A second method uses AcroPrep Advance filter plates with Mustang® IEX membrane to separate a mixture of three different proteins. In each case, the utilization of a filter plate-based strategy allows for rapid screening of multiple purification schemes. Fractionation and protein purification methods developed within the plate can be used for optimization of larger-scale purifications.

Discussion

Protein purification strategies using AcroPrep Advance filter plates with a combination of media provide:

1. A detailed protocol for the efficient fractionation of a protein sample using a mixed-mode chromatography resin loaded in a filter plate.
2. An optimized, rapid protocol for the efficient fractionation of a protein sample using anion and cation exchange membranes.
3. A simple protocol for using minimal sample volume to conserve precious samples.
4. An example for protocol development of small-scale protein purification.

Resin-Based Protein Purification

A wide variety of chromatography resins are available with various ligands (IEX, affinity and mixed-mode) to isolate proteins based on specific physical and chemical properties. A stepwise exposure of a protein sample to multiple chromatography resins allows for successive enrichment of a target protein. Native proteins typically require chromatography screening to determine which are best suited for a specific target protein. The combination of a filter plate and chromatography resin is an ideal format to analyze binding and elution characteristics for a protein of interest. The protocol below describes separation of three proteins using HEA HyperCel, a mixed-mode resin, along with the AcroPrep Advance filter plate. This is an example of one separation procedure that can be employed in an overall purification process.

Materials and Methods

- AcroPrep Advance filter plate with 1.2 μm Supor membrane, PN 8039
- HEA HyperCel resin, PN 20250-033
- Vacuum manifold, PN 5017
- Vacuum/pressure pump, PN 13157

Table 1

Buffer	Composition
Binding Buffer	Carbonate buffer, 150 mM NaCl, pH 10
Elution Buffer 1	20 mM Tris HCl, pH 7.0
Elution Buffer 2	20 mM sodium acetate, pH 5.0
Elution Buffer 3	20 mM sodium acetate, pH 4.0

Protocol for Fractionation with HEA HyperCel Resin

1. Wash appropriate volume of HEA HyperCel resin a minimum of two times with a total of at least 20 resin volumes of binding buffer in a conical tube (removes storage buffer). Centrifuge at 1,000 x g for 2-5 minutes.
2. Prepare 50% (vol/vol) resin slurry by adding an equal volume of binding buffer.
3. Place a collection plate underneath the filter plate. Quickly pipette 100 μL of resin slurry (50 μL resin) into the filter plate. Mix slurry frequently to avoid settling and inconsistent volume delivery to the plate.
4. Remove excess liquid from the well by applying 10-20 in. Hg (0.34-0.68 bar) vacuum for 10-15 seconds. Use these settings for all fluid collection steps.
5. Load 100 μL protein sample on to the resin. (For 50 μL of HEA HyperCel resin bed, 210 μg of total protein was added.)
6. Mix contents thoroughly using a plate mixer for 20 minutes at room temperature to facilitate binding.

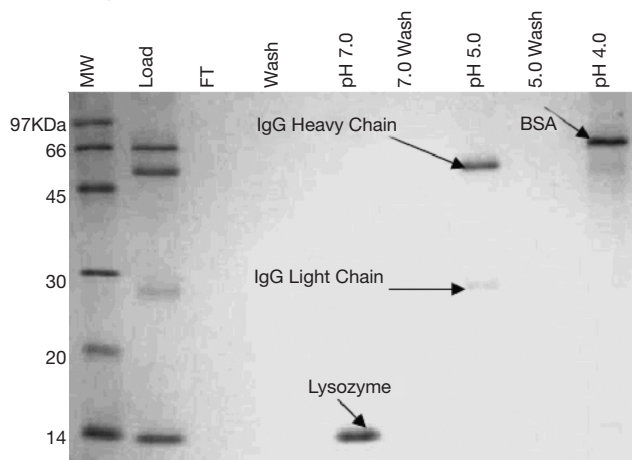
7. Collect flow through fraction (containing unbound proteins) with vacuum. Save fraction for analysis.
8. Wash resin at least twice with a total of 10x resin volumes of equilibration buffer (e.g., 250 μL x 2). Each addition of buffer is mixed for 5 minutes before evacuating.
9. Elute retained proteins with buffers having progressively decreasing pH (Table 1). Add 3x resin volume of Elution Buffer 1 (1 x 150 μL).
10. Wash the resin bed with 10 bed volumes (2 x 250 μL) of pH 7.0 buffer to avoid carryover of protein from Elution 1 to Elution 2.
11. Elute next set of proteins with two additions of 3x bed volume of Elution Buffer 2 (2 times x 150 μL). Collect each elution as a separate fraction.
12. Elute final set of proteins with two additions of 3x bed volume of Elution Buffer 3 (2 times x 150 μL).

Results

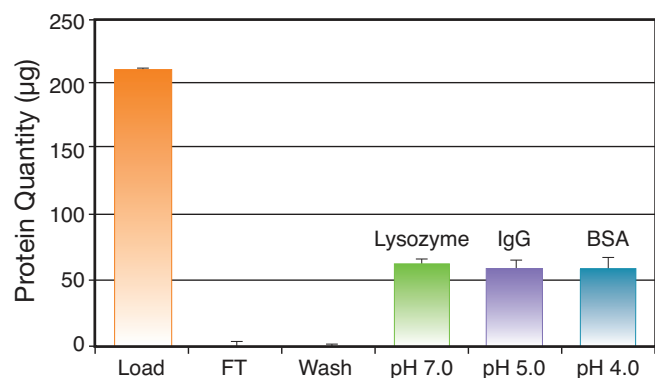
A protein sample containing lysozyme, human IgG and bovine serum albumin was loaded into all wells of the filter plate containing HEA HyperCel resin. SDS-PAGE analysis of each fraction and the starting material are shown in Figure 1. The absence of protein in the flow through and wash fractions indicates that all three proteins bind, while each elution fraction contains a single distinct protein. Additional washes between each step prevent carryover. Over 85% of total protein loaded into the wells is recovered in the elution fractions as shown in Figure 2. While not evidenced on the SDS gel, some protein most likely collects in the wash fractions between elutions and a small fraction remains bound to the resin.

Figure 1

Clear Separation of Protein Mixture in Three Fractions



2-6 μg of total reduced protein loaded onto 12% SDS-PAGE run with glycine buffer. Lanes: MW = molecular weight markers; Load = protein mixture; FT = flow through; Wash = wash; pH 7.0, 5.0 and 4.0 = elution buffer pH fractions. Coomassie Brilliant Blue G-250 stain.

Figure 2*Efficient Recovery of Loaded Protein Sample*

Average total protein assay results (Bradford reagent) for indicated fractions; error bars show standard deviation. Load = total protein applied to HEA HyperCel resin; FT = flow through; Wash = wash fraction; pH 7.0, 5.0 and 4.0 indicate pH of elution fraction. 24 replicates were used to show reproducibility.

Discussion

Fractionation of a complex mixture can be achieved with HEA HyperCel resin and subsequently used for downstream analysis or applications. While the method presented here used a combination of three proteins to demonstrate the performance of HEA HyperCel resin, more complex mixtures can be fractionated with this mixed-mode resin. In addition to HEA HyperCel resin, a variety of chromatography resins from Pall can be used to fractionate protein mixtures. Traditional chemically-defined chromatography resin (e.g., IEX and mixed-mode) are generally used for stepwise complexity reduction while affinity media results in a higher purity protein sample in a single step. If necessary, purity can be increased with the inclusion of a second IEX, mixed-mode or size exclusion chromatography to remove contaminants from the final protein sample. As with any protein purification strategy, optimization of binding and elution conditions is required to yield a desired result. The 96-well filter plate provides a convenient format to optimize conditions, as well as perform small-scale purifications.

Membrane-Based Protein Purification

To overcome mass transfer limitations associated with conventional resin-based chromatography, membrane chromatography was developed to obtain better flow distribution, fast flow rates, and increased accessibility for rapid purification of target proteins or removal of contaminants. These attributes translate into higher throughputs and reduced processing times. Pall offers two IEX membranes with strong IEX ligands, quaternary amine and sulfonic functional groups on Mustang Q (strong anion) and Mustang S (strong cation) membranes respectively. Pall's AcroPrep Advance 96-well filter plate with Mustang Q or S membrane is ideal for use in small scale, primary protein purification applications. The following protocol describes a purification strategy for each of the devices.

Materials and Methods

- AcroPrep Advance filter plates with Mustang Q membrane, 350 µL, PN 8071
- AcroPrep Advance filter plates with Mustang Q membrane, 1 mL, PN 8171
- AcroPrep Advance filter plates with Mustang S membrane, 350 µL, PN 8072
- AcroPrep Advance filter plates with Mustang S membrane, 1 mL, PN 8172
- Vacuum manifold, PN 5017 (optional)
- Vacuum/pressure pump, PN 13157 (optional)

Table 2*Buffers for Mustang Q Membrane Protein Separation*

Buffer	Composition
Loading Buffer	50 mM Tris, pH 8.8
Elution Buffer 1	50 mM Tris, 0.1 M NaCl, pH 8.8
Elution Buffer 2	50 mM Tris, 0.4 M NaCl, pH 8.8

Table 3*Buffers for Mustang S Membrane Protein Separation*

Buffer	Composition
Loading Buffer	50 mM sodium acetate, pH 4.5
Elution Buffer 1	50 mM sodium acetate, 0.2 M NaCl, pH 4.5
Elution Buffer 2	50 mM sodium acetate, 0.4 M NaCl, pH 4.5
Elution Buffer 3	50 mM sodium acetate, 1 M NaCl, pH 4.5

Protocol

Mustang Q Membrane Protein Mixture Separation

1. Pre-wet AcroPrep Advance filter plate with Mustang Q membrane with 300 µL/well of Loading Buffer.
2. Place filter plate on collection plate and centrifuge at 1,000 x g for 5 minutes to remove Loading Buffer. All subsequent centrifugation will occur at 1,000 x g for 5 minutes.
3. Load 200 µL/well of protein mixture to filter plate and incubate on shaker for 10 minutes.
4. Place filter plate on clean collection plate and centrifuge. Retain flow through fraction containing unbound protein.
5. Wash with 200 µL/well Loading Buffer, place filter plate on clean collection plate, centrifuge and retain flow through.
6. Add 200 µL/well Elution Buffer 1, incubate on shaker for 10 minutes.
7. Place filter plate on clean collection plate and centrifuge. Retain eluate.
8. Repeat steps 6-7.
9. Add 200 µL/well Elution Buffer 2, incubate on shaker for 10 minutes.

10. Place filter plate on clean collection plate and centrifuge. Retain eluate.
11. Repeat steps 9-10.
12. Analyze collected fractions by SDS-PAGE.

Mustang S Membrane Protein Mixture Separation

1. Pre-wet AcroPrep Advance filter plate with Mustang S membrane with 300 μ L/well of Loading Buffer.
2. Place filter plate on collection plate and centrifuge at 1,000 x g for 5 minutes to remove Loading Buffer. All subsequent centrifugation will occur at 1,000 x g for 5 minutes.
3. Load 200 μ L/well of protein mixture to pre-wet filter plate with Mustang S membrane and incubate on shaker for 10 minutes.
4. Place filter plate on clean collection plate and centrifuge. Retain flow through fraction containing unbound protein.
5. Wash with 200 μ L/well Loading Buffer, place filter plate on clean collection plate, centrifuge and retain flow through.
6. Add 200 μ L/well Elution Buffer 1, incubate on shaker for 10 minutes.
7. Place filter plate on clean collection plate and centrifuge. Retain eluate.
8. Repeat steps 6-7.
9. Add 200 μ L/well Elution Buffer 2, incubate on shaker for 10 minutes.
10. Place filter plate on clean collection plate and centrifuge. Retain eluate.
11. Repeat steps 9-10.
12. Add 200 μ L/well Elution Buffer 3, incubate on shaker for 10 minutes.
13. Place filter plate on clean collection plate and centrifuge. Retain eluate.
14. Repeat steps 12-13.
15. Analyze collected fractions by SDS-PAGE.

Results

Performance of Mustang Q membrane is illustrated by separating a three protein mixture using a salt elution strategy. SDS-PAGE analysis of fractions (Figure 3) shows a clear separation of each protein.

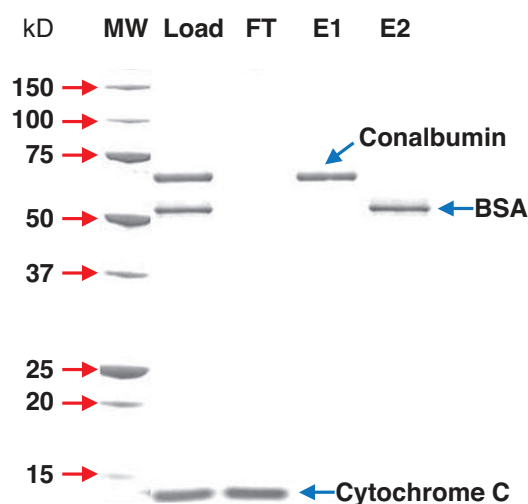
- Cytochrome C (12 kD) does not bind to the Mustang Q membrane at pH 8.8 (Lane FT)
- Conalbumin (78 kD) is eluted with the addition of 0.1 M NaCl (Lane E1)
- Albumin (67 kD) is eluted with 0.4 M NaCl (Lane E2)

Similar levels of protein separation are seen with Mustang S membrane in AcroPrep Advance filter plates, as seen by SDS-PAGE analysis of these fractions (Figure 4).

- All three tested proteins bind to the Mustang S membrane in Na Acetate, pH 4.5
 - Trypsinogen (24 kD) elutes first with 0.2 M NaCl (Lane E1)
 - Cytochrome C (12 kD) requires higher salt and elutes with 0.5 M NaCl (Lane E2)
 - Lysozyme 14.4 kD requires 1.0 M NaCl for elution (Lane E3)
- AcroPrep Advance filter plates with Mustang IEX membranes have demonstrated robust charge-based protein separations allowing for rapid protein purification.

Figure 3

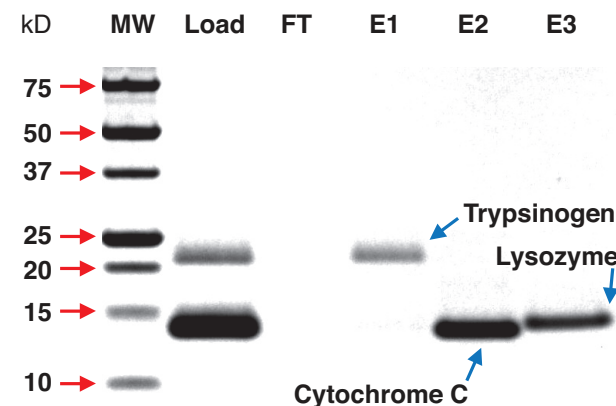
Complete Separation of Proteins with AcroPrep Advance Filter Plates with Mustang Q Membrane



1.25-3.75 μ g of total protein (not reduced) loaded onto 12% SDS-PAGE. Lanes: MW = molecular weight markers; Load = protein mixture; FT = flow through; E1 and E2 = 0.1 and 0.4 M eluate fractions. GelCode stain used.

Figure 4

Complete Separation of Proteins with Mustang S Membrane



1.25-3.75 μ g of total protein (not reduced) loaded onto 12% SDS-PAGE. Lanes: MW = molecular weight markers; Load = protein mixture; FT = flow through; E1 and E2 = 0.1 and 0.4 M eluate fractions. GelCode stain used.

Membrane-Based Protein Purification

(continued)

Discussion

AcroPrep Advance filter plates with Mustang IEX membranes have several performance advantages over resin-based IEX purification. Increased flow rates and better flow distribution allow for rapid binding and elution of biomolecules, and wash steps are immediate. Large membrane pores increase accessibility to the IEX chemistries, either for purification or contaminant removal. In addition, membranes have reduced hold-up compared to the resin, thus increasing wash efficiency and yielding more concentrated elutes. These factors combine to create an efficient platform for protein purification. When reviewing protein purification strategies using IEX chemistries, it is highly recommended that the performance of the membrane chromatography is analyzed. Pall's Mustang membrane is available in syringe filters and capsules to easily scale up purification developed in the AcroPrep Advance filter plate platform.

Conclusion

Three methods for fractionation of protein samples have been demonstrated using AcroPrep Advance filter plates. The first method combines an AcroPrep Advance filter plate with 1.2 μm Supor membrane with chromatography resin for small scale batch-mode protein separations. HEA HyperCel resin, capable of hydrophobic and IEX interactions, is used as a model to separate a three-protein mixture. This technique can be applied using a variety of sample types and optimized with Pall chromatography resin ideally suited to meet purification requirements. Purification strategies should be developed based on known physical and chemical characteristics of the target molecule, such as net charge, hydrophobicity and affinity for metals or ligands. Ion exchange, mixed-mode and affinity chromatography resins can then be screened to determine ideal candidates for purification of the target molecules.

Fractionation of additional three-protein mixtures was achieved using Mustang Q and S membranes. In both cases, proteins are bound to the membrane and eluted with salt. Each fraction contained one protein which could be used for downstream applications. In contrast to filter plates combined with chromatography resin, AcroPrep Advance filter plates with Mustang membrane are ready-to-use separation devices.

Summary

- AcroPrep Advance filter plates with Supor membrane are an ideal tool for small-scale protein purification with chromatography resin. This membrane has very low protein binding and does not clog in the presence of chromatography resin.
- Mustang Q and Mustang S membranes rapidly and efficiently fractionate proteins from complex protein samples.
- Optimization of binding and elution conditions for target molecules are easily achieved in the multi-well format of AcroPrep Advance filter plates.
- The protocols provided are easy to use with minimal sample quantity and processing time required.

Related Literature

All listed literature is available online at www.pall.com/lab in Pall's Literature Library.

- Poster: An Efficient Alternative for Purification of Antibodies Using a Synthetic Liquid
- Poster: Membrane Adsorbers as a Tool for Rapid Purification of Gene Therapy Products
- Technical Article: Mustang Chromatography Units Offer High Binding Capacities and Fast Flow Rates
- Poster: A New Method for Antibody Purification
- Poster: Phosphopeptide Enrichment Using IMAC HyperCel Resin
- Application Note: Efficient Depletion and Fractionation of Human Plasma Samples Using Pall Sample Prep Tools, PN 33548
- Poster: Use of Heparin HyperD[®] M Affinity Resin for Batch Mode Protein Purification From Plasma
- Application Note: Purification of Polyhistidine-Tagged Recombinant Proteins With IMAC HyperCel Resin Using AcroPrep 96-well Filter Plates or Nanosep[®] Centrifugal Devices, PN 33546

Related Products Available From Pall

AcroPrep 384-well Filter Plates for superior performance in high throughput sample preparation applications.

AcroWell™ 96-well Filter Plates exhibit high binding capacities for proteins and nucleic acids.

Centrifugal Devices concentrate and purify samples of < 50 µL to 60 mL with efficient recovery and low non-specific binding.

Chromatography Resins simplify protein purification and fractionation for biomarker discovery, proteomics research and drug development.



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