2.1.1 Introduction

Complexity reduction in samples is an important step in facilitating access to the low abundant proteins of interest for disease research and diagnostics. This process for human serum and plasma frequently includes the depletion of high abundant proteins such as albumin and IgG in combination with other fractionation techniques prior to 2D gel electrophoresis or LC-MS/MS detection.

Pall currently offers several solutions for abundant protein separation or removal. Options include affinity chromatography resins as well as complete kits for removal of multiple abundant proteins (albumin, IgG) in one step.

Enchant™ Protein Separation Kits rapidly deplete abundant proteins and unmask low abundant biomarkers from serum and plasma samples. Alternatively, these kits can be used to collect the abundant protein fractions for further analysis.

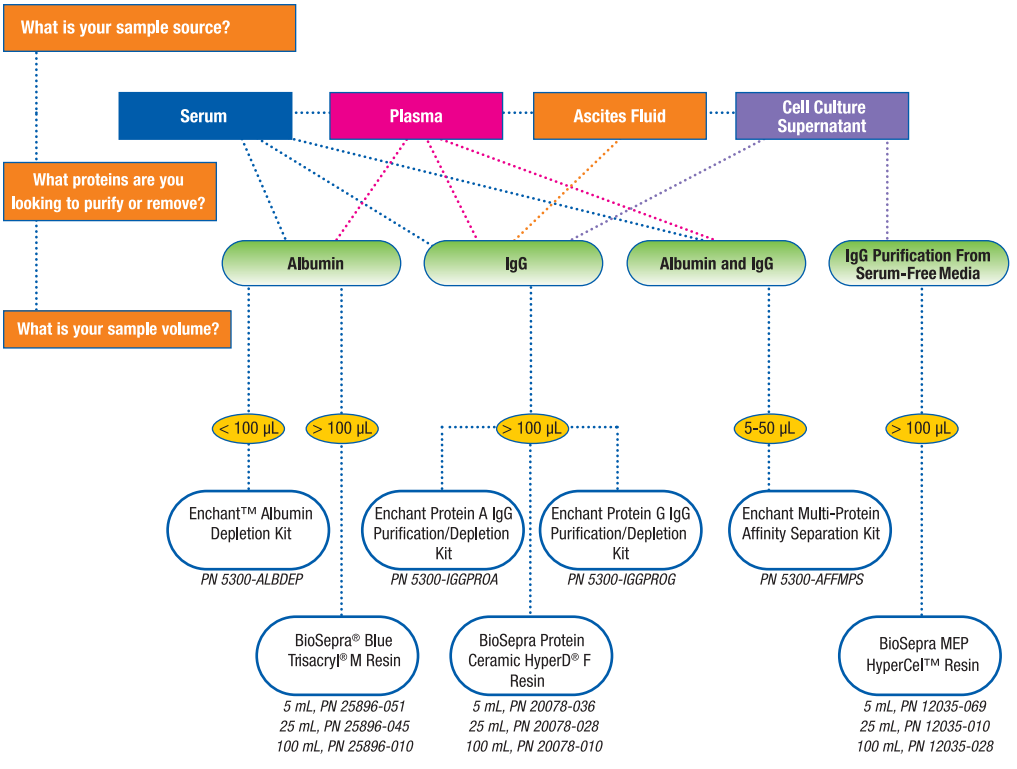
Figure 2.1
Enchant Protein Separation Kits

Abundant Protein Separation Quick Selection Guide

	Enchant™ Multi-Protein Affinity Separation Kit	Enchant Albumin Depletion Kit	Enchant Protein A IgG Purification/Depletion Kit	Enchant Protein G IgG Purification/Depletion Kit
Target Proteins				
Albumin	•	•		
IgG	•		•	•
Specificity				
> 80%		•		
> 90%			•	•
> 95%	•			
Ligand				
Single Chain Antibodies	•			
Protein A			•	
Protein G				•
Cibacron* Blue		•		
Sample Volume				
5-50 µL	•	•		
5-100 µL		•		
> 100 µL			•	•
Sample Type				
Serum	•	•	•	•
Plasma	•	•	•	•
Ascites Fluid	•	•	•	•
Cell Culture Supernatant	•	•	•	•
Species				
Human	+++	+++	+++	+++
Rat		++	+	++
Mouse			+++	+++
Goat		++	+	+++
Horse			+	+++
Cow		++	+	+++
Sheep			+	+++
Cat			+++	+
Dog			+++	+
Donkey			++	+++
Guinea Pig			+++	+
Hamster			++	++
Pig			+++	+
Rabbit			+++	+++
Rhesus Monkey			+++	+++

Key: +++ = High Binding, ++ = Medium Binding, + = Low Binding, none = No Binding

Choose the Best Protein Removal Product for Your Needs



2.1 – Section 2.1.2

2.1.2 Enchant™ Albumin Depletion Kit

This kit contains all necessary reagents to process 0.01-0.1 mL of serum or plasma. Specifications are summarized in Table 2.1. The albumin depleting discs are supplied in dehydrated form with a Cibacron* Blue-based support. When hydrated with a buffer or directly with the sample, it forms a gel-based slurry. The resulting slurry is equivalent to 0.2 mL of resin. The easy five-step protocol allows you to remove ≥ 2 mg of albumin from each sample processed (see Figure 2.2).

The Enchant Albumin Depletion Kit has been optimized for use with human serum albumin (HSA) from plasma or serum samples. In addition, this kit can be optimized for use with other species including bovine, calf, goat, and rat. This kit is not recommended for use with mouse. The Enchant Albumin Depletion Kit can also be used in tandem with the Enchant IgG Purification Kit for removal of IgG and albumin from serum or plasma samples. Alternatively, for depletion of both albumin and IgG from a single sample, Pall recommends the use of our new Enchant Multi-Protein Affinity Separation Kit. Refer to Section 2.1.4, page 33 for more information.

Table 2.1
Specifications of the Enchant Albumin Depletion Kit

Specification	Parameter
Nanosep® Centrifugal Device	
0.45 µm GHP Membrane	Hydrophilic polypropylene
Nanosep Device Filtrate Receiver	Polypropylene Polypropylene
Effective Membrane Area	0.28 cm^2
Dimensions, Overall Length (with Cap)	4.5 cm (1.8 in.)
Capacities Maximum Sample Volume Filtrate Receiver Volume Hold-up Volume (Membrane/Support)	 0.5 mL 0.5 mL < 0.005 mL
Operating Temperature Range	0-40 °C (32-104 °F)
pH Range	3-14
Maximum Centrifugal Force	14,000 x g
Centrifuge Type	Fits rotors that accept 1.5 mL tubes
Albumin Depletion Disc	
Binding Capacity	Each albumin depleting disc can remove ≥ 2 mg of albumin from diluted serum or plasma samples
Mechanism of Action	Cibacron Blue-based dehydrated support
Storage Conditions	Store at room temperature
Shelf Life	One year from date of purchase, if stored properly. (Refer to package for exact expiration date.)

Protocol for Enchant™ Albumin Depletion Kit

Tip: *The Enchant Albumin Depletion Kit has been designed for depleting albumin from plasma or serum samples. If depletion of multiple proteins from a sample is desired, Pall recommends the use of the Enchant Multi-Protein Affinity Separation Kit (see Section 2.1.4, page 33). However, if using the albumin dye-based system is preferred, this kit can be used in tandem with the Enchant IgG Purification Kit (see Section C, page 13).*

A. Materials Required

1. Materials supplied in the kit:

- **a.** Nanosep® devices with 0.45 µm GHP membrane, 25 complete columns
- **b.** Additional filtrate tubes, 25
- **c.** Albumin-depleting discs, 25
- **d.** Binding/wash buffer, 6.25 mL

2. Additional materials needed:

- **a.** Micro-centrifuge; 1.5-2.0 mL tubes, angle rotor, variable speed up to 12,000 x g maximum. Refrigeration not required.
- **b.** Optional (needed if depleting IgG before albumin) – Enchant IgG Purification Kit (PN 5300-IGGPROG or PN 5300-IGGPROA).

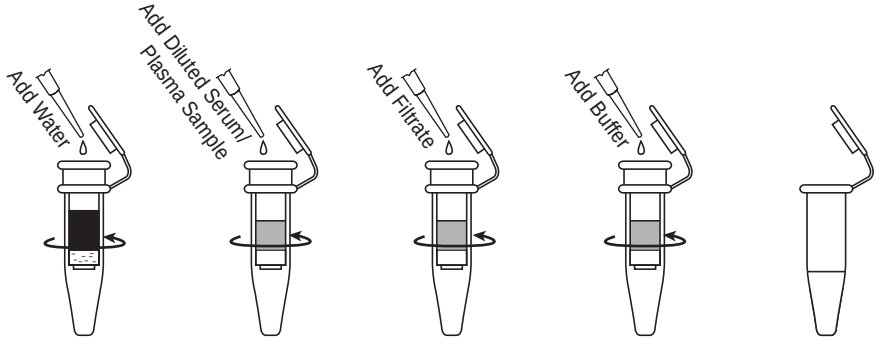
B. Albumin Depletion Using the Enchant Kit

1. Serum/plasma sample preparation

It is important not to exceed the binding capacity of each albumin depleting disc. Each disc will bind ≥ 2 mg of human serum albumin and requires a load volume of at least 0.05 mL. For highly concentrated serum or plasma samples, dilute sample with the binding/wash buffer. For diluted samples, > 0.05 mL can be applied directly to the Enchant albumin depleting spin column without dilution. If using serum from species other than human, refer to the FAQ Section (page 17) for buffer guidelines.

Figure 2.2

Methodology: Remove ≥ 2 mg Albumin in Just Five Easy Steps



Step 1: Hydrate albumin depleting disc and centrifuge to remove excess liquid.

Step 2: Add serum/ plasma sample to hydrated column. Incubate and centrifuge.

Step 3: Collect filtrate and add back to the column. Incubate and centrifuge.

Step 4: Add binding/ wash buffer and spin. Wash sample 1-4 times.

Step 5: Pool fractions for downstream analysis or elute bound fraction.

2. Albumin depletion protocol

a. Place one albumin depleting disc into a Nanosep® MF centrifugal device.

b. Add 0.38 mL of high purity water to each Nanosep centrifugal device containing the albumin depleting disc. Vortex for five seconds. The disc should be fully hydrated in less than 30 seconds.

c. Centifuge the albumin depleting column at 12,000 x g for 1 minute.

Tip: *Make sure you counterbalance the centrifuge.*

d. Discard the filtrate.

e. Apply between 0.05-0.1 mL of diluted albumin containing sample. Typical sample volumes have a total volume of 0.1 mL but only contain ≤ 0.03 mL of serum.

Tip: *When sample is added to the Enchant™ albumin depleting column, a slurry does not form, it just becomes wet.*

f. Incubate sample in the albumin depleting column for 2 minutes.

g. Centrifuge the albumin depleting column at 12,000 x g for 1 minute.

h. Recover filtrate and add back to the albumin depleting column.

i. Incubate sample in the albumin depleting column for another 2 minutes.

j. Centrifuge the albumin depleting column at 12,000 x g for 1 minute.

k. Retain the filtrate for downstream analysis.

l. Replace used Nanosep filtrate tube with a new filtrate tube.

m. Add 0.05 mL of the binding/wash buffer to the albumin depleting column. This will release any unbound proteins.

n. Centrifuge the albumin depleting column at 12,000 x g for 1 minute. Retain filtrate.

o. Analyze retained filtrate fractions by SDS-PAGE.

p. To recover bound albumin, proceed to Step 3.

q. Dispose of column. Do not re-use for another sample.

Tip: *A single wash of the Enchant albumin depleting column is typically sufficient for obtaining adequate amounts of the desired protein sample. If further washes are required, repeat Step 2 as needed and combine desired fractions. If additional washes are needed, you can order additional Nanosep filtrate tubes (PN FD001X34).*

3. Recovery of bound albumin

a. Add 0.2 mL of any gel loading buffer diluted to 1x (example: 0.02 M sodium phosphate, 0.5 M sodium thiocyanate, pH 7.2) to the albumin depleting spin column.

Tip: *High salt concentrations will remove the bound albumin (example: 500 mM salt solution).*

b. Centrifuge at 12,000 x g for 1 minute.

c. Retain the filtrate.

d. Repeat Step 3 if desired. Replace filtrate tube each time.

e. Analyze filtrates by SDS-PAGE.

f. Dispose of column. Do not re-use for another sample.

C. Serial Depletion of IgG and Albumin From Serum/Plasma Using the Enchant™ IgG Purification Kit (PN 5300-IGGPROA or 5300-IGGPROG) in Conjunction with the Enchant Albumin Depletion Kit (PN 5300-ALBDEP)

Tip: *For efficient, simultaneous removal of human albumin and IgG with up to 0.05 mL of sample, the new Enchant Multi-Protein Affinity Separation Kit (PN 5300-AFFMPS), based on an immunochemical affinity ligand, is highly recommended (see Section 2.1.4, page 33).*

If use of a dye-based method for albumin depletion as part of a combined albumin/IgG removal is preferred, then the protocol below is recommended. This protocol combines the use of the Enchant Albumin Depletion Kit (PN 5300-ALBDEP) with the Enchant IgG Purification Kit (PN 5300-IGGPROA or 5300-IGGPROG).

The IgG Purification Kit should be used first since it can process larger sample volumes than the Albumin Depletion Kit. The binding capacity of the affinity columns in the Enchant IgG Purification Kit allows for removal of IgG from 1 to 2 mL of plasma/serum. The total IgG content of serum is typically 10-15 mg/mL, while the specific IgG of interest only accounts for 2-5% of this total. Protein A or Protein G IgG Purification Kits can be used depending on the species the sample was isolated from and the antibody isotype that needs to be depleted. Albumin is the most abundant protein in serum at 30-40 mg/mL. The Albumin Depletion Kit can process 0.01-0.1 mL of serum or plasma. Each disc can bind ≥ 2 mg of albumin.

All kit components should be at same temperature to avoid bubbles, which could prevent flow.

Start with IgG depletion.

1. Dilute 1 mL of sample 1:1 with binding buffer (supplied in the IgG Purification Kit) before applying to the column.

Tip: *Plasma samples will become cloudy/opaque after dilution with the binding buffer. This is a result of lipoprotein precipitation. If this occurs, centrifuge the sample at 10,000 x g for 15 minutes (or until clear).*

2. Equilibrate by washing with 5 mL of binding buffer. Allow buffer to pass through column by gravity flow.

3. Add diluted sample to Protein A or Protein G affinity column and allow it to flow through the column.

4. Collect 1 mL fractions and set aside for analysis. Wash the column by passing 5-15 mL of binding buffer through the column by gravity flow.

5. Collect 1 mL fractions and set aside for analysis. Typically the IgG depleted sample is distributed between 4 fractions. By measuring OD at 280 nm, fractions with the highest protein content can be pooled together and processed for albumin depletion. The concentration of albumin in these pooled fractions is typically around 10 mg/mL.

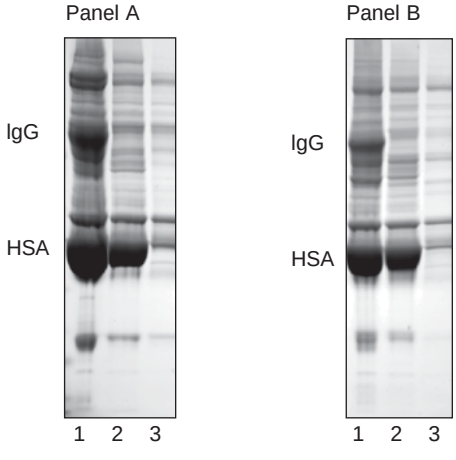
6. Continue with albumin depletion. Follow Step B2 on page 12.
7. The bound IgG can be eluted from the column using the elution buffer supplied with the kit. Eluted fractions can consequently be neutralized with alkaline buffers and desalted using the desalting columns provided with the kit. By measuring absorbance at 280 nm of the eluted IgG fractions, the amount of IgG that was removed by the column can be quantified.
8. The Protein A and Protein G columns can be regenerated and reused.

Application Data for Enchant™ Albumin Depletion Kit

Effective Depletion of HSA and IgG from Plasma and Serum Samples

By combining the Enchant IgG Purification and Albumin Depletion Kits in a serial process, it is possible to remove IgG and albumin from serum or plasma. This double depletion process is illustrated in Figure 2.3 for human serum and plasma. The data clearly shows the serial removal of IgG and albumin.

Figure 2.3



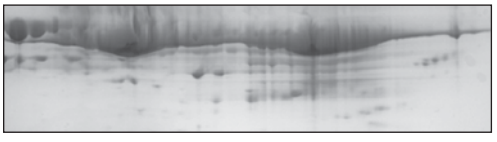
1 mL of human plasma and serum was processed using the Enchant Protein A IgG Purification Kit and then 0.03 mL of the column flowthrough was treated with the Enchant Albumin Depletion Kit. Samples were loaded onto 4-12% SDS-PAGE gels and resolved in MOPS/SDS running buffer under non-reducing conditions. Proteins were visualized with colloidal Coomassie Blue. Left Panel A shows results for human plasma and right Panel B shows results for human serum. Lane 1 in both panels shows the serum and plasma starting samples. Lane 2 shows IgG removal with the Protein A column. Lane 3 shows subsequent removal of albumin from both samples.*

Visualize Low Abundant Proteins with Effective Albumin Depletion

One of the objectives of depleting abundant proteins, such as albumin and IgG from a sample, is to increase the relative concentration of low-abundance proteins for analytical process like 2DGE. An example of the increased visualization of low-abundance proteins in plasma is shown in Figure 2.4. In Panel B, the 2DGE gel clearly shows that there are many more proteins visible in the region where albumin would have resolved.

Figure 2.4

Panel A, pI 4-7 Region of Plasma 2DGE
Before Albumin Depletion



Panel B, pI 4-7 Region of Plasma 2DE
After Albumin Depletion

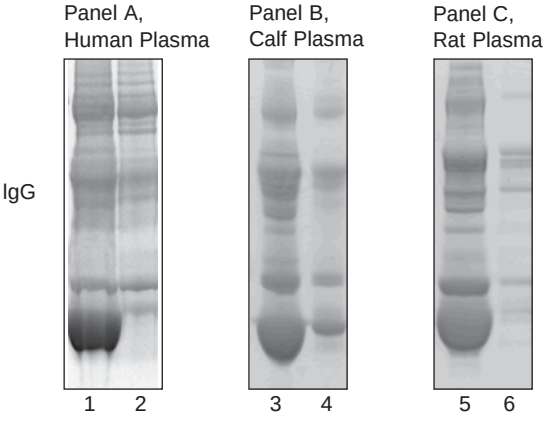
Human plasma (0.02 mL) was diluted with 0.03 mL of binding buffer and added to pre-swelled albumin depletion resin in a Nanosep® GHP centrifugal device. The sample was incubated for 10 minutes at room temperature then spun at 12,000 x g for 1 minute. Filtrate was recovered, added back to the resin, incubated for 2 minutes, and spun again. The recovered filtrate and starting material were analyzed by 2DGE by focusing in the first dimension on a pH 4-7 IPG strip then resolving the second dimension on an 8-16% tris-glycine gel under reducing conditions. Proteins were visualized with colloidal Coomassie Blue.*

Panel A shows the whole plasma sample and Panel B shows the improved resolution of protein bands after depletion of albumin.

Versatile Purification and Depletion of IgG and Albumin from Multiple Species

Albumin is a major component of mammalian serum or plasma and shows some structural conservation across species. The Cibacron* Blue resin (Enchant™ Albumin Depletion Kit) used in this depletion process can also bind albumin from species other than human. Data for calf and rat plasma depletion is shown in Figure 2.5. Although blue dye-based ligands like the one used in the Enchant Albumin Depletion Kit will bind albumin from most species with moderate to high affinity, they can also bind to other serum and plasma proteins in some species.

Figure 2.5



Plasma samples were processed using the Enchant Albumin Depletion Kit according to the protocol provided. The influent and effluent samples were loaded onto 4-12% SDS-PAGE gels and run under non-reducing conditions in MOPS/SDS running buffer. Resolved proteins were visualized by staining with colloidal Coomassie Blue.

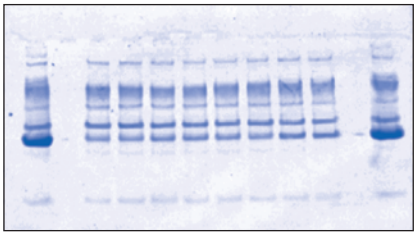
Panels A-C show plasma samples from human, calf, and rat species. Lanes 1, 3, and 5 show the undepleted plasma sample. Lanes 2, 4, and 6 show plasma after depletion.

Consistent Depletion of Albumin from Serum or Plasma Samples

The Enchant™ Albumin Depletion Kit provides consistent depletion from sample to sample. Eight independent samples of serum and plasma were processed using the Enchant Albumin Depletion Kit. The serum and plasma influents plus 8 samples were resolved by SDS-PAGE and then transferred to FluoroTrans® W membrane. The membrane was stained with SYPRO* Ruby protein stain and visualized on the GE Healthcare Storm* 860. A summary of the data generated is shown in Figure 2.6. Panel A is serum and Panel B is plasma. In Panel C, quantitative fluorescence densitometry of albumin bands visualized with the SYPRO Ruby stain are summarized.

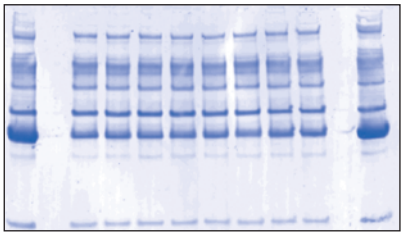
Figure 2.6

Panel A, Serum Albumin Depletion



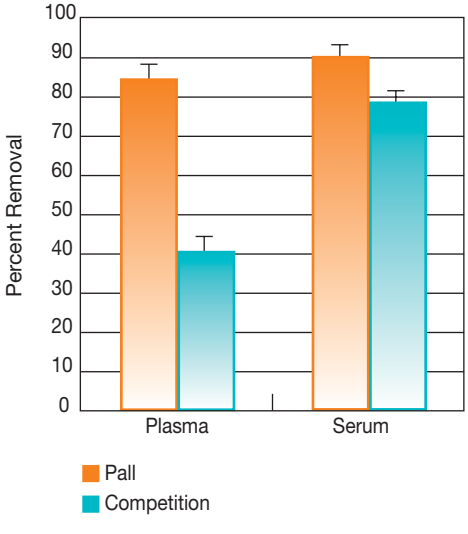
Serum Influent | Serum Effluent Replicates 1-8 | Serum Influent

Panel B, Plasma Albumin Depletion



Plasma Influent | Plasma Effluent Replicates 1-8 | Plasma Influent

Panel C, Quantitation of Albumin Depleted Samples by Scanning Fluorescence Densitometry



Percent albumin reduction was calculated by quantitating the fluorescent signal (Storm, GE Healthcare) of primary HSA band for both the influent and effluent. Error bars indicate the standard deviation between duplicate experiments.

FAQ and Troubleshooting for Enchant™ Albumin Depletion Kit

1. Do I need to store the Enchant Albumin Depletion Kit in a cold room?

   No. The Enchant Albumin Depletion Kit can be stored at room temperature. The kit should not be frozen.

2. Does pH affect the binding of the Enchant albumin depleting spin column?

   Yes. The optimal pH range for albumin binding is 6.0-9.0.

3. Do I need to desalt my sample prior to using the kit?

   Yes. Binding of albumin is salt dependent. The samples have to be desalted prior to applying to the albumin depleting spin column. The Nanosep® UF centrifugal devices offer a superior platform for sample desalting (see Section 2.4.2, page 154). If dialysis is preferred, we recommend the following buffers: human and swine samples (25 mM Tris, 75 mM NaCl, pH 7.5), and bovine, goat, calf, and rat samples (25 mM Tris, 25 mM NaCl, pH 7.5). This kit is not recommended for mouse species.

4. Can the same wash/binding buffer be used for all species of serum?

   No. The Enchant Albumin Depletion Kit was optimized for the removal of human serum albumin. Depending on the species, a different wash/binding buffer may be needed. For bovine, calf, rat, and goat species, we recommend a wash/binding buffer of 25 mM Tris, pH 7.2.

5. Does my sample need to be free of particulate matter prior to adding to the albumin depleting spin column?

   Yes. For optimal performance of the Enchant albumin depleting spin columns, the sample should be clarified by microfiltration. The Nanosep MF centrifugal devices provide a convenient platform for removing particulates prior to processing (see Section 2.5.3, page 210).

6. How do I remove the albumin from the resin?

   You can elute the bound albumin from the resin by referring to Section C on page 13 of this protocol.

7. Is the effluent sample from the Enchant IgG Protein A or Protein G Purification Kit compatible with binding to the albumin depletion discs?

   You can use the collected peak fraction from the IgG depleted fractions directly in albumin depletion. Desalting is optional and does not seem necessary for human IgG. It may be necessary to optimize this step for species other than human where the binding to the albumin depletion resin may not be as strong.

8. How much of the effluent from an Enchant IgG Protein A or Protein G Purification Kit can you load onto an albumin depletion disc?

   It is important not to exceed the binding capacity of each albumin depleting disc. Each disc will bind ≥ 2 mg of human serum albumin and requires a load volume of at least 0.05 mL. Since larger volumes (minimum 1.0 mL) are run on the Enchant IgG purification columns, the sample will be diluted up to 1:4 in the flowthrough fraction. You can load up to 0.1 mL of the sample per albumin depleting disc, which should contain approximately 1 mg of albumin. This is well within the capacity of the resin and will give efficient albumin depletion. You can concentrate the fractions by 100-150% using a Nanosep UF centrifugal device with a 10K MWCO membrane (see Section 2.4.2, page 154) and use 0.1 mL of the retentate to load approximately 2-3 mg of albumin.

Ordering Information for Enchant™ Albumin Depletion Kit

Part Number	Description	Pkg
5300-ALBDEP	Enchant Albumin Depletion Kit (25 Nanosep® 0.45 µm GHP centrifugal devices, 25 Nanosep filtrate tubes, 25 albumin-depleting discs, 6.25 mL binding/wash buffer)	25 samples

2.1.3 Enchant™ IgG Purification Kit

Effective sample preparation and purification are the most critical aspects of protein analysis. Although there are numerous techniques for purifying proteins, affinity purification is one of the most robust methods for identifying proteins of interest. Affinity purification utilizes immobilized ligands that employ binding specificity to various types of proteins from complex mixtures.

Protein A and Protein G are cell wall proteins that have specificity for the Fc portion of certain immunoglobulins. Protein A is a 43,000 dalton protein that is produced by the bacteria *Staphylococcus aureus* and contains four binding sites to the Fc regions of IgG. Protein G is produced from group *G Streptococci* and has two binding sites for the Fc region of IgG. Both proteins have been widely characterized for their affinity to various types of immunoglobulins.

The Enchant IgG Purification Kit utilizes immobilized Protein A or Protein G crosslinked to 6% beaded agarose in gravity filtration columns. They can be used for small-scale purification of IgG from serum/plasma or tissue culture samples. In addition, the IgG depleted fraction of serum/plasma can be combined with the Enchant Albumin Depletion Kit to serially remove IgG and albumin. Specifications are summarized in Table 2.2.

Table 2.2
Specifications of the Enchant IgG Purification Kit

Specification	**Parameter**
Enchant IgG Purification Kit	
Binding Capacity	
Protein A Columns	11-19 mg of human IgG/mL of gel and 6-8 mg of mouse IgG/mL of gel
Protein G Columns	10-15 mg of human IgG/mL of gel
Storage Conditions	Store at 4 °C upon receipt. Affinity and desalting columns should be kept upright. Shipping occurs at ambient temperature.
Shelf Life	One year from the date of purchase, if stored properly.
Enchant IgG Purification Kit Components	
Protein A Kit	
Protein A Affinity Purification Columns	
Quantity	5 columns
Solid Support	Protein A covalently crosslinked to 6% beaded agarose
Column Volume	1 mL. Columns are supplied in an aqueous slurry containing 0.02% sodium azide (bacteriocide).
IgG Binding Buffer (Contains EDTA as preservative)	
Quantity	1 L
pH	8.0

Table 2.2 (continued)
Specifications of the Enchant IgG Purification Kit

Specification	Parameter
Enchant™ IgG Purification Kit Components	
Protein A Kit	
IgG Elution Buffer	
Quantity	500 mL
pH	2.8
Gel Filtration Desalting Columns	
Quantity	5 columns
Solid Support	Porous cellulose beads
Particle Size	40-100 µm
Column Volume	5 mL
Void Volume	1.75 mL
Molecular Weight Cut-off (MWCO)	5,000 daltons
Protein G Kit	
Protein G Affinity Purification Column	
Quantity	1 column
Solid Support	Protein G crosslinked to 6% agarose
Column Volume	2 mL. Columns are supplied in an aqueous slurry containing 0.02% sodium azide (bacteriocide).
IgG Binding Buffer [Contains 0.02% sodium azide (bacteriocide)]	
Quantity	240 mL
pH	5.0
IgG Elution Buffer	
Quantity	120 mL
pH	2.8
Gel Filtration Desalting Columns	
Quantity	5 columns
Solid Support	Porous cellulose beads
Particle Size	40-100 µm
Column Volume	5 mL
Void Volume	1.75 mL
MWCO	5,000 daltons

Protocol for Enchant™ IgG Purification Kit

A. Materials Supplied in the Kit

1. Protein A Kit

a. Protein A affinity purification columns

(1) Quantity: 5 columns

(2) Solid support: Protein A covalently crosslinked to 6% beaded agarose

(3) Column volume: 1 mL

(4) Columns are supplied in an aqueous slurry containing 0.02% sodium azide

b. IgG Binding Buffer (Protein A)

(1) Quantity: 1 L

(2) pH: 8.0

Tip: *Contains EDTA as preservative.*

c. IgG Elution Buffer (Protein A)

(1) Quantity: 500 mL

(2) pH: 2.8

d. Gel filtration desalting columns

(1) Quantity: 5 columns

(2) Solid support: porous cellulose beads

(3) Particle size: 40-100 µm

(4) Column volume: 5 mL

(5) Void volume: 1.75 mL

(6) MWCO: 5,000 daltons

2. Protein G Kit

a. Protein G affinity purification column

(1) Quantity: 1 column

(2) Solid support: Protein G crosslinked to 6% agarose

(3) Column volume: 2 mL

(4) Columns are supplied in an aqueous slurry containing 0.02% sodium azide

b. IgG Binding Buffer (Protein G)

(1) Quantity: 240 mL

(2) pH: 5.0

(3) Contains 0.02% sodium azide

c. IgG Elution Buffer (Protein G)

(1) Quantity: 120 mL

(2) pH: 2.8

d. Gel filtration desalting columns
- **(1)** Quantity: 5 columns
- **(2)** Solid support: porous cellulose beads
- **(3)** Particle size: 40-100 µm
- **(4)** Column volume: 5 mL
- **(5)** Void volume: 1.75 mL
- **(6)** MWCO: 5,000 daltons

B. Materials Not Supplied with the Kit

1. Collection tubes 1-2 mL volume
2. Rack or stand for stabilization of the affinity column
3. Centrifuge tubes (15 mL plastic)
4. Suitable centrifuge with a fixed rotor to fit 15 mL tubes and able to spin at 10,000 x g under refrigerated conditions
5. 1 M Tris base, pH 9.5 for neutralization
6. 0.1 M citric acid, pH 3.0 (adjust pH with NaOH) for column regeneration
7. 0.02% sodium azide for column storage
8. Optional—Enchant™ Albumin Depletion Kit (PN 5300-ALBDEP) needed if depleting albumin as well as IgG

C. IgG Purification Using the Enchant Kit (Protein A)

It is important to note that when purifying antibodies, different types of immunoglobulins may exhibit different binding affinity for Protein A. Use the table on pages 30-31 to aid in the selection of the appropriate affinity column. If the antibody isotype is unknown, a test experiment can be performed.

1. Protein A affinity column equilibration and sample preparation
   - **a.** Bring all kit components and sample to same temperature. Procedure can be carried out either at room temperature or in a cold room. If all components are not at the same temperature, air bubbles in the column could result.
   - **b.** Dilute sample (serum, plasma, ascites fluid, or tissue culture supernatant) 1:1 with Protein A binding buffer before applying to the Protein A affinity column.

Tip: *Plasma samples will become cloudy/opaque after dilution with the Protein A binding buffer. This is a result of lipoprotein precipitation. If this occurs, centrifuge the sample at 10,000 x g for 15 minutes (or until clear).*

   - **c.** Open the Protein A affinity column by removing the top cap first. Decant the storage buffer. Remove the cap on the bottom of the column. Place Protein A affinity column in a standard test tube or test tube holder.

Tip: *Retain caps for storage and column regeneration.*

   - **d.** Equilibrate the Protein A affinity column. Pipette 5 mL of Protein A binding buffer into the Protein A affinity column. Allow the binding buffer to pass through the column. Collect binding buffer in standard test tube.

2. IgG depletion with Protein A

a. Dilute 1 mL of sample 1:1 with Protein A binding buffer before applying to Protein A affinity column.

Tip: *Plasma samples will become cloudy/opaque after dilution with the Protein A binding buffer. This is a result of lipoprotein precipitation. If this occurs, centrifuge the sample at 10,000 x g for 15 minutes (or until clear). Apply cleared supernatant to the Protein A affinity column.*

b. Apply at least 1 mL of diluted sample to the Protein A affinity column and allow sample to pass through the column.

c. Collect 1 mL fractions and set aside for analysis.

d. Add 10-15 mL of Protein A binding buffer to the column to wash IgG depleted sample through.

e. Continue to collect 1 mL fractions.

f. Measure the absorbance of each fraction at a wavelength of 280 nm (A_{280}). The fractions with the highest absorbance will be the IgG depleted samples. This will typically be 2-4 fractions.

3. IgG purification with Protein A

a. Transfer Protein A affinity column to second test tube and apply at least 1 mL of diluted sample to the Protein A affinity column and allow sample to pass through the column.

b. Collect the filtrate solution in a test tube and set aside for analysis of binding efficiency.

c. Add 10-15 mL of Protein A binding buffer to the column to wash the Protein A affinity column.

d. To verify that all unbound proteins are washed away, collect filtrate in 2 mL fractions. Measure their A_{280} readings. These fractions should have absorbance readings that are the same as the binding buffer.

4. IgG elution with Protein A

a. Add 5 mL of Protein A elution buffer to the Protein A affinity column to recover the bound IgG.

b. Collect the filtrate in numbered collection tubes. For collection purposes, it is important to know where your sample is in the column. The gel bed holds approximately 1 mL of solution. If you have 1 mL of sample, then the entire sample will fit into the gel bed. As the sample passes through the column, the first milliliter that comes through the column will contain some of the elution buffer and some of the purified IgG. The next 2-3 fractions will contain the majority of the purified IgG.

Tip: *We recommend that you collect the sample in 0.5 mL to 1.0 mL fractions.*

5. Sample neutralization

a. To neutralize the purified fractions, add either 0.1 mL of Protein A binding buffer or 0.05 mL of 1 M Tris, pH 9.5 to each 1 mL fraction.

Tip: *The advantage of using the 1 M Tris solution is that it will allow you to keep your samples in smaller volumes.*

6. Identify fractions containing purified IgG

a. Zero a spectrophotometer set at 280 nm. Use 1 mL of Protein A elution buffer with 50-100 µL of neutralization buffer to set the spectrophotometer to a zero reading.

b. Measure the absorbance of each fraction at the 280 nm reading.

c. The fractions with the highest absorbance readings will contain your purified IgG.

d. Pool only the purified fractions with the highest absorbance readings.

Tip: *You can read the absorbance of the fractions before or after neutralization.*

7. Protein A affinity column regeneration and storage

a. Wash it with 8 mL of 0.1 M citric acid, pH 3.0 (adjust pH with NaOH).

b. To store the Protein A affinity column, wash it with 5.0 mL of a 0.02% solution of sodium azide in water. Recap the column when 1-2 mL of water remains above the disc at the top of the column. Recap column by replacing the bottom cap first. Store column upright at 4 °C.

D. IgG Purification Using the Enchant™ Kit (Protein G)

1. Protein G affinity column equilibration and sample preparation

a. Bring all kit components and sample to same temperature. This procedure can be performed either at room temperature or in a cold room. If all components are not at the same temperature, air bubbles in the column could result.

b. Dilute sample (serum, plasma, ascites fluid, or tissue culture supernatant) 1:1 with Protein G binding buffer before applying to the Protein G affinity column.

Tip: *Plasma samples will become cloudy/opaque after dilution with the Protein G binding buffer. This is a result of lipoprotein precipitation. If this occurs, centrifuge the sample at 10,000 x g for 15 minutes (or until clear).*

c. Open the Protein G affinity column by removing the top cap first. Decant the storage buffer. Remove the cap on the bottom of the column. Place Protein G affinity column in a standard test tube or test tube holder.

Tip: *Retain caps for storage and column regeneration.*

d. Pipette 5 mL of Protein G binding buffer to the Protein G affinity column. Allow the binding buffer to pass through the column. Collect binding buffer in standard test tube. This will equilibrate the Protein G affinity column.

2. IgG depletion with Protein G

a. Dilute 2 mL of sample 1:1 with Protein G binding buffer before applying to Protein G affinity column.

Tip: *Plasma samples will become cloudy/opaque after dilution with the Protein G binding buffer. This is a result of lipoprotein precipitation. If this occurs, centrifuge the sample at 10,000 x g for 15 minutes (or until clear). Apply cleared supernatant to the Protein G affinity column.*

b. Apply at least 5 mL of diluted sample to the Protein G affinity column and allow sample to pass through the column.

c. Collect 1 mL fractions and set aside for analysis.

d. Add 10-15 mL of Protein G binding buffer to the column to wash IgG depleted sample off.

e. Continue to collect 1 mL fractions.

f. Measure the A_{280} of each fraction. The fractions with the highest absorbance reading will contain the IgG depleted sample. This will typically be 2-4 fractions.

3. IgG purification with Protein G

a. Transfer the Protein G affinity column to a second test tube and apply at least 5 mL of diluted sample to the Protein G affinity column and allow sample to pass through the column.

b. Collect the filtrate solution in a test tube and set aside for analysis of binding efficiency.

c. Add 10 mL of Protein G binding buffer to the column to wash the Protein G affinity column.

d. To verify that all unbound proteins are washed away, collect filtrate in 2 mL fractions. Measure their A_{280} readings. The last fractions should have absorbance readings that are the same as the Protein G binding buffer.

4. IgG elution with Protein G

a. Pipette 6 mL of elution buffer to the Protein G affinity column to recover the bound IgG.

b. Collect the filtrate in numbered collection tubes. For collection purposes, it is important to know where in the column your sample is. The gel bed holds approximately 2 mL of solution. If you have 2 mL of sample, then the entire sample will fit into the gel bed. As the sample passes through the column, the first 2 mL that pass through the column will contain some of the elution buffer and some of the purified IgG. The next fractions will contain the majority of the purified IgG.

Tip: *We recommend that you collect the sample in 0.5 mL to 1.0 mL fractions.*

5. Sample neutralization

a. Prepare a high ionic strength alkaline neutralization buffer. 1 M phosphate, pH 7.5-9.0 or 1 M Tris, pH 7.5-9.0 are sufficient.

b. If using a neutralization buffer with a pH of 7.5, add 0.1 mL to each 1 mL fraction. If using a neutralization buffer with a pH of 9.0, add 0.05 mL to each 1 mL fraction.

6. Identify fractions containing IgG

- **a.** Zero a spectrophotometer set at 280 nm. Use 1 mL Protein G elution buffer with 0.05-0.1 mL of neutralization buffer to zero the spectrophotometer.
- **b.** Measure the A_{280} of each fraction.
- **c.** The fractions with the highest absorbance readings will contain the purified IgG.
- **d.** Pool only the purified fractions with the highest absorbance readings.

7. Protein G affinity column regeneration and storage

- **a.** To regenerate the Protein G affinity column, wash it with 5 mL of Protein G elution buffer.
- **b.** To store the Protein G affinity column, equilibrate it with 5 mL of binding buffer with 0.02% sodium azide. Recap the column when 1-2 mL of buffer remains above the disc at the top of the column. Recap column by replacing bottom cap first. Store upright at 4 °C.

E. Sample Desalting

1. Bring desalting columns to room temperature. It is important that the column, sample, and buffer are all at the same temperature.

- **a.** Prepare 100 mL of exchange buffer.
- **b.** Remove the top cap from the desalting column and pour off storage solution.
- **c.** Remove the bottom cap of the desalting column. Add 10 mL of desired desalting buffer and allow it to pass completely through the desalting column.
- **d.** Prepare a series of collection tubes (test tubes) numbered 1-10.
- **e.** Pipette 1 mL of IgG sample to the desalting column and collect the first 1 mL of sample into the first numbered collection tube.
- **f.** When the sample drains down to the disc at the top of the gel bed, add desalting buffer to the column and continue collecting the samples in 0.5 mL fractions. Collect each 0.5 mL fraction in separate collection tubes.
- **g.** To identify which fractions contain the desalted, purified IgG, measure each fraction's absorbance reading at 280 nm and compare it with the desalting buffer.
- **h.** The fractions with the high absorbance values can then be pooled.
- **i.** After collecting the antibody, wash the desalting column with an additional 15 mL of desalting buffer with 0.02% sodium azide. Cap the desalting column when approximately 2 mL of buffer remains above the disc at the top of the column. Store the column upright at 4 °C.

F. Serial Depletion of IgG and Albumin from Serum/Plasma Using the Enchant™ Albumin Depletion Kit (PN 5300-ALBDEP) in Conjunction with the Enchant IgG Purification Kit (PN 5300-IGGPROA or PN 5300 IGGPROG)

Tip: *For efficient removal of albumin and IgG, the new Enchant Multi-Protein Affinity Separation Kit (PN 5300-AFFMPS), based on an immunochemical affinity ligand, is highly recommended (see Section 2.1.4, page 33). As an alternative, the following protocol can be used for serial removal.*

If use of a dye-based method for albumin depletion as part of a combined albumin/IgG removal is preferred, then the protocol below is recommended. This protocol combines the use of the Enchant™ Albumin Depletion Kit (PN 5300-ALBDEP) with the Enchant IgG Purification Kit (PN 5300-IGGPROA or 5300-IGGPROG).

The IgG Purification Kit should be used first since it can process a larger sample volumes than the Albumin Depletion Kit. The binding capacity of the affinity columns in the Enchant IgG Purification Kit allows for removal of IgG from 1 to 2 mL of plasma/serum. The total IgG content of serum is typically 10-15 mg/mL, while the specific IgG of interest only accounts for 2-5% of this total. Protein A or Protein G IgG Purification Kits can be used depending on the species the sample was isolated from and the antibody isotype that needs to be depleted. Albumin is the most abundant protein in serum at 30-40 mg/mL. The Albumin Depletion Kit can process 0.01-0.1 mL of serum or plasma. Each disc can bind ≥ 2 mg of albumin.

All kit components should be at the same temperature to avoid bubbles, which could prevent flow.

Start with IgG depletion.

1. Dilute 1 mL of sample 1:1 with binding buffer (supplied in the IgG Purification Kit) before applying to the column.

Tip: *Plasma samples will become cloudy/opaque after dilution with the binding buffer. This is a result of lipoprotein precipitation. If this occurs, centrifuge the sample at 10,000 x g for 15 minutes (or until clear).*

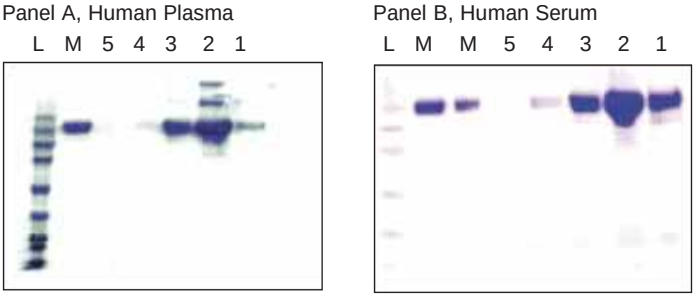
2. Equilibrate by washing with 5 mL of binding buffer. Allow buffer to pass through column by gravity flow.
3. Add diluted sample to Protein A or Protein G affinity column and allow it to flow through the column.
4. Collect 1 mL fractions and set aside for analysis. Wash the column by passing 10-15 mL of binding buffer through the column by gravity flow.
5. Collect 1 mL fractions and set aside for analysis. Typically the IgG depleted sample is distributed between 4 fractions. Fractions with the highest protein content can be pooled together and processed for albumin depletion. The concentration of albumin in these pooled fractions is typically around 10 mg/mL.
6. Continue with albumin depletion. Follow Step B2 of the Enchant Albumin Depletion Protocol on page 12.
7. The bound IgG can be eluted from the column using the elution buffer supplied with the kit. Eluted fractions can consequently be neutralized with alkaline buffers and desalted using the desalting columns provided with the kit. By measuring absorbance at 280 nm of the eluted IgG fractions, the amount of IgG that was removed by the column can be quantified.
8. The Protein A and Protein G columns can be regenerated and reused.

Application Data for Enchant™ IgG Purification Kit

Effective Affinity Purification of IgG with Protein A

Protein A can be used to purify human IgG from plasma and serum samples. Figure 2.7 shows the efficient recovery of IgG from the Protein A affinity column.

Figure 2.7

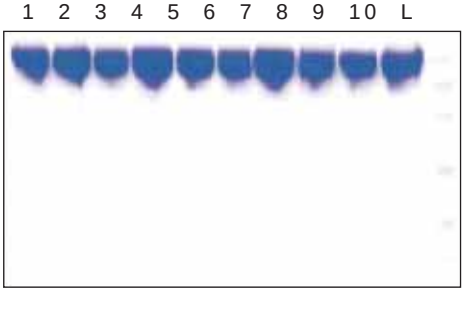


Panel A: Human plasma samples were affinity purified with the Enchant Protein A IgG Purification Kit. Panel B: Human serum samples were affinity purified with the Enchant Protein A IgG Purification Kit. Both purified samples were eluted in five 1 mL fractions, resolved by SDS-PAGE gel electrophoresis, and stained with Coomassie Blue. Lanes 1-5 show the eluted fractions with purified IgG in Lane M. Molecular weight markers were resolved in Lane L. The gels were run under non-reducing conditions.*

Regenerated Columns Provide Efficient Purification of IgG

Due to the high stability of Protein A and its linkage to the affinity support, it is possible to re-use the affinity column for purification of IgG. An example of 10 rounds of affinity purification of IgG using the regeneration protocol described above is summarized in Figure 2.8. The SDS-PAGE analysis clearly shows a highly reproducible recovery of IgG from a human serum sample.

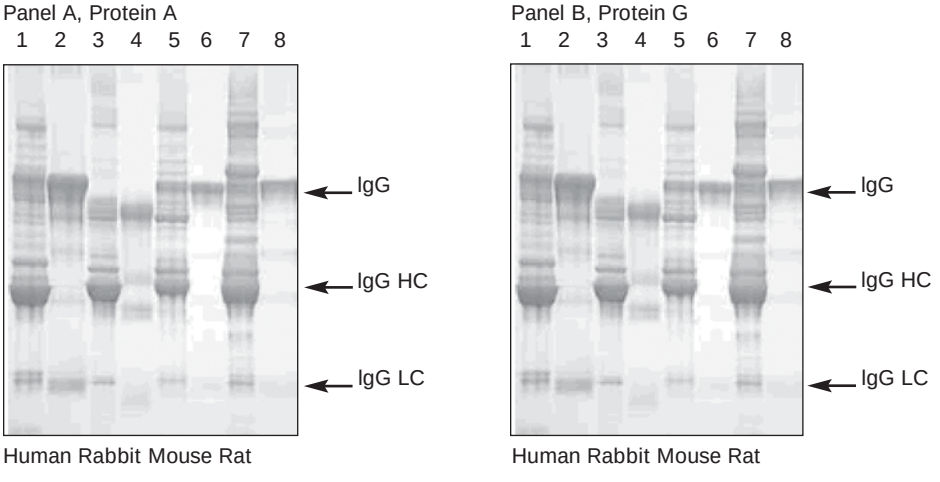
Figure 2.8



Human serum samples were processed using the Enchant Protein A IgG Purification Kit. IgG was effectively purified 10 times using a regenerated Protein A column. The resulting pooled peak fractions were analyzed on a SDS-PAGE gel (Lanes 1-10) and stained with Coomassie Blue.*

Purification of Different Species' IgGs Using Enchant™ Protein A and Protein G IgG Purification Kit
The Enchant purification kit can be used with a range of species for the purification of IgG from serum or plasma. An example of each affinity ligand purification of IgG from human and rat plasma and serum from rabbit and mouse is shown in Figure 2.9. Panel A shows Protein A and Panel B shows Protein G.

Figure 2.9



One mL of plasma (human, rat) or serum (rabbit, mouse) was processed according to manufacturer's instructions. The plasma or serum starting material (1 µL Lanes 1, 3, 5, 7) and one eluate from each species with an A280 of approximately 1.0 (10 µL Lanes 2, 4, 6, 8) were loaded onto 4-12% SDS-PAGE gels and resolved under non-reducing conditions in MOPS/SDS running buffer. Proteins were visualized with colloidal Coomassie Blue staining. Efficient purification occurred with each species processed. Panel A shows purification by Protein A and Panel B shows Protein G.*

FAQ and Troubleshooting for Enchant™ IgG Purification Kit

1. How do I select which affinity column will be optimal for purifying my antibody of interest?

   There are differences in the affinity of Protein A and Protein G for antibodies depending on species and isotype. The following chart provides a guideline for selecting the proper affinity column based on the antibody and isotype being purified.

Species	Antibody Class	Protein A	Protein G
Human	Total IgG	+++	+++
Human	IgG1	+++	+++
Human	IgG2	+++	+++
Human	IgG3	+	+++
Human	IgG4	+++	+++
Human	IgM	+	None
Human	IgD	None	None
Human	IgE	++	None
Human	IgA	+	None
Human	IgA1	+	None
Human	IgA2	+	None
Human	Fab	+	+
Human	ScFv	+	None
Mouse	Total IgG	+++	+++
Mouse	IgG1	+	++
Mouse	IgG2a	+++	+++
Mouse	IgG2b	+++	+++
Mouse	IgG3	+++	+++
Mouse	IgM	None	None
Rat	Total IgG	+	++
Rat	IgG1	+	++
Rat	IgG2a	None	+++
Rat	IgG2b	None	+
Rat	IgG2c	+++	+++
Horse	Total IgG	+	+++
Horse	IgG(ab)	+	None
Horse	IgG(c)	+	None

(continued)

Species	Antibody Class	Protein A	Protein G
Horse	IgG(T)	None	+++
Cow	Total IgG	+	+++
Cow	IgG1	+	+++
Cow	IgG2	+++	+++
Goat	Total IgG	+	+++
Goat	IgG1	+	+++
Goat	IgG2	+++	+++
Sheep	Total IgG	+	+++
Sheep	IgG1	+	+++
Sheep	IgG2	+++	+++
Cat	Total IgG	+++	+
Chicken	Total IgY	None	None
Dog	Total IgG	+++	+
Donkey	Total IgG	++	+++
Guinea Pig	Total IgG	+++	+
Hamster	Total IgG	++	++
Pig	Total IgG	+++	+
Rabbit	Total IgG	+++	+++
Rhesus Monkey	Total IgG	+++	+++

Key: Strong Binding = +++, Medium Binding = ++, Weak Binding = +, No Binding = None

2. Can I neutralize my Protein A purified fractions?

   Yes. To neutralize the purified 1 mL fractions, add either 0.1 mL of binding buffer or 0.05 mL of 1 M Tris, pH 9.5. The advantage of using the 1 M Tris solution will allow you to keep your samples in smaller volumes.

3. Can I neutralize my Protein G purified fractions?

   Yes. Prepare a neutralization buffer. 1 M phosphate, pH 7.5-9.0 or 1 M Tris, pH 7.5-9.0 are sufficient. If using a neutralization buffer with a pH of 7.5, add 0.1 mL to each 1 mL fraction. If using a neutralization buffer with a pH of 9.0, add 0.05 mL to each 1 mL fraction.

4. What is the gravity flow rate of the desalting columns?

   The flow rate of the desalting columns is approximately 0.1 mL per minute. Flow on the desalting column will stop if the sample drains down to the disc on the top of the column bed. Do not allow the gel bed in the column to dry out.

5. What is the binding capacity of the Protein A and Protein G affinity columns?

   The total IgG content of serum is typically 10-15 mg/mL. The binding capacity of the affinity columns is enough to purify 1-2 mL of serum.

6. Can I reuse my Protein A and Protein G affinity purification columns?

   Yes. The Protein A and Protein G affinity purification columns can be washed and stored. Each column can be used for 10 purifications without loss of binding capacity. See protocol section for column regeneration and storage procedures (pages 24 and 26).

7. Is the flowthrough sample from the Enchant™ IgG Protein A or Protein G Purification Kit compatible with binding to the albumin depletion discs?

   You can use the collected peak fraction from the IgG depleted flowthrough fractions directly in albumin depletion. Desalting is optional and does not seem necessary for human samples. It may be necessary to optimize this step for species other than human where the binding to the albumin depletion resin may not be as strong.

8. How much of the flowthrough from an Enchant IgG Protein A or Protein G Purification Kit can you load onto an albumin depletion disc?

   It is important not to exceed the binding capacity of each albumin depletion disc. Each disc will bind ≥ 2 mg of human serum albumin and requires a load volume of at least 0.05 mL. Since larger volumes (minimum 1.0 mL) are run on the Enchant IgG purification columns, the sample needs to be diluted up to 1:4 in the flowthrough fraction. You can load up to 0.1 mL of the sample recovered from the Enchant IgG Purification Kits. It should contain approximately 1 mg of albumin, which is well within the binding capacity of the resin and will give efficient albumin depletion. To increase throughput, reduce the volume of the flowthrough sample by 100-150% using a Nanosep® UF centrifugal device with a 10K MWCO membrane (see Section 2.4.2, page 154) and use 0.1 mL of the retentate to load approximately 2-3 mg of albumin.

Ordering Information for Enchant IgG Purification Kit

Part Number	Description	Pkg
5300-IGGPROA	Enchant Protein A IgG Purification Kit (5 Protein A affinity purification columns, 5 desalting columns, 1 liter Protein A binding buffer, 500 mL Protein A elution buffer)	50 purifications
5300-IGGPROG	Enchant Protein G IgG Purification Kit (1 Protein G affinity purification column, 5 desalting columns, 240 mL Protein G binding buffer, 120 mL Protein G elution buffer)	10 purifications

Section 2.1.4 Enchant™ Multi-Protein Affinity Separation Kit

Over the past decade, the interest in proteomics research has increased dramatically. Meaningful proteomics data rely on the development of reproducible and rapid methods. Achieving this methodology has been as challenging as the biological studies. Sample preparation has received a great deal of focus with particular emphasis on plasma and serum, two of the most readily accessible biological fluids. The concentrations of individual proteins in these samples range from > 10 mg/mL to < 1 pg/mL. As a result, the most abundant proteins often mask other proteins present in relatively low concentrations. Removal of the most abundant proteins is one of the few options for scientists interested in detecting the moderate to low abundancy proteins using mass spectrometry (MS) or two dimensional gel-based (2D gel) proteomic analytical methods. The removal of abundant proteins must be balanced with the desire to minimize sample handling to limit protein loss while maintaining reproducibility and ease-of-use characteristics.

In this section, a new kit for the depletion of human serum albumin (HSA) and human IgG is described. The kit utilizes a protein-based specific capture method to achieve maximal depletion of target proteins and minimal loss of other proteins. Depletion of HSA and IgG is typically > 98%.

Depletions take approximately 20 minutes from start to finish (see Figure 2.10, page 36). The Enchant Multi-Protein Affinity Separation Kit uses Nanosep® devices (loaded with HSA and/or IgG depleting resins), which are disposable, to eliminate the possibility of cross-contamination. Each column is designed for depletion of 50 µL of human plasma or serum, resulting in more depleted samples per column than most other commercially available kits. The increased amount of protein allows for greater flexibility in the number and type of analysis performed after the depletion step. The resins are mixed by the user so it is easy to alter the ratios of HSA and IgG depletion resin when working with unusual samples containing higher or lower than normal concentrations of these proteins (e.g., specific diseased states). Additionally, the use of specific ligands for the capture of HSA and IgG overcomes the limitations of the commonly used blue dyes (e.g., Cibacron* Blue) for albumin removal. These dyes are known to capture many other proteins. Protein A, used for antibody capture, shows widely varying degrees of affinity for the antibody isotypes and sub-types. This results in incomplete removal of some sub-types of IgG.

A protocol is described for serial or combined depletion of these two abundant serum/plasma proteins under native or denaturing conditions. The latter being a novel application of these stable immunochemical ligands leading to reduced, non-specific binding of serum/plasma proteins to the retained target protein. This new ligand-based depletion method is highly specific, disposable, cost-effective, and flexible. Specifications are summarized in Table 2.3.

Table 2.3
Specifications of the Enchant™ Multi-Protein Affinity Separation Kit

Specification	Parameter
Binding Capacity	> 98% removal of albumin and IgG from 50 µL of serum/plasma diluted 1:5
Maximum Sample Volume	50 µL
Sample Type and Species	Human serum or plasma
Specificity	> 90% of proteins bound during fractionation will be target protein when the eluate is measured by SDS-PAGE
Storage Conditions	Store at 4 °C
Shelf Life	One year from the date of purchase, if stored properly
Centrifugal Device Dimensions Outside Diameter (Maximum) Overall Length (Fully Assembled)	 Fits 1.5 mL microcentrifuge rotors 4.5 cm (1.8 in.)
Recommended Centrifugal Force	800-900 x g (3,000 rpm)
Anti-HSA Fractionation Resin Volume	10 mL. Resin is supplied in an aqueous slurry containing 0.02% (w/v) sodium azide.
Anti-IgG Fractionation Resin Volume	10 mL. Resin is supplied in an aqueous slurry containing 0.02% (w/v) sodium azide.
Binding/Wash Buffer Volume pH	 20 mL 7.4
Kit Components	
Nanosep® Centrifugal Device	
Quantity Filter Media Sample Reservoir Membrane Support Base Filtrate Receiver	24 complete columns 0.45 µm GHP membrane (hydrophilic polypropylene) Polypropylene Polypropylene Polypropylene

Protocol for Enchant™ Multi-Protein Affinity Separation Kit

A. Materials Required

1. Materials supplied in the kit (PN 5300-AFFMPS):

a. Nanosep® devices with 0.45 µm GHP membrane (hydrophilic polypropylene)

b. Anti-HSA fractionation resin. Volume: 10 mL. Resin is supplied in an aqueous slurry containing 0.02% (w/v) sodium azide.

c. Anti-IgG fractionation resin. Volume: 10 mL. Resin is supplied in an aqueous slurry containing 0.02% (w/v) sodium azide.

d. Binding/wash buffer volume: 20 mL

2. Additional materials needed:

a. Micro-centrifuge: 1.5-2.0 mL volume tubes, angle rotor, variable speed up to 12,000 x g. Refrigeration not required.

b. If doing depletion under denaturing conditions: 9 M urea, 2% (v/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) in 50 mM Tris, pH 9.0

c. If eluting bound IgG and/or HSA: 0.1 M glycine HCL, pH 2.3

d. To neutralize eluate: 1 M Tris-HCl, pH 8

B. Depletion of Albumin and IgG from Plasma or Serum

1. Sample preparation

a. Native conditions

(1) Plasma or serum samples should be diluted 1:5 or 1:4 in binding buffer. To dilute 5-fold, add 0.2 mL of binding/wash buffer to 0.05 mL of serum or plasma.

(2) Following dilution, keep the samples on ice until they are loaded into the Nanosep centrifugal device packed with the fractionation resin(s).

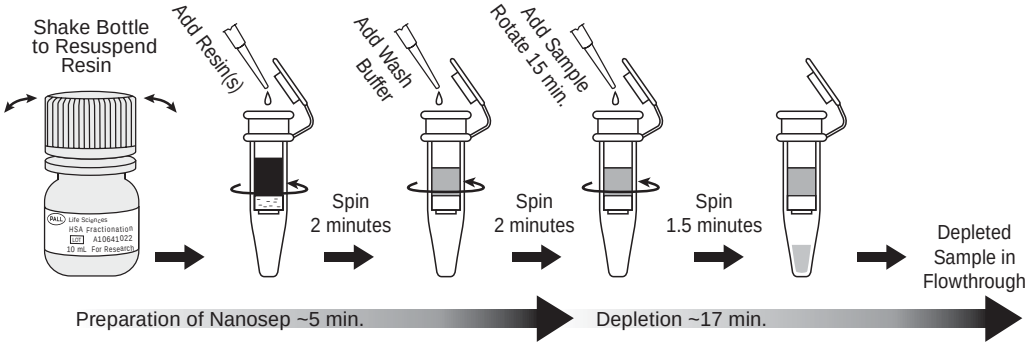
Tip: *Visually inspect samples. If there is significant precipitate in the sample, a filtration or centrifugation step is recommended prior to fractionation (see Table 2.4). This can be done before or after sample dilution. The overall performance and reproducibility of the fractionation and other downstream methods will be improved.*

Table 2.4
Product Recommendations

Sample Volume	Device Part	Part Number
100-150 mL	Serum Acrodisc® Syringe Filter	4525
0.05-0.5 mL	Nanosep Centrifugal Device	ODGHPC34
Up to 0.3 mL	AcroPrep™ 96 Filter Plate	5030

Tip: *Serum and plasma samples do not go through freeze-thaw cycles well. There are changes that occur in the proteins as well as an increased incidence of cryoprecipitates. It is recommended that samples be aliquoted to minimize freeze-thaw cycles if they will be used more than once.*

Figure 2.10
Schematic of Depletion Methods



b. Denaturing conditions.
It is well known that abundant proteins, such as albumin (a natural carrier protein) and to a lesser extent IgG, when removed from a serum or plasma sample, will co-purify with other proteins. There is increased interest in studying this population of proteins since they may have a biological significance in various physiological states. However, most applications of standard depletion for complexity reduction desire minimal loss of moderate to low abundance proteins. In addition, in all bead-based approaches, the possibility of some non-specific binding to the ligand or the support is fairly high. To minimize both of these potential problems, we have developed a protocol for ligand-specific depletion of both human albumin and IgG from serum or plasma in the presence of urea, a chaotropic agent, and CHAPS, a strong zwitterionic detergent widely used in Proteomics, prepared in Tris buffer at pH 9.0.

(1) Make a fresh batch of 9 M urea + 2% (w/v) CHAPS in 50 mM Tris, pH 9.0, using high quality water. Alternatively, the solution can be made in advance and stored in aliquots at -20 ºC.

Tip: *Older preparation of urea stored at 4°C or room temperature can result in carbamylation of proteins during sample handling. This can lead to charge trains in the isoelectric focusing step of two dimensional gel electrophoresis and the formation of mass covalent adducts, which can make MS analysis much more difficult.*

(2) The spin columns are designed for depletion of IgG and albumin from 0.05 mL of serum or plasma. To this amount of sample, add 0.063 mL of 9 M urea + 2% (v/v) CHAPS in 50 mm Tris, pH 9.0, to give a final concentration of 5 M urea. Incubate at room temperature for 30 minutes.

(3) Add 0.136 mL of binding buffer for a final volume of 0.25 mL with a final urea concentration of 2.25 M. Store samples at room temperature for use within 8 hours or store frozen at -20 ºC.

Tip: *Visually inspect samples prior to dilution. If there is significant precipitate in the sample, a filtration or centrifugation step is recommended prior to fractionation (see Table 2.4).*

2. Single protein fractionation (IgG or HSA)

a. Ensure that the anti-IgG or anti-HSA fractionation resin is in a homogenous suspension prior to pipetting into the Nanosep® centrifugal device. To minimize the formation of bubbles in the bottle, gently roll the bottle between the palms of your hands.

b. Pipette 0.4 mL of the appropriate fractionation resin into a Nanosep centrifugal device. Centrifuge at 800-900 x g (3,000 rpm) for 2 minutes and discard filtrate.

Tip: *Draw 0.4 mL of resin up into the pipette tip and dispense back into the bottle. Draw another 0.4 mL up into the pipette tip and dispense that into the Nanosep centrifugal device.*

If your samples contain higher than normal amounts of IgG or HSA, additional resin can be added to the Nanosep spin filter to accomodate the greater target protein load.

c. Wash 1 time with binding/wash buffer. Add 0.4 mL of binding/wash buffer and vortex. Centrifuge at 800-900 x g (3,000 rpm) for 2 minutes and discard wash solution.

d. Add 0.25 mL of diluted sample (diluted 1:5 in binding/wash buffer prepared in Step B1a on page 35 for native conditions or Step B1b on page 36 for denaturing conditions) to the resin-packed Nanosep centrifugal device.

e. Mix well by vortexing and then tumble end-over-end for 15 minutes at room temperature.

f. Centrifuge at 800-900 x g (3,000 rpm) for 1.5 minutes and collect flowthrough. The flowthrough is your albumin or IgG depleted sample.

g. Wash the resin 3-4 times with 0.4 mL of binding/wash buffer and vortex. Use an A_{280} measurement after last wash to ensure that no more protein is being washed off. Retain collected fractions for further analysis.

h. To elute the bound protein, add 0.25-0.4 mL of 0.1 M glycine HCl, pH 2.3 (not included in the kit).

i. Mix well by vortexing and tumble end-over-end for 15 minutes at room temperature.

j. Centrifuge at 800-900 x g (3,000 rpm) for 1.5 minutes and collect the eluted fraction. The bound protein will be present in the eluate fraction.

k. To neutralize eluate, add 0.04 mL (1/10 volume) of 1 M Tris-HCl, pH 8 to bring pH back to neutral (~pH 7).

l. For improved recovery, repeat the elution steps above one more time.

3. Multi-protein fractionation (IgG and HSA)

a. Ensure that the anti-IgG and anti-HSA fractionation resins are in a homogenous suspension prior to pipetting into the Nanosep centrifugal device. To minimize the formation of bubbles in the bottle, gently roll the bottle between the palms of your hands.

b. Pipette 0.4 mL of the IgG fractionation resin into a Nanosep centrifugal device. Centrifuge at 800-900 x g (3,000 rpm) for 2 minutes and discard filtrate.

Tip: *Draw 0.4 mL of resin up into the pipette tip and dispense back into the bottle. Draw another 0.4 mL up into the pipette tip and dispense that into the Nanosep® centrifugal device.*

If your samples contain higher than normal amounts of IgG, additional resin can be added to the Nanosep spin filter to accomodate the greater target protein load.

c. Pipette 0.4 mL of the HSA fractionation resin into the same Nanosep centrifugal device. Centrifuge at 800-900 x g (3,000 rpm) for 2 minutes and discard filtrate.

d. Wash 1 time with binding/wash buffer. Add 400 µL of binding/wash buffer and vortex. Centrifuge at 800-900 x g (3,000 rpm) for 2 minutes and discard wash solution.

e. Add 0.25 mL of diluted sample (diluted 1:5 in binding/wash buffer prepared in Step B1a on page 35 for native conditions or Step B1b on page 36 for denaturing conditions) to the resin packed Nanosep centrifugal device.

f. Mix well by vortexing and then tumble end-over-end for 15 minutes at room temperature.

g. Centrifuge at 800-900 x g (3,000 rpm) for 1.5 minutes and collect the flowthrough. The flowthrough is the albumin and IgG depleted sample.

h. Wash the resin 3-4 times with 0.4 mL of binding/wash buffer and vortex. You can measure the light absorption after the last wash at A280 nm (A_{280}) to ensure that no more protein is being washed off. Retain collected fractions for further analysis.

i. To elute the bound proteins, add 0.25-0.4 mL of 0.1 M glycine HCl, pH 2.3 (not included in kit).

j. Mix well by vortexing and tumble end-over-end for 15 minutes at room temperature.

k. Centrifuge at 800-900 x g (3,000 rpm) for 1.5 minutes and collect the eluted fraction. The bound proteins will be present in the eluate fraction.

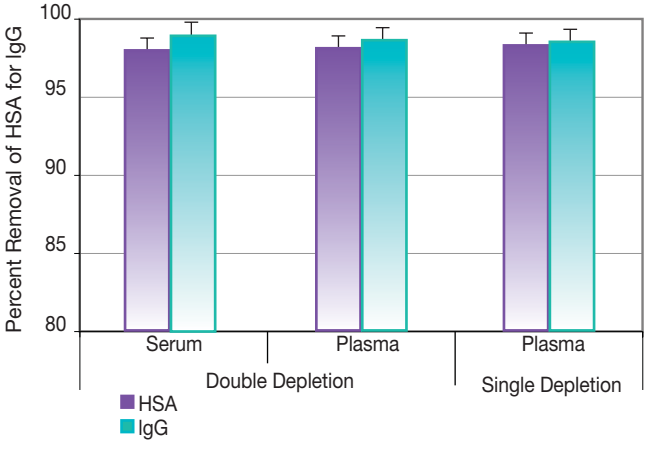
l. To neutralize the eluate, add 0.04 mL (1/10 volume) of 1 M Tris-HCl, pH 8 to bring pH back to neutral (~pH 7).

m. For improved recovery, repeat the elution steps above one more time.

Application Data for Enchant™ Multi-Protein Affinity Separation Kit

HSA and human IgG were independently measured by ELISA. The average of 4-9 replicates, spanning multiple resin lots, is plotted. Error bars represent one standard deviation from the mean. Plasma from single donor is used for single depletion. Pooled plasma and serum is used for double depletion experiments.

Figure 2.11
Efficient Depletion of HSA and IgG from Plasma

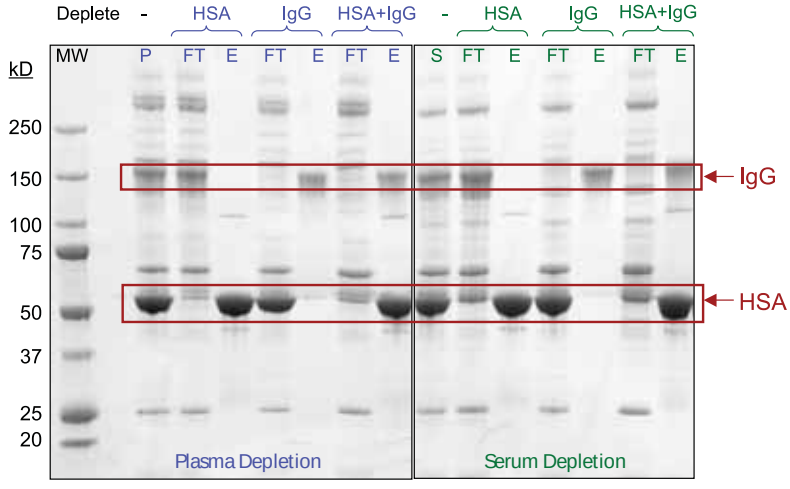


ELISA data indicate very efficient depletion (> 98%) using the Enchant Multi-Protein Affinity Separation Kit. Efficient depletion was obtained after both single protein and double protein depletion.

The albumin/IgG depletion efficiency was evaluated using the Enchant Multi-Protein Affinity Separation Kit for pooled serum and plasma samples. The resulting flowthrough (FT) and elution fractions (E) were anlyzed on ID SDS-PAGE gels. A typical result is shown in Figure 2.12. This data clearly shows the specificity of the two coupled resins in the kit is very good for single or dual depletion.

Figure 2.12

High Specificity of Ligands for the Targeted Protein

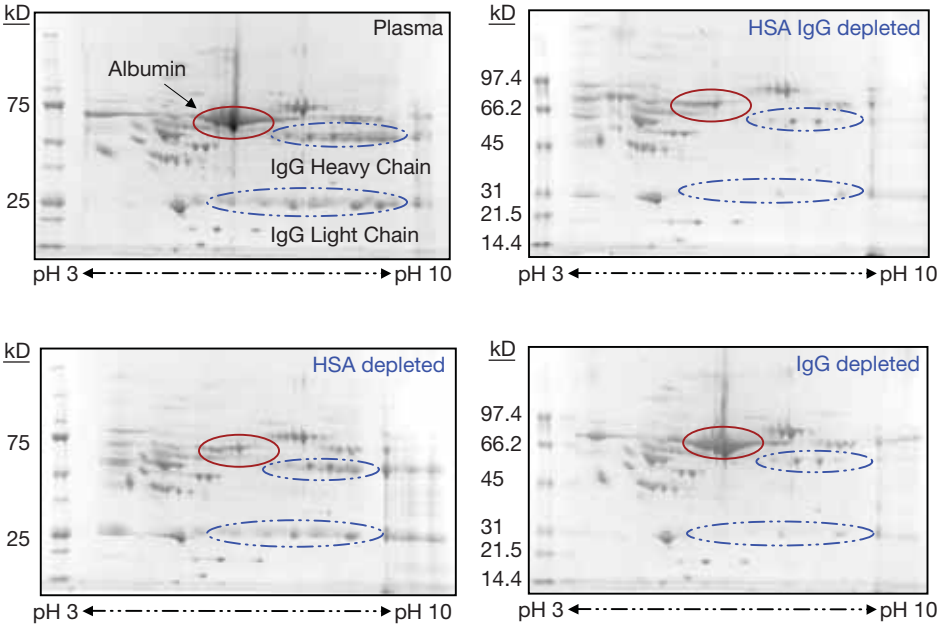


Anti-HSA and IgG coupled resin were placed in Nanosep® GHP spin filters and processed for depletion as described in the protocol section. Samples were analyzed under non-reducing conditions using 4-12% Bis-Tris linear gradient gels (Criterion, BioRad) and stained with colloidal Coomassie Blue. Panel A, human pooled serum lanes: 1) Precision Plus Standards (BioRad); 2) blank; 3) pooled plasma (~ 15 µg protein); 4) HSA depleted FT; 5) HSA depletion eluate (E); 6) IgG depleted FT; 7) IgG depletion eluate (E); 8) HSA and IgG depletion FT; 9) HSA and IgG depletion eluate (E); and 10) purified IgG and HSA. Panel B, human pooled plasma: 1) pooled human serum (~ 15 µg protein); 2) HSA depletion FT; 3) HSA depletion eluate (E); 4) IgG depletion FT; 5) IgG depletion eluate (E); 6) HSA and IgG depletion FT; 7) HSA and IgG depletion combined eluate (E); and 8) purified IgG and HSA.*

Depletion of abundant proteins, such as HSA and IgG, from complex samples can lead to increased visualization of less abundant proteins in 2DE gel analysis. Several examples of 2DE gel analysis of single and dual depleted samples are shown in Figure 2.13 for pooled human plasma. The data clearly shows that single or dual depletion leads to better resolution of proteins in the area of the 2DE pattern masked by the presence of HSA or IgG.

Figure 2.13

Increase Resolution of Low Abundant Proteins:
2–Dimensional IEF + SDS-PAGE Analysis of Plasma Before and After Single and Double Depletion



Two-dimensional gel electrophoresis (2DGE) analysis of human plasma following single and double depletion of HSA and IgG using the Enchant™ Multi-Protein Affinity Separation Kit. Starting material and depleted filtrate samples with between 100 and 200 µg total protein were analyzed by focusing in the first dimension on 11 cm, pH 3-10 non-linear IPG strip and resolving in the second dimension on a 4-12% Bis-Tris gel (Criterion, BioRad) under reducing conditions. Proteins were visualized with colloidal Coomassie Blue.*

Ordering Information for Enchant™ Multi-Protein Affinity Separation Kit

Part Number	Description	Pkg
5300-AFFMPS	Enchant Multi-Protein Affinity Separation Kit (24 Nanosep® for centrifugal devices, 5 mL anti-HSA resin, 5 mL anti-IgG resin, 20 mL wash buffer, 20 mL elution buffer)	24 samples

References for Enchant Multi-Protein Affinity Separation Kit

1. Sinz, A., Bantscheff, M., Mikkat, S., Ringel, B., Drynda, S., Kekow, J., et al. (2002). Mass spectrometric proteome analysis of synovial fluids and plasmas from patients suffering from rheumatoid arthritis and comparison to reactive arthritis or osteoarthritis. *Electrophoresis*, (23), 3445–3456.
2. Iman-Sghiouar, N., Laude-Lemaire, I., Labas, V., Pflieger, D., LeCaer, J.P., Caron, M., et al. (2002). Subproteomics analysis of phosphorylated proteins: application to the study of B-lymphocyctes from a patient with Scott syndrome. *Proteomics*, (2), 828–838.
3. Vejda, S., Posovszky, C., Zelzer, S., Peter, B., Bayer, E., Gelbmann, D., et al. (2002). Plasma from cancer patients featuring a characteristic protein composition mediates protection against apoptosis. *Mol. Cell Proteomics*, (1), 387–393.
4. Hinerfield, D., Innamorati, D., Pirro, J., & Tam, S.W. (2004). Serum plasma depletion with chicken immunoglobulin Y antibodies for proteomics analysis from multiple mammalian species. *J. Biomol. Tech.*, (15), 184–190.

2.1.5 BioSepra® Chromatography Resins

Both immobilized Protein A and immobilized Cibacron* Blue are frequently used for binding IgG and albumin. For situations when a complete kit is not needed, Pall offers bottled chromatography resins for abundant protein binding.

Resins available include: Protein A Ceramic HyperD® for IgG purification or removal and Blue Trisacryl® M for the depletion of albumin. Properties of these resins are highlighted in Table 2.5 and Table 2.6. Pall also offers a unique mixed-mode resin, MEP HyperCel™, which can be used as an alternative to Protein A for IgG purification or depletion. Properties are highlighted in Table 2.7.

Refer to Sections 2.2.3.4, page 100 (Protein A resin), 4.2.3, page 325 (Blue Trisacryl M resin), and 2.2.4.1, page 112 or 4.2.6, page 350 (MEP HyperCel resin) for detail protocols.

Figure 2.14
Molecular Structure of Blue Trisacryl M Resin

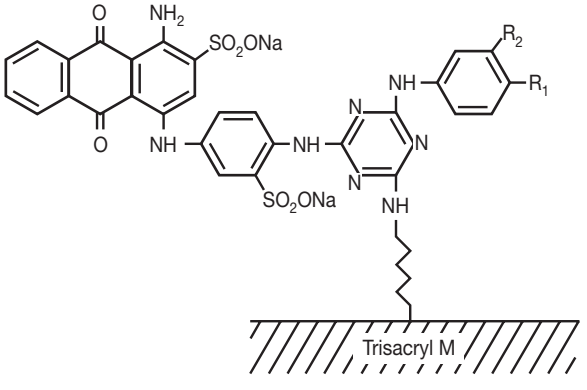


Figure 2.15
Protein Adsorption and Elution Mechanism on MEP HyperCel Resin

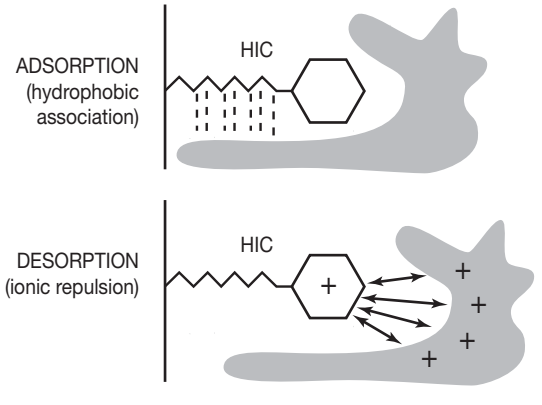
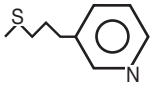


Figure 2.16
MEP HyperCel™ Mimetic Affinity Ligand



4-Mercapto-Ethyl-Pyridine
(4-MEP)
pka-4.8

Table 2.5
Properties of Blue Trisacryl® M Affinity Resin

Specification	Parameter
Particle Size	40-80 µm (M)
Exclusion Limit (daltons)	10^7
Immobilized Dye Loading	4 µmoles/mL gel
Human Serum Albumin (HSA) Binding Capacity	10-15 mg/mL
Bovine Serum Albumin (BSA) Binding Capacity	5-7 mg/mL
pH Stability	1-11
Pressure Stability	Up to 3 bar (300 kPa, 44 psi)

Table 2.6
Properties of Protein A Ceramic HyperD® F Resin

Specification	Parameter
Particle Size	50 µm (average)
Ligand	Recombinant Protein A resin
Ligand Loading	4-5 mg/mL
Human IgG Capacity (10% Breakthrough at 100 cm/h Linear Velocity)*	> 30 mg/mL
Working pH	2-11
Cleaning pH	2-13
Volume Changes Due to pH and Ionic Strength	Non-compressible
Pressure Stability	Up to 70 bar (7,000 kPa, 1015 psi)

**Determined using 10 mg/mL human IgG in phosphate buffered saline (PBS), pH 7.4; Elution with 0.1 M sodium citrate, pH 2.5. Column: 4.6 ID x 100 mm.*

Table 2.7
Properties of MEP HyperCel™ Resin

Specification	Parameter
Particle Size	80-100 µm
Bead Composition	Cellulose
Ligand	4-mercapto ethyl pyridine
Ligand Density	70-125 µmoles/mL media
Working pH Range	3-12
Cleaning pH Range	3-14
Pressure Range	< 3 bar (300 kPa, 44 psi)
Dynamic Binding Capacity for Human IgG	
pH 8.0	> 30 mg/mL media
pH 7.0	25
pH 6.0	15
pH 5.0	5
Salt Impact on Dynamic Binding Capacity for Human IgG	
None	> 30 mg/mL media
0.2 M	> 30
0.6 M	> 30
1.0 M	> 30
Species Affinity	
Human Polyclonal IgG	32 mg/mL media
Murine IgG_1	37
Murine IgG_{2a}	34

Ordering Information for BioSepra® Chromatography Resins

Part Number	Description	Pkg
25896-051	Blue Trisacryl® M	5 mL
25896-045	Blue Trisacryl M	25 mL
25896-010	Blue Trisacryl M	100 mL
25896-028	Blue Trisacryl M	1000 mL
12035-069	MEP HyperCel™	5 mL
12035-010	MEP HyperCel	25 mL
12035-028	MEP HyperCel	100 mL
12035-036	MEP HyperCel	1000 mL
20078-036	Protein A Ceramic HyperD® F	5 mL
20078-028	Protein A Ceramic HyperD F	25 mL
20078-010	Protein A Ceramic HyperD F	100 mL
20078-044	Protein A Ceramic HyperD F	1000 mL

2.2.1 Introduction

Sample complexity reduction is an important first step to facilitating access to the low abundant proteins of interest for clinical research and diagnostics. The process for human serum and plasma frequently includes the depletion of highly abundant proteins such as albumin and IgG in combination with other fractionation technologies, such as ion exchange or affinity separation, prior to 2D gel or LC-MS/MS analyses.

Pall currently offers several solutions for small-scale chromatographic protein fractionation (see Table 2.8). Options include chromatography resin for affinity capture, ion exchange separation, and mixed-mode capture resins. Pall also offers membrane chromatography solutions, i.e., Mustang® membranes. All chemistries are available for use in several forms including standard chromatography columns, disposable columns, spin filters, and multi-well filter plates.

Table 2.8
Protein Fractionation Quick Selection Guide

Media	Description	Formats Available	Separation Mechanism
Ion Exchange			
Q Ceramic HyperD® 20	Resin	Bottled resin	Strong anionic exchanger; binds negatively charged target
Q Ceramic HyperD F	Resin	Bottled resin	Strong anionic exchanger; binds negatively charged target
Mustang Q	Membrane unit	Syringe column, multi-well filter plate	Strong anionic exchanger; binds negatively charged target
S Ceramic HyperD 20	Resin	Bottled resin	Strong cationic exchanger; binds positively charged target
S Ceramic HyperD F	Resin	Bottled resin	Strong cationic exchanger; binds positively charged target
Mustang S	Membrane unit	Syringe column, multi-well filter plate	Strong cationic exchanger; binds positively charged target
DEAE Ceramic HyperD F	Resin	Bottled resin	Weak anion exhanger
CM Ceramic HyperD F	Resin	Bottled resin	Weak cationic exchanger
Affinity			
Heparin	Resin	Bottled resin	Direct binding to targets with an affinity for Heparin
Lysine	Resin	Bottled resin	Direct binding to targets with an affinity for Lysine
IMAC	Resin	Bottled resin	Binds tagged proteins using an immobilized metal compound

Table 2.8 (continued)
Protein Fractionation Quick Selection Guide

Media	Description	Formats Available	Separation Mechanism
Mixed Mode			
MEP	Resin	Bottled resin	Uses several binding mechanisms including hydrophobic interactions
HA Ultrogel®	Resin	Bottled resin	Mixed mode
Size Exclusion			
Ultrogel AcA	Resin	Bottled resin	Separates targets by size

Table 2.9
Available Separation Columns

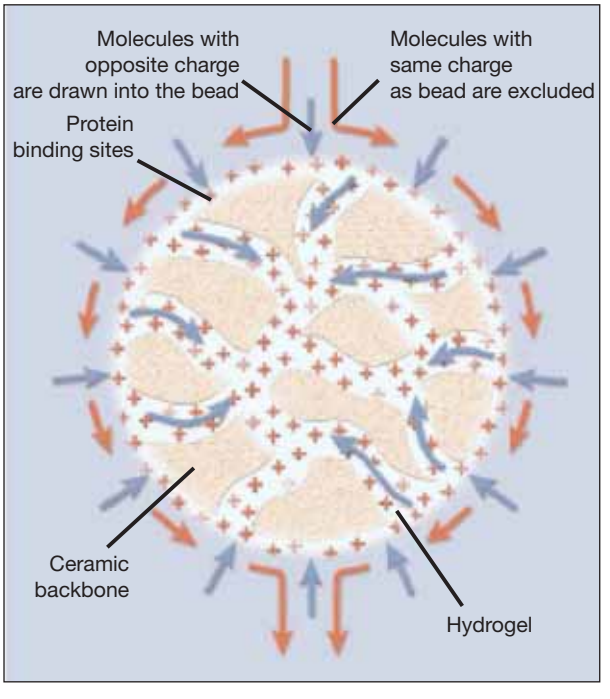
Description	Column Volume	Available from Pall
Glass Chromatography Column	Varies	No
Disposable Chromatography Column	Varies	No
Spin Filter	≤ 1 mL	Yes
Deep Well Multi-Well Filter Plate	96 x ≤ 1 mL	Yes
Multi-Well Filter Plate	96 x < 350 µL	Yes

2.2.2 Q, S, DEAE, and CM Ion Exchange Media

2.2.2.1 Ceramic HyperD® Ion Exchange Resin

BioSepra® Ceramic HyperD ion exchangers employ a high-capacity hydrogel polymerized within the gigapores of a rigid ceramic bead. As shown in Figure 2.17, this design combines the desirable characteristics of a soft, high-capacity hydrogel with the dimensional stability of a rigid ceramic bead. Ceramic HyperD resins do not shrink or swell with changes in pH or salt concentration. Abundant ion exchange sites in the hydrogel are highly accessible to protein molecules. Proteins diffuse rapidly within the hydrogel, increasing the efficiency of protein binding as seen in Table 2.10. This mechanism of mass-transfer – known as enhanced diffusion – allows the resin to operate free of the operational constraints typically encountered with conventional macroporous ion exchange resins.

Figure 2.17
Ceramic HyperD® Resins–'Gel in a Shell' Design



Ceramic HyperD resins deliver outstanding dynamic binding capacity and exceptional dimensional stability due to their unique 'gel in a shell' design.

Table 2.10
Protein Binding Capacity of Ceramic HyperD® Ion Exchange Resins in a Nanosep® GHP Centifugal Device

Media	mg/Device*	mg/Unit Media**
Q Ceramic HyperD	3.4	68.9
DEAE Ceramic HyperD	3.1	62.4
HyperD-S Ceramic	4.5	90.4
HyperD-M Ceramic	4.5	89.0

**A Nanosep GHP device was filled with 0.05 mL of packed bed volume of HyperD ion exchange resins. For anion ion exchange, 2 mL of a 5 mg/mL BSA solution in 25 mM Tris HCl, pH 8.5 was passed through the resin bed, followed by extensive washing with loading buffer and then elution with 1 M NaCl in loading buffer. The eluted protein recovered was estimated by measuring absorbance at 280 nm. For cation ion exchange, a 5 mg/mL Lysozyme solution was used in 10 mM MES-NaOH, pH 5.8.*

***Capacity per mL of packed bed volume.*

The Enhanced Diffusion Concept
Traditional macroporous ion exchangers operate on the basis of classical pore diffusion. Unfortunately, pore diffusion is typically characterized by a rapid decrease in binding capacity with increased flow rates. In contrast, the unique structure of the Ceramic HyperD resin supports a rapid mechanism of enhanced diffusion, thus overcoming the classical flow rate dependence. Since proteins are bound throughout the gel-filled pore, not merely at the interior surface of the pore, total binding capacity is enhanced. Binding of protein within the hydrogel is illustrated by the electron micrograph in Figure 2.18. The hydrogel carries an extraordinarily high concentration of ion exchange functional groups: 150-400 µeq/mL. The average distance between charged sites on the hydrogel is ~20 Å. Thus, a protein molecule within the gel is simultaneously in contact with a large number of ion exchange sites. It remains in contact with a similar number of sites no matter where it moves within the three-dimensional structure of the hydrogel. As a result, the protein is energetically unconstrained and may migrate freely. Protein diffuses rapidly within the hydrogel to give a homogeneous distribution, facilitating uptake of additional material from solution. Under binding conditions, strong, attractive electrostatic forces between the highly substituted hydrogel and the protein drive entry of protein into the gel.

In this section, the focus will be on methods development and scouting protocols employing small gravity flow, single, and multi-well devices describing small-scale protein pre-fractionation. Larger small-scale purification in a gravity flow and packed columns for use on a liquid chromatography instrument are described in Section 4.2.2, page 310.

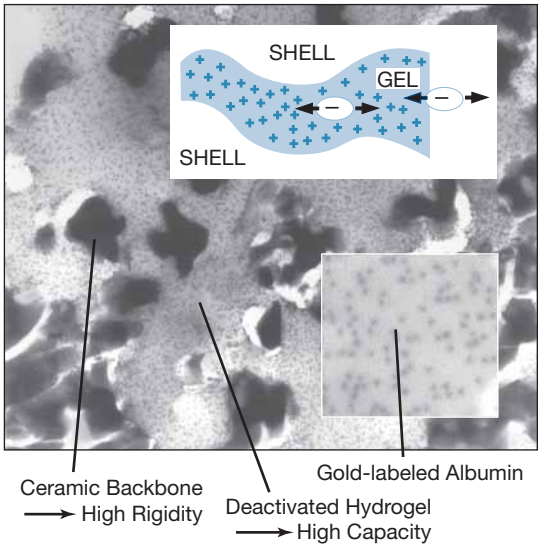
Ceramic HyperD ion exchange resins are available in a variety of chemistries and grades. Table 2.11 highlights the common ion exchangers available, along with their main properties.

Table 2.11
Properties of Ceramic HyperD® Ion Exchange Resin

Type of Ceramic HyperD Resin	Q	S	Q	S	DEAE	CM
Grade	20	20	F	F	F	F
Average Particle Size (µm)	~20	~20	~50	~50	~50	~50
Dynamic Binding Capacity (mg/mL) 10% Breakthrough at 200 cm/h	BSA 85*	Lysozyme 85**	BSA 85*	Lysozyme 75**	BSA 85*	IgG 60***
Amount of Ionic Groups (µeq/mL)	250	150	250	150	200	250-400
Working pH	2-12	2-12	2-12	2-12	2-12	2-12
Cleaning pH	1-14	1-14	1-14	1-14	1-14	1-14
Volume Changes Due to pH and Ionic Strength	Non-compressible					
Pressure Resistance	20 grade: 200 bar (20,000 kPa, 2,901 psi)		F grade: > 70 bar (7,000 kPa, 1,015 psi)			

**Sample: 5 mg/mL BSA in 50 mM Tris-HCl buffer, pH 8.6.*
***Sample: 5 mg/mL lysozyme in 50 mM sodium acetate, pH 4.5.*
****Sample: 5 mg/mL Human IgG in 50 mM sodium acetate, 100 mM NaCl, pH 4.7.*

Figure 2.18
Structure of Ceramic HyperD Ion Exchange Resins



A cross section through the bead shows binding of gold-labeled albumin. Notice that the hydrogel completely fills the pores within the ceramic shell and that gold-labeled albumin – visible as dense black dots – is distributed throughout the hydrogel.

Protocol for Ceramic HyperD® Ion Exchange Resin

A. Materials Required

1. Container/column for ion exchange resin, one of the following:

a. Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922)

b. Nanosep® MF centrifugal device with 0.45 µm GHP membrane (PN ODGHPC34)

c. Choose either:

(1) AcroPrep™ 96 filter plate, 350 µL well, 0.45 µm GHP membrane (PN 5030); 0.45 µm Supor® membrane (PN 5029); 1.2 µm Supor membrane (PN 5039); or

(2) AcroPrep 96 filter plate, 1 mL well, 0.45 µm GHP membrane (PN 5054)

d. Glass column 6.6 mm ID x 10 cm length, 1-2 mL volume (e.g., OmniFit PN 006CC-06-10-AF)

2. Needed if filter plate is used:

a. Collection plates [e.g., 96 well polypropylene V bottom, 0.5 mL (Axygen PN P96450V) or 1.64 mL round bottom (Axygen PN PDW20)]

b. Adhesive plate sealing film

c. AcroPrep 96-well plate sealing cap mat (PN 5230)

3. Separation apparatus (if filtration device is used)

a. Source of vacuum 25.4-50.8 cm Hg (10-20 in. Hg) (PN 5017); or

b. Centrifuge fitted with a swinging bucket rotor

4. Degassed* 50% (v/v) slurry of the HyperD ion exchange resin

5. Degassed* suitable buffer, such as 50 mM Tris HCl pH 8.5 (anion exchange) or 50 mM sodium acetate, pH 4.5 (cation exchange) with an ionic strength in the range of 4-5 mS/cm as measured with a conductivity meter. Depending on how some buffers are made up and adjusted to their final pH, it may or may not be necessary to adjust the ionic strength to the indicated range with NaCl.

Tips on Handling Ceramic HyperD Resin:
*For packed columns, use only degassed liquids. *Degassing is not necessary for batch mode methods.*

Some BioSepra® media are supplied as concentrated slurries and may be difficult to resuspend. Do not use magnetic stir bars with BioSepra media as they can damage the beads. Also, these resins are quite dense and settle quickly. When adding slurry to any device, mix well between additions. If it is necessary to prepare a 50% (v/v) slurry, use a clean spatula to remove some of the packed media and transfer to a graduated glass cylinder containing buffer. DO NOT add any media back to the storage bottle to avoid contamination of the bulk media. Thoroughly mix and allow settling. Note the volume of settled resin. Decant the supernatant and add back an equal volume of buffer to make a 50% (v/v) slurry.

For packed columns, removal of fines may be necessary. Prepare the slurry in desired buffer, mix, and allow to settle for approximately 5 minutes or for enough time that the beads have settled but small particles are still in the solution. Decant off the suspension of fine particles and add fresh buffer and re-mix. Repeat the process until particles settle within 5 minutes and leave a clear supernatant.

B. Packing Ceramic HyperD® Ion Exchange Resin

1. Gravity flow column format

- **a.** Equilibrate column, 50% gel slurry, and buffer solution at room temperature.
- **b.** Secure a bottom cap on the column tip and clamp the column [1-5 mL bed volume column (e.g., Pierce PN 29922)] upright in a laboratory stand.
- **c.** Add a sufficient volume of degassed buffer to the column to fill it up to the reservoir (wide-mouth) portion, and then gently tap the end and side of the column to dislodge any air bubbles.
- **d.** Float a porous disc on top of the liquid within the column.
- **e.** Using the reverse end of a Pasteur pipette or reverse end of a serum separator (e.g., Pierce PN 69710), push the disc evenly to the bottom of the column.
- **f.** Decant most of the liquid from the empty column, being sure to avoid getting air bubbles in the tip region of the column below the inserted disc. Place the column back in its stand with bottom cap still in place.
- **g.** Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 1-2 mL.
- **h.** Allow gel to settle in the column for at least 5 minutes.
- **i.** Position a second porous disc on top of the settled gel bed by floating it on the liquid within the column and pushing it down to just above the settled gel. Leave 1-2 mm of space between the top of the gel bed and the top disc. Do not compress the gel bed.
- **j.** Wash the inside top part of the column with buffer to remove residual gel that may have remained along the sides during packing.
- **k.** Packed column is now ready for storage at 4 °C or immediate use.

Tip: *Store the packed column upright at 4 °C with the gel bed submerged under 1-2 mL of buffer and a top column cap securely in place. Sodium azide added to the storage buffer to a concentration of 0.02% (w/v) will help prevent microbial growth. Always remove the top cap before the bottom cap to avoid drawing air bubbles down into the gel bed. Prevent air bubbles from forming in the gel bed by using only degassed buffer and sample solutions. Degassing involves subjecting a solution to vacuum to remove excess dissolved air. Use of too high a vacuum can lead to evaporation of any solvent from the solution. Check the final volume after degassing and, if necessary, add more solvent or water to return to original volume.*

- **l.** Refer to Section C on page 55 for pre-fractionation use instructions.

2. Nanosep® spin filter column format

- **a.** Remove the spin filter from the Nanosep collection tube and place in a rubber stopper (with a suitable hole to form a tight fit to the spin filter) on the vacuum flask. Apply a low vacuum of 5-10 mm Hg.

b. Mix the 50% Ceramic HyperD® ion exchange slurry (washed in bulk with buffer to remove the storage solution) and quickly pipette 0.4 mL of the slurry to the Nanosep® device.

Tip: *This volume of slurry will give a 0.2 mL packed volume ion exchange bed in the device with a saturation capacity of up to 12-15 mg protein. For ion exchange pre-fractionation applications, up to 50% of this amount can be loaded without compromising the chromatographic performance of the resin. Larger volumes of resin in this device should be avoided since it will limit the volume of sample that can be loaded to < 0.2 mL and will not allow for good mixing of the media in this device format.*

c. After final addition, allow the vacuum to remove the liquid from the resin bed, which should now partially fill the Nanosep device.

d. Replace the spin filter in the collection tube for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 ºC for no more than one week. If they will be stored for > 1 week, add just enough buffer plus 0.02% sodium azide or equivalent preservative so that the resin looks wet (approximately 200 µL/well, depending on resin volume), and store at 4 °C.*

e. Refer to Section C on page 55 for pre-fractionation use instructions.

3. AcroPrep™ multi-well filter plate format

a. Wash the Ceramic HyperD ion exchange media with 5 column volumes (CV) of buffer to remove the 1 M NaCl, 20% ethanol storage buffer. Adjust to a final 50% (v/v) slurry.

b. Place an AcroPrep 96 filter plate (350 µL or 1 mL well volume) on a suitable vacuum manifold (PN 5017) with a collection plate underneath.

c. Mix the 50% slurry and quickly pipette 0.35 mL to the 350 µL multi-well plate. Rapidly follow with a second and third volume of slurry for the 1.0 mL volume plate.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

d. After final addition, allow the vacuum to remove the excess liquid from the resin bed, which should partially fill the well of the AcroPrep 96 filter plate.

e. The plate is ready for immediate use. To store the plate, add approximately 200 µL of buffer per well, then cover. Excess buffer will need to be removed (by vacuum or centrifugation) immediately before use.

Tip: *Since all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 ºC for no more than one week.*

f. Refer to Section C on page 55 for pre-fractionation use instructions

4. Glass column for pre-fractionation application

Tip: *Adjust bed height and resin volume to suit the specific application.*

a. Equilibrate column, degassed 50% gel slurry, and degassed buffer solution to room temperature.

b. Attach the bottom end fitting on to the column and clamp upright in a laboratory stand.

c. Add 1 mL of degassed buffer to the column to cover the bottom frit, and then gently tap the end and side of the column to dislodge any air bubbles.

d. Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 1-2 mL.

e. Allow gel to settle in the column for at least 5 minutes. If fine, slowly settling particles are seen, remove them.

f. Position the adjustable height, top fitting onto the column. Gently screw the top fitting down on to the settled gel bed. This should displace the air from the top fitting in the column. Do not over compress the gel bed.

g. Place the column on a suitable chromatography system and pump liquid up though the column at 1 mL/min for 2-3 minutes to displace any trapped air. Reverse the flow and equilibrate the column for at least 10 CV at up to 10 mL/min.

h. Packed column is now ready for immediate use or storage at 4 °C for up to a week removed from the LC system, with end caps in place to seal the chromatography tubing fittings, preventing fluid loss from the column. If it will be stored for > 1 week at 4 °C, the column should be filled with 1-2 CV of buffer plus 0.02% sodium azide or equivalent preservative.

i. Refer to Section C below for fractionation use instructions.

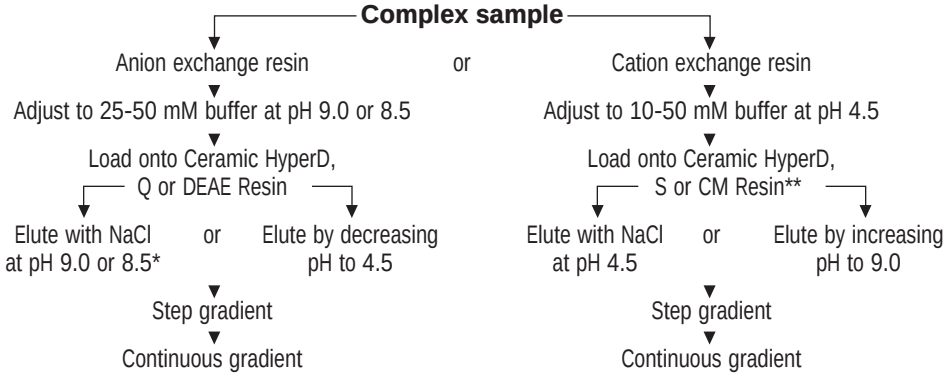
C. Ion Exchange Pre-fractionation

The following approaches can be used:

- Change the pH of the buffer.
- Introduce a counter ion into the buffer in the form of a salt.

Changes in pH result in changes in the charge state of the proteins in the sample or bound to the resin. If they become neutral or acquire the same charge as the ion exchange support, they will either not bind or elute. The presence of a small counter-ion will compete for binding to the resin, either inhibiting the protein capture or causing elution. The two approaches are useful in developing appropriate pre-fractionation strategies in multiple formats (see Table 2.12). The number of steps in a stepwise elution (i.e., number of different pHs or salt concentrations) or the beginning and end of the gradient will largely be determined by the expected range of pI in the sample, the complexity of the sample, and the total number of fractions desired.

Table 2.12
Summary of Pre-fractionation Options for HyperD® Ion Exchange Resin



**Elution may require several steps, such as pH and salt linked together to achieve efficient recovery of bound material.*

***Due to the high ligand density within the hydrogel of the CM beads, it may be necessary to include some NaCl in the initial binding buffer to improve adsorption. Earlier studies have shown that 50 mM sodium acetate plus 100 mM NaCl is optimal for adsorption of IgG and other model protein. For proteins other than IgG, if maximal capacity is required, it should be tested in the presence of 75-100 mM NaCl in the loading buffer at pH 4.5.*

1. Gravity flow column format

- **a.** Prepare a 1-2 mL column as described above.
- **b.** Wash the Ceramic HyperD ion exchange resin with 5 CV of buffer to remove the storage buffer.
- **c.** Allow the liquid to drain from the column and load the sample up to a 2 mL volume onto the column.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.12.*

- **d.** Collect the column effluent in 1 mL fractions. Measure A_{280} to locate the protein peak.

Tip: *Unretained protein rapidly elutes from the column and should be found in the first three fractions. Some slight dilution of the sample will occur during elution. If necessary, the sample can be concentrated in a centrifugal UF spin filter, such as a Nanosep® or Microsep™ centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).*

- **e.** After unbound protein has flowed through the column, the column should be washed with 5 CV of loading buffer or until A_{280} is at baseline before elution begins.
- **f.** Retained fractions can then be eluted by a series of buffer pH steps, serial salt steps up to 1.0 M, or a combination of both. The volume of each elution should be at least 2 CV to avoid “carry over” between steps in the elution. Minimal volume is recommended to minimize sample dilution. Multiple fractions can be collected and tested for protein concentration.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF spin filter with a 10K MWCO membrane (see Section 2.4, page 152).*

g. After the last pH or salt step, tightly bound material can be eluted with 1% (w/v) sodium dodecyl sulfate (SDS) in buffer and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141).

h. Methods for column regeneration can be found in product insert and ion exchange purification section (see Section 4.2.2, page 310). After an SDS detergent elution, the column should be discarded.

2. Nanosep® spin filter column format

a. Prepare the spin column as described above.

b. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to remove excess fluid from the packed bed.

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.1-0.4 mL, depending on total protein concentration and amount of resin) on to the top of the dry packed bed in the Nanosep device.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.12.*

e. Place Nanosep device in the collection tube.

Tip: *At this stage, the sample and resin should be mixed and allowed to remain in contact for 5-10 minutes at room temperature to improve binding.*

f. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to pass the sample through the ion exchange resin bed.

g. Filtrate in the collection tube will be the unretained flowthrough fraction.

h. After proteins in the flowthrough fraction are collected, the column should be washed with 5 CV of loading buffer before elution is attempted.

i. Retained fractions can then be eluted by a series of buffer pH gradient steps, serial salt steps up to 1.0 M, or a combination of both. The volume of each elution should be at least 0.2 mL to avoid “carry over” between steps in the elution. Minimal volume is recommended to minimize sample dilution.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF spin filter, such as a Nanosep or Microsep™ centrifugal device, with a 10K MWCO membrane (see Section 2.4, page 152).*

j. After the last pH or salt step, tightly bound material can be eluted with 1% (w/v) SDS in buffer and recovered by acetone precipitation or detergent removal using SDR HyperD resin (see Section 2.3, page 141).

k. Methods for column regeneration can be found in product insert and ion exchange purification section (Section 4.2.2, page 310). After an SDS detergent elution, the column should be discarded.

3. AcroPrep™ multi-well filter plate format

Tip: *For prolonged incubations or incubations using solutions that reduce surface tension (e.g., detergents, alcohols, and acetonitrile) in 96-well plates, it may be necessary to seal the bottom of the plate to prevent leakage. This can be tested in advance with just the solutions in the chosen plates.*

An adhesive plate sealer can be used on the top of the wells to prevent cross contamination during vigorous shaking or evaporation during prolonged or warm incubations. For sealing, you can use a plastic, self-adhesive plate sealer (e.g., Sigma EASYseal) or a cap-mat (PN 5230).*

a. Prepare the packed multi-well filter plate as described above.

b. Centrifuge the multi-well plate plus collection plate in a suitable swinging bucket rotor at 400 x g for 2 minutes to remove excess fluid from the packed bed. Vacuum can also be used if preferred.

Tip: *As an alternative to centrifuging, a vacuum manifold can be used. See Section 6.3 of the Appendix for more detail.*

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (40-400 µL, depending on total protein concentration and resin volume) on to the top of the dry packed bed in the AcroPrep™ 96 multi-well filter plate.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.12. The amount of sample loaded should be < 50% of the static binding capacity of the resin.*

e. The sample and well contents should be thoroughly mixed and kept in contact for 30 minutes at room temperature to improve binding.

Tip: *To minimize buffer leakage and loss due to evaporation, use an adhesive plate sealer or cap-mat (PN 5230) on the top of the plate and incubate in a moisture-resistant bag or container.*

The shaker table used should be able to agitate the plate to keep the chromatography media and sample suspended within the well on the plate. Control the mixing so sample and resin do not come in contact with the top sealing sheet.

f. Place the sealed plate on top of the collection plate to catch any filtrate. Tap the plate to dislodge any liquid near the top of the plate and then carefully and slowly remove the top sheet.

g. Centrifuge the multi-well plate and collection plate in a swinging bucket rotor at 400 x g for 2 minutes to pass the sample through the ion exchange resin bed.

h. Filtrate recovered from the collection plate will be the unretained fraction.

i. After unbound proteins are collected, the resin should be washed with 5 CV of loading buffer before elution begins.

j. Retained fractions can then be eluted by a series of buffer pH steps, serial salt steps up to 1.0 M, or a combination of both. The volume of each elution should be at least 2 CV to avoid “carry over” between steps in the elution. Minimal volume is recommended to minimize sample dilution.

Tip: *Sample concentration at this stage is best carried out in an AcroPrep 96-well UF filter plate with a 10K MWCO membrane (PN 5034) (see Section 2.4, page 152).*

k. After the last pH or salt elution step, tightly bound material can be removed with 1% (w/v) SDS in buffer and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

4. Chromatography glass column format

a. Prepare 1-2 mL column as described above.

b. Load the sample up to a 2 mL volume onto the column at 1 mL/min. flow rate. Monitor the effluent at 280 nm.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.12.*

c. Collect the column effluent in 1 mL fractions. Measure absorbance at 280 nm to locate the protein peak.

Tip: *Protein rapidly elutes from the column and should be found in the first three fractions. Some slight dilution of the sample will occur during elution. If necessary, the sample can be concentrated in a centrifugal UF spin filter, such as a Nanosep® or Microsep™ centrifugal device, with a 10K MWCO UF membrane.*

d. Retained fractions can then be eluted by a linear gradient of buffer pH, a linear gradient up to 1.0 M salt, or a combination of both.

e. The volume of the gradient should be at least 10 CV. Fractions of 1 mL should be collected.

f. After protein has eluted, the column can be regenerated by 5 CV of 1.0 M NaCl followed by equilibration back to the initial buffer conditions.

g. Some slight dilution of the sample will occur during elution.

Tip: *If necessary, the samples can be concentrated in a centrifugal UF spin filter, such as a Nanosep or Microsep centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).*

Application Data for Ceramic HyperD Ion Exchange Resin

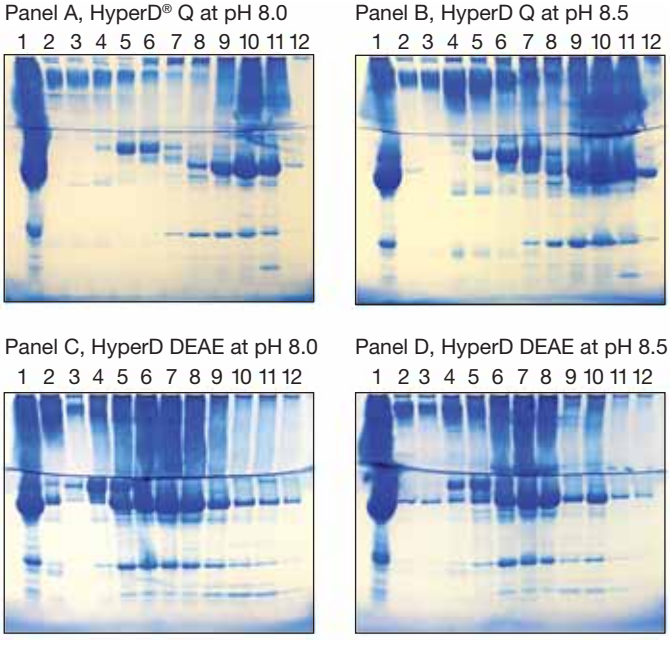
Pre-fractionation of Human Plasma on HyperD Ion Exchange Resins in a Nanosep GHP Centrifugal Device Format by Serial Salt Step Elution

Methods development for pre-fractionation of complex samples on ion exchange resins in a small centrifugal spin filter, such as a Nanosep GHP device, can be a useful approach for small amounts (1-2 mg) and low number samples (< 12). In this format, up to 0.2 mL of packed bed resin can be employed and are able to retain and resolve up to 6.0-7.5 mg protein. Protein binding capacity using purified proteins was measured in this device and clearly shows that these resins exhibit > 65 mg/mL media binding capacity. This is comparable to that measured in a chromatography column format (see Table 2.12).

Resins in the Nanosep GHP Centrifugal Spin Filter Format

Pre-fractionation of human plasma in Nanosep GHP devices with Q and DEAE Ceramic HyperD ion exchange resin was carried out using a serial salt step elution protocol. Two loading buffer pHs were chosen, 8.0 and 8.5, to assess their impact on the pre-fractionation achieved on the two ion exchange surfaces. Eluted samples were acetone precipitated and re-suspended into 1X SDS-PAGE sample buffer and then resolved on SDS-PAGE gels stained with colloidal Coomassie* Blue. Gel images are shown in Figure 2.19.

Figure 2.19



Human plasma was diluted 1:20 in loading buffer, clarified by centrifugation or filtration, and loaded onto HyperD® anion exchange resins in Nanosep® GHP devices as described in the Protocol section. After a wash with loading buffer, the retained material was serially eluted with loading buffer plus increasing amounts of NaCl up to 1.0 M. The flowthrough plus wash and the eluted fractions were concentrated by acetone precipitation and resuspended into 1X SDS-PAGE sample buffer. The fractions were resolved by SDS-PAGE and stained with colloidal Coomassie Blue.*

Panel A, HyperD Q pH 8.0; Panel B, HyperD Q pH 8.5; Panel C, HyperD DEAE pH 8.0; Panel D, HyperD DEAE pH 8.5. Lane 1, Control plasma; 2. Flowthrough plus wash; 3. 25 mm NaCl eluate; 4. 50 mM eluate; 5. 75 mM eluate; 6. 100 mM eluate; 7. 125 mM eluate; 8. 150 mM eluate; 9. 175 mM eluate; 10. 200 mM eluate; 11. 300 mM eluate; and 12. 1000 mM eluate.

Anion exchange on the Q Ceramic HyperD or DEAE Ceramic HyperD resins showed excellent retention of proteins at pH 8.5 with some resolution between IgG and albumin (see boxes in panels A-D). There did appear to be some selectivity differences between Q and DEAE in the resolution and elution positions of albumin, being more retained and eluting later on the Q surface. This is not surprising when comparing strong vs. weak anion exchange. For proteomics applications, setting up the ion exchange fractionation in a 96-well filter plate can be carried out very easily. This step can be combined with an upstream depletion step for even better protein separation.

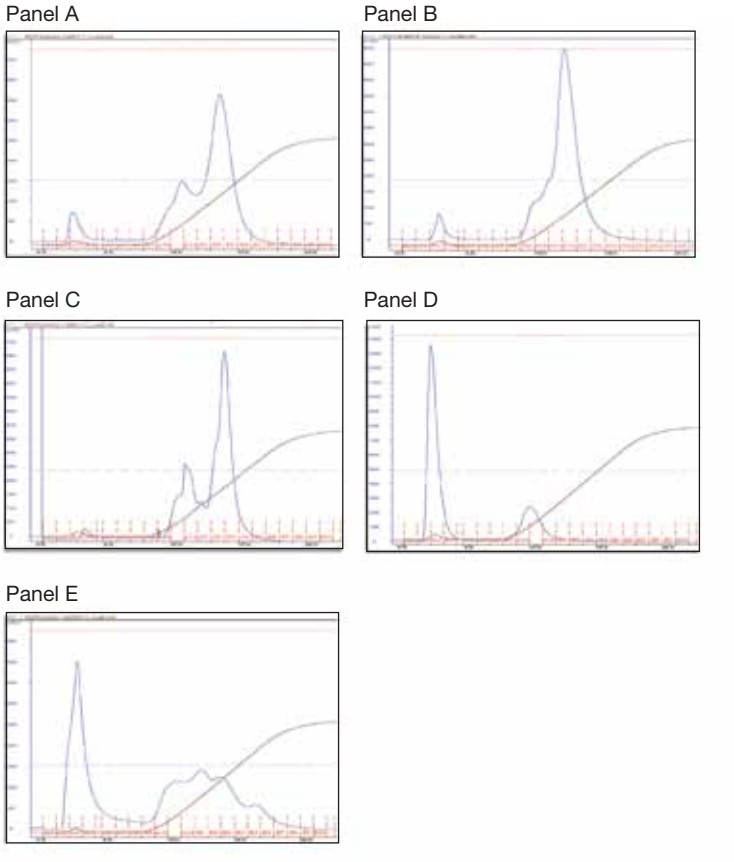
HPLC Column Based Pre-fractionation of Human Plasma with Ceramic HyperD® Ion Exchange Resins

For large-scale pre-fractionation, Ceramic HyperD ion exchange resins were packed into 1 mL glass columns as described in the Protocol section on page 53. Human serum, clarified by centrifugation at 12,000 x g for 10 minutes or filtered with a Serum Acrodisc® syringe filter (PN 4525) was diluted 1:4 with loading buffer and put onto the column. After sample loading, the column washed back to baseline OD and the retained material was eluted with a linear NaCl gradient (0 to 0.5 M in 10 CV) followed by a ramp from 0.5 to 1.0 M NaCl for 5 CV. Representative HPLC chromatograms are shown in Figure 2.20. The results indicate that under the conditions tested, none of the resins give baseline resolution of the many protein components in serum, as expected for such a complex sample. The best resolution was seen on the small particle size Q Ceramic HyperD 20 resin, while CM Ceramic HyperD F resin showed a greater degree of resolution than the strong S cation chemistry. Similar results would be obtained if pH gradient or steps were used to elute bound proteins rather than salt. However, pH elution has the advantage of salt-free fractions for the next step in preparation or analysis. These data suggest that the separation obtained by ion exchange would be useful as a complexity reduction step for many applications. This is comparable to that measured in a chromatography column format (see Table 2.11, page 51).

Methods development for pre-fractionation of complex samples on ion exchange resins in a small centrifugal spin filter, such as a Nanosep® GHP device, can be a useful approach for small amounts (1-2 mg) and low number samples (< 12). In this format, up to 0.2 mL of packed bed resin can be employed and are able to retain and resolve up to 6.0-7.5 mg protein. Protein binding capacity using purified proteins was measured in this device and the results are summarized in Table 2.10, page 50. The data demonstrates binding capacities of > 65 mg/mL.

Figure 2.20

HPLC Pre-fractionation of Whole Human Plasma on HyperD® Ion Exchange Packed Columns



Human plasma (0.5 mL) diluted 1:4 with loading buffer was clarified by centrifugation and then injected onto a 1 mL packed column prepared as described in the Protocol section. The column was operated at room temperature at 1 CV per minute flow rate and retained material was eluted in a 10 CV linear gradient up to 0.5 M NaCl followed by a step to 1.0 M NaCl for 5 CV. Note: only the 0-0.5 M NaCl gradient is shown. No significant protein elution was seen in the 0.5-1.0 M final NaCl gradient.

Panel A, HyperD F Q at pH 8.5, Panel B, HyperD F DEAE at pH 8.5, Panel C, HyperD Q-20 at pH 8.5, Panel D, HyperD F S at pH 5.8 and Panel E, HyperD F CM at pH 5.8.

Ordering Information for Ceramic HyperD® Ion Exchange Resin

Part Number	Description	Pkg
20040-051	Q Ceramic HyperD 20	5 mL
20040-044	Q Ceramic HyperD 20	25 mL
20040-036	Q Ceramic HyperD 20	100 mL
20040-028	Q Ceramic HyperD 20	500 mL
20040-010	Q Ceramic HyperD 20	1000 mL
20066-098	Q Ceramic HyperD F	5 mL
20066-031	Q Ceramic HyperD F	25 mL
20066-023	Q Ceramic HyperD F	100 mL
20066-015	Q Ceramic HyperD F	1000 mL
20038-055	S Ceramic HyperD 20	5 mL
20038-048	S Ceramic HyperD 20	25 mL
20038-030	S Ceramic HyperD 20	100 mL
20038-022	S Ceramic HyperD 20	500 mL
20038-014	S Ceramic HyperD 20	1000 mL
20062-089	S Ceramic HyperD F	5 mL
20062-030	S Ceramic HyperD F	25 mL
20062-022	S Ceramic HyperD F	100 mL
20062-014	S Ceramic HyperD F	1000 mL
20067-070	DEAE Ceramic HyperD F	5 mL
20067-039	DEAE Ceramic HyperD F	25 mL
20067-021	DEAE Ceramic HyperD F	100 mL
20067-013	DEAE Ceramic HyperD F	1000 mL
20050-084	CM Ceramic HyperD F	5 mL
20050-035	CM Ceramic HyperD F	25 mL
20050-027	CM Ceramic HyperD F	100 mL
20050-019	CM Ceramic HyperD F	1000 mL

References for Ceramic HyperD® Ion Exchange Resin

1. Duval, M., et al. (1993). *Job Life Sciences*, (316), 1463.
2. Boschetti, E., et al. (1996). *J. Biochem. Biophys. Meth.*, (32), 15.
3. Coffman, J.L., & Boschetti, E. (1998). *Bioseparation & Bioprocessing*, 1, 157.
4. Necina, R., Amatschek, K., & Jungbauer, A. (1998). *Biotechnology and Bioengineering*, 60(6), 689.
5. Hanser, E., & Mollerup, I. (1998). *J. Chromatography*, A827, 259.
6. Hahn, R., et al. (1998). *J. Chromatography*, A795, 277.
7. Moure, F., Rendueles, M., & Diaz, M. (1999). ECCE2 (Second European Congress of Chemical Engineering), Montpellier.
8. Fernandez, A., et al. (1996). *J. Chromatography*, A746, 169.
9. Boschetti, E., & Jungbauer, A. (2000). *Separation Science & Technology*, 2, 535.
10. Riedel, K.U., et al. (1995). Eur. *J. Biochem.*, (231), 742.
11. Jouanneau, Y., et al. (2000). Eur. *J. Biochem.*, (267), 780.
12. Sookkheo, B., et al. (2000). *Protein Expression and Purification*, (20), 142.
13. Couriol, C., et al. (2000). *Chromatographia*, 52(7–8), 495.

2.2.2.2 Mustang® Ion Exchange Membrane

The high capacity Mustang Q and S membranes, made from polyethersulfone (PES) material modified with anion and cation exchange chemistry, have been put into an AcroPrep™ 96-well plate format. Mustang ion exchange membranes deliver efficient and rapid flow rates with a convective pore structure resulting in processing times that are much shorter and more efficient than conventional resin-based technology. Mustang devices have throughputs of up to 100 times that of traditional bead-based media with no associated loss of capacity. This multi-well format can be used to carry out parallel, high throughput ion exchange pre-fractionation of complex samples, such as serum or plasma. The multi-well ion exchange plate specifications are summarized in Table 2.13 for Mustang Q and S chemistries. This convenient methods development format can be directly scaled up to syringe-based Acrodisc® devices (see Section 4.1, page 297) and larger capsules with Mustang Q membrane for large-volume bioprocess applications. These 96-well plate and Nanosep® centrifugal device applications are equivalent to batch-mode chromatography using stepwise elution conditions with a great deal of flexibility in the number of steps employed by the user.

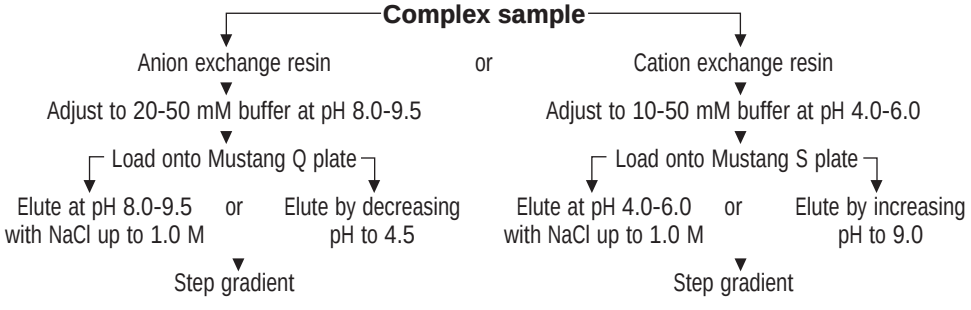
Table 2.13
Specifications of the AcroPrep 96 Filter Plates with Mustang Ion Exchange Membrane

Specification	Parameter
Materials of Construction	
Membrane	Mustang Q or S modified Supor® PES
Device	Polypropylene
Effective Membrane Area	0.25 cm^2
Dimensions	
Length	12.78 cm (5.03 in.)
Width	8.51 cm (3.35 in.)
Height (Plus Lid)	1.66 cm (0.655 in.)
Height (without Lid)	1.44 cm (0.565 in.)
Tip Length	0.53 cm (0.21 in.)
Capacity/Well	
Maximum Well Volume	0.35 mL
Recommended Volume	0.30 mL
Maximum Centrifugal Force	3,000 x g
Centrifuge	Swinging bucket rotor
Operating Vacuum	25.4 cm Hg (10 in. Hg)

The capture of proteins for ion exchange is typically done in the presence of low salt. Due to the nature of Mustang membranes, they are more salt sensitive than the same chemistries on beads. This should be considered when choosing the buffers for protein capture and elution. In addition, they should not be overloaded with protein since the separation under these conditions is much less reproducible and predictable. For

proteomics applications where the goal is complexity reduction in user-defined number of fractions, the binding step is often performed at relatively low stringency so the majority of proteins will bind. Then stepwise elution is used to selectively elute specific subsets of proteins, based on their pI (pH elution) or charge density (salt elution). Elution based on pI is achieved with buffers of increasingly higher stringency (lower pH for anion exchange and higher pH for cation exchange). For salt-based elution (most common for traditional ion exchange chromatography), a buffer with increasing salt concentrations is used. Thus proteins elute from the ion exchange surface due to a change in charge state so they are no longer capable of stable salt bridge formation with the ion exchange chemistry (pH elution) or displacement due to the presence of a small counter ion in the form of salt. The two approaches are useful in developing appropriate pre-fractionation strategies in multi-well formats and are summarized in Table 2.14. When downstream steps are salt sensitive, pH elution is preferred since this eliminates the need for a salt removal step. The number of intervals in a stepwise elution (i.e., number of different pH or salt concentrations) is largely determined by the expected pI range in the sample, the complexity of the sample, and the total number of fractions desired. Either strategy or a combination of both can be applied to the purification of components from a complex sample in the AcroPrep™ 96 filter plate.

Table 2.14
Summary of Pre-fractionation Options for Mustang® Ion Exchange Membrane in an AcroPrep 96 Filter Plate



In this application section, protocols will be described for pre-fractionation of complex samples employing multi-well, small-volume devices.

Protocol for Mustang Ion Exchange Membrane

Collection of fractions from Mustang ion exchange plates can be done under vacuum or in a centrifuge. Both methods are described below. Experience indicates that fraction collection in a centrifuge is somewhat more reproducible even though it takes slightly more time.

A. Materials Required

1. AcroPrep 96 filter plate, 350 µL and 1 mL volume filter plates with Mustang Q and S membranes. For specifications, see Table 2.13.
2. Collection plates [e.g., Axygen 96 well polypropylene V bottom, 0.5 mL (P96450V) or round bottom, 1.64 mL (PDW20)]

3. High purity binding/equilibration buffer such as 25 mM Tris HCl, pH 8.5 (Mustang® Q membrane) and 10 mM MES-NaOH, pH 5.5 (Mustang S membrane)
4. High purity elution buffers
5. Separation apparatus
   a. Source of vacuum capable of 25.4 cm Hg (10 in. Hg) (vacuum manifold, PN 5017); or
   b. Centrifuge fitted with a swinging bucket rotor with multi-well plate adapters and able to spin up to 3,000 x g

B. Vacuum Manifold Processing

1. During use, hold the plate so that the outlets on the bottom of the plate are not touched to prevent liquid flow due to wicking. The easiest way to accomplish this is to place the Mustang plate on a receiver plate.
2. Place an appropriately sized receiver plate into the vacuum manifold.
3. Place the filter plate in the vacuum manifold. Add 0.2 mL equilibration buffer.
4. Apply vacuum to manifold to initiate liquid flow. Recommended vacuum is minimum required to initiate flow, up to 25.4 cm Hg (10 in. Hg) for 96-well plates. DO NOT exceed 38.1 cm Hg (15 in. Hg). DO NOT leave the plate under vacuum for longer then necessary or it will dry out and protein will be unrecoverable.
5. Release vacuum and discard the first filtrate. Gently tap the plate to remove any hanging droplets.

Tip: *Do not release vacuum by pulling the corner of the plate as it will degrade the manifold gasket.*

6. Add the sample to the plate.
7. Place a receiver plate into the vacuum manifold sized to collect the volume of the filter plate.
8. Apply vacuum to manifold to initiate liquid flow, as directed above. Use minimal vacuum to achieve flow and maximize the protein-membrane interaction time.
9. Release vacuum from the manifold and recover unbound proteins (flow through sample) from the collection plate.

C. Processing by Centrifugation in a Swinging Bucket Rotor

1. Place the AcroPrep™ 96 filter plate on top of a receiver plate sized to collect the appropriate volume from the filter plate.
2. Insert the two plates into a standard multi-well plate swinging bucket rotor assembly.
3. Place a duplicate pair of plates matching the weight of the test plate (add water to the receiver plate and match weight of the test plate).

Tip: *An imbalance can result with a single test plate if no counter balancing plate is used. If different volumes of sample are used in multiple plates, they will need to be balanced in pairs by addition of water to empty wells.*

4. Centrifuge at 500 x g for 2 minutes.

Tip: *The centrifugal force and time parameters can be varied to optimize the filtration rate of fluids in contact with the well of the plate.*

D. General Fractionation Protocol

Tip: *Although the binding capacity of the Mustang® membrane is quite high per unit volume, there are only 2 layers of membrane in the current Mustang AcroPrep™ 96-well plate. As a result, the net binding capacity is limited. We recommend loading < 0.2 mg protein per well. If over capacity, the binding is much less reproducible. It is very important to allow the protein sample sufficient time to interact with the membrane during the fraction collection step. For this reason, low vacuum and low centrifugation speeds are highly recommended.*

1. Place an appropriately sized collection plate into the vacuum manifold.
2. Place a Mustang ion exchange membrane plate onto the vacuum manifold.
3. Pipette 0.3 or 0.5-0.8 mL of equilibration/loading buffer into the well of the plate (depending on plate capacity).

Tip: *At this stage, loading buffer optimization should be carried out as suggested in Table 2.14. The 96-well filter plate facilitates replicates and parallel testing of many variables in one experiment.*

4. Discard the equilibration buffer from the collection plate.
5. Add up to 0.3 or 0.8 mL equilibration/binding buffer to the wells.

Tip: *The total protein load on these plates should be < 0.2 mg per well (equivalent to 2-3 µL whole plasma) in a minimum of 20 µL. Dilute samples if necessary. For best results samples should be prefiltered with an Acrodisc® syringe device or an AcroPrep 96 filter plate, 0.45 µm, with GHP membrane (see Section 2.5, page 198); or hard spun (5-10 minutes in microfuge at maximum speed) to remove particulate material.*

6. Collect the unbound proteins [flowthrough fraction (FT)] as indicated above.

Tip: *The vacuum should be controlled to allow sufficient time for protein interaction with the mustang membrane surface. We recommend at least 1-2 minutes.*

7. For best results, after liquid has passed through the membrane, release vacuum and reload the filtrate into the wells of the plate and repeat Step 6 above.

Tip: *This recycling should be optimized since the Mustang ion exchange membranes exhibit very rapid adsorption/desorption kinetics due to the efficient convective pore structure of the modified PES microporous membrane.*

8. Add 0.1-0.3 or 0.5-0.8 mL of equilibration buffer to the wells and repeat Steps 5-7 to wash away residual unbound proteins.

Tip: *At this stage, these wash fractions can be retained, combined with the flowthrough fractions, or discarded. If the protein concentration of the combined flowthrough and wash is too dilute, the sample can be concentrated with an AcroPrep 96 filter plate with a 10K MWCO UF membrane (see Section 2.4, page 152).*

9. Elute bound proteins using salt or pH change as described in Table 2.14. Combinations of both variables should be considered to fine tune elution conditions when necessary.
10. Fractions are eluted by adding 0.1-0.3 or 0.2-0.8 mL of elution buffer to the wells and repeating Steps 5-7 above. Each step should be saved in a separate collection plate and processed for analysis.

11. After the final NaCl or pH elution step, tightly bound material can be eluted with 1% (w/v) sodium dodecyl sulfate (SDS) in water and, if necessary, recovered by acetone precipitation or SDR detergent removal (see Section 2.3, page 141). Other strong denaturants compatible with downstream steps can also be used. After an SDS detergent elution, the plate should be discarded.

Application Data for Mustang® Ion Exchange Membrane

Mustang Ion Exchange Membrane Protein Binding Capacity in an AcroPrep™ 96 Filter Plate
The current Mustang ion exchange membrane in an AcroPrep filter plate contains two layers of modified Supor® PES membrane and can be used to pre-fractionate a complex sample, such as serum or plasma. To estimate protein binding capacity of ion exchange membrane, it is common practice to use saturation binding with purified standard proteins, such as BSA for an anion ion exchange and lysozyme for a cation ion exchange surface. An example of a study to measure the BSA binding across a whole Mustang Q AcroPrep plate is summarized in Table 2.15. The results showed a high degree of reproducibility across the 96 wells of the plate and a saturation binding capacity of 0.17 mg/well under these conditions, which corresponds to 24.4 mg/mL of Mustang Q membrane volume. This protein binding capacity is sufficient for small scale fractionation (e.g., proteomics applications) and purification methods development to investigate purification parameters for larger scale processes employing Mustang Q and S Acrodisc® syringe filters (see Sections 4.1.2, page 298 and 4.1.3, page 303).

Table 2.15
Saturation BSA Binding Capacity for Mustang Q in a 350 µL AcroPrep 96 Filter Plate on a Vacuum Manifold

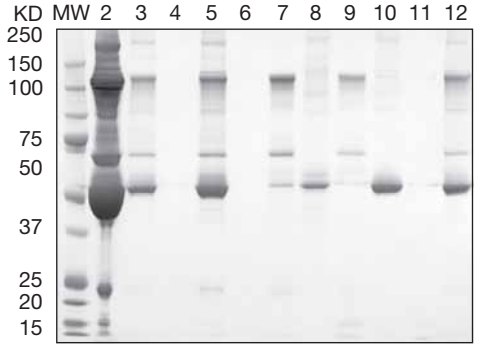
Binding Capacity (mg BSA/well)*				
Membrane	**Elution #1 (+/- SD)**	**Elution #2 (+/- SD)**	**Total**	**mg BSA /mL media****
Mustang Q	0.16 ± 5.9%	0.01 ± 7.3%	0.17	24.3

**Binding capacity was measured using 5 mg/mL BSA in 25 mM Tris HCl, pH 8.5 as follows: a) equilibrate the membrane with the above buffer; b) add 0.3 mL of the BSA sample and adjust vacuum to give a flow time of 2 minutes; c) repeat process 5 times to load a total of 7.5 mg BSA per well, sufficient to saturate the binding sites on the Mustang membrane; d) wash with 10 x 0.3 mL of the loading buffer. Check absorbance at 280 nm of final filtrate on a plate reader to confirm eluate at baseline absorbance; and e) elute the bound BSA with 2 x 0.3 mL of 1 M NaCl in the above buffer. Measure eluted protein absorbance at 280 nm. Calculate BSA bound from the pooled fractions.*

***Membrane volume/well calculated to be 0.007 mL, assuming an area of 0.25 cm^2, and a membrane thickness of 0.014 cm with 2 layers per well.*

Pre-fractionation of Plasma by Mustang® Ion Exchange Membrane-based Chromatography
Resolution of complex samples such as plasma or serum by ion exchange chromatography leading to pre-fractionation can be investigated in the AcroPrep™ 96 filter plate. This methodology can be easily adapted to small volumes of samples and carried out in parallel using the multi-well plate format. An example of a preliminary bind and elute study to examine binding of plasma proteins to Mustang Q membrane in an AcroPrep filter plate is shown in a 1D SDS-PAGE in Figure 2.21. Binding conditions were varied while elution for all samples was done at pH 2.5. Under these circumstances a 1D gel will only show the most abundant proteins, but will give a sense of the separation possibilities. The results show that at physiological pH and ionic strength [PBS Lane 5 and 6, flowthrough (FT), and eluate], very little protein is retained as expected. When the ionic strength is lowered, some proteins are retained (Lane 8, eluate). When the capture is performed at pH 10 in the absence of salt, almost all proteins are bound. While in the presence of salt, some of the proteins show up in the FT fraction. Thus, the control of sample complexity in collected fractions occurs not only by elution buffer choice but also by capture conditions. This illustrates the utility of membrane-based ion exchange to rapidly explore a fractionation strategy in a multi-well filter plate. Some preliminary experiments to determine the optimal capture and elution conditions for appropriate complexity reduction are highly recommended.

Figure 2.21
Pre-fractionation of Human Serum on Mustang Q Membrane in an AcroPrep 96 Filter Plate



Pooled human serum proteins were captured on a Mustang Q membrane (AcroPrep 96 filter plate). The conditions for protein binding were varied in both pH (pH 10 carbonate buffer and pH 7 phosphate buffer, representing low and moderate stringency, respectively) and in the absence or presence of 150 mM NaCl. In all cases, bound proteins were eluted at pH 2.5 for SDS-PAGE analysis. Molecular weight markers and 1 µL of pooled human serum are in Lanes 1 and 2. The flowthrough (FT) and eluate (E) samples alternate from Lane 3-12, beginning with FT. Lane 3 = FT and Lane 4 = E from 10 µL of neat serum, which is not recommended due to poor reproducibility. Lanes 5 and 6 are FT and E from pH 7 + 150 mM NaCl binding. Lanes 7 and 8 are FT and E from pH 7, no salt capture conditions. Lanes 9 and 10 are FT and E from pH 10 + 150 mM NaCl binding. Lanes 11 and 12 are FT and E from pH 10, no salt capture conditions.

Ordering Information for Mustang® Ion Exchange Membrane

AcroPrep™ 96 Filter Plate, 350 µL Well

Part Number	Description	Pkg
5047	Mustang Q membrane, natural	10/pkg
5048	Mustang S membrane, natural	10/pkg

AcroPrep 96 Filter Plate, 1 mL Well

Part Number	Description	Pkg
5062	Mustang Q membrane, natural	5/pkg
5063	Mustang S membrane, natural	5/pkg

2.2.3 Affinity Separations – Chromatography Resins

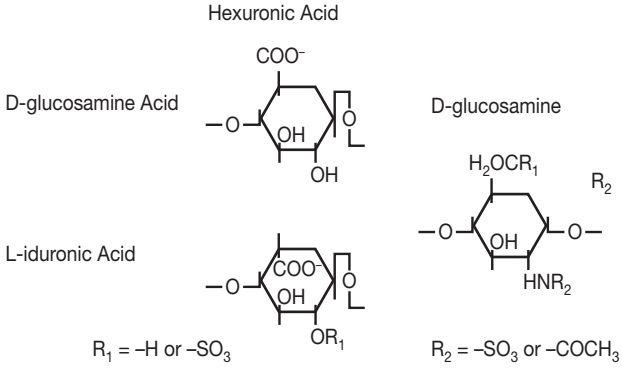
2.2.3.1. Heparin HyperD® M Resin

Heparin is a mucopolysaccharide consisting of a repeating dimer of hexuronic acid and D-glucosamine (see Figure 2.22) with a molecular weight range of 5,000-30,000. Immobilized heparin has two main modes of interaction with proteins:

- As an affinity ligand, e.g. in its interaction with growth factors and antithrombin III; and
- As a cation exchanger due to its high content of anionic sulphate groups. For example, in its interaction with nucleic acid-binding proteins where it mimics the similarly polyanionic structure of the nucleic acid.

Gradient elution with salt is most commonly used in both cases. As individual proteins often bind by a unique combination of affinity and ion exchange, even small differences between bound proteins can result in good purifications.

Figure 2.22
Heparin Structure



Structure of a heparin polysaccharide, consisting of alternating hexuronic acid and D-glucosamine residues. The hexuronic acid can either be D-glucuronic acid (top) or its C-5 epimer, L-iduronic acid.

Heparin HyperD M resin provides high binding capacity and high flow rates, ideal for small- and large-scale applications. A summary of the properties of the heparin affinity resin is presented in Table 2.16. In this section, the application focus will be on small-scale pre-fractionation in single- and multi-well devices with scale-up into gravity flow columns. Larger scale protocols employing a liquid chromatography workstation in glass columns are described in purification applications Section 4.2.7, page 359.

Table 2.16
Properties of Heparin HyperD® in Resin

Specification	Parameter
Particle Size	80 µm (average)
Ligand	Porcine Heparin
Ligand Loading	5-10 mg/mL
Human Antithrombin III (10% Breakthrough)*	25 mg/mL
pH Stability	3-13
Pressure Stability	Up to 70 bar (7,000 kPa, 1,015 psi)

**Determined using human ATIII at 72.5 µL/mL in 20 mM Tris HCl, 0.3 M NaCl, pH 7.4. Elution with 20 mM Tris HCl, 2 M NaCl, pH 7.4 at a flow rate of 600 cm/h, 10 cm bed height.*

Protocol for Heparin HyperD M Resin

A. Materials Required

1. Container/column for resin, one of the following:
   - **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922)
   - **b.** Nanosep® MF centrifugal device with 0.45 µm GHP membrane (PN ODGHPC34)
   - **c.** Choose either:
     - **(1)** AcroPrep™ 96 filter plate, 350 µL well, 0.45 µm GHP membrane (PN 5030); 0.45 µm Supor® membrane (PN 5029); 1.2 µm Supor membrane (PN 5039); or
     - **(2)** AcroPrep 96 filter plate, 1 mL well, 0.45 µm GHP membrane (PN 5054)
2. Needed if filter plate is used:
   - **a.** Collection plates [e.g., 96-well polypropylene V-bottom, 0.5 mL (Axygen PN P96450V) or 1.64 mL round-bottom (Axygen PN PDW20)]
   - **b.** Adhesive plate sealing film
   - **c.** AcroPrep 96-well plate sealing cap mat (PN 5230)
3. Separation apparatus (if filtration is used)
   - **a.** Source of vacuum 25.4-50.8 cm Hg (10-20 in. Hg) (PN 5017); or
   - **b.** Centrifuge fitted with a swinging bucket rotor
4. Degassed 50% (v/v) slurry of the Heparin HyperD M resin
5. Degassed suitable buffer, such as 25 mM Tris HCl pH 8.5, 0.3 M NaCl, pH 7.4

Tips on Handling Heparin HyperD M Resin:
For packed columns, use only degassed liquids. Degassing is not necessary for batch mode methods.

Some BioSepra® media are supplied as concentrated slurries and may be difficult to resuspend. Do not use magnetic stir bars with BioSepra media as they can damage the

beads. Also, these resins are quite dense and settle quickly. When adding slurry to any device, mix well between additions. If it is necessary to prepare a 50% (v/v) slurry, use a clean spatula to remove some of the packed media and transfer to a graduated glass cylinder containing degassed water or buffer. DO NOT add any media back to the storage bottle to avoid contamination of the bulk media. Thoroughly mix and allow settling. Note the volume of the packing. Decant the supernatant and add back an equal volume of buffer to make a 50% (v/v) slurry.

For packed columns, removal of fines may be necessary. Prepare the slurry in desired buffer, mix, and allow to settle for approximately 5 minutes or for enough time that the beads have settled but small particles are still in the solution.

B. Packing Heparin HyperD® M Resin

1. Gravity flow column format

- **a.** Equilibrate column, degassed 50% gel slurry, and degassed buffer solution to room temperature.
- **b.** Secure a bottom cap on the column tip and clamp the column [1-5 mL bed volume column (e.g., Pierce PN 29922)] upright in a laboratory stand.
- **c.** Add a sufficient volume of degassed buffer to the column to fill it up to the reservoir (wide-mouth) portion, and then gently tap the end and side of the column to dislodge any air bubbles.
- **d.** Float a porous disc on top of the liquid within the column.
- **e.** Using the reverse end of a Pasteur pipette or reverse end of a serum separator (e.g., Pierce PN 69710), push the disc evenly to the bottom of the column.
- **f.** Decant most of the liquid from the empty column, being sure to avoid getting air bubbles in the tip region of the column below the inserted disc. Place the column back in its stand with bottom cap still in place.
- **g.** Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 1-2 mL.
- **h.** Allow gel to settle in the column for at least 5 minutes.
- **i.** Position a second porous disc on top of the settled gel bed by floating it on the liquid within the column and pushing it down to just above the settled gel. Leave 1-2 mm of space between the top of the gel bed and the top disc. Do not compress the gel bed.
- **j.** Wash the inside top part of the column with buffer to remove residual gel that may have remained along the sides during packing.
- **k.** Packed column is now ready for storage at 4 °C or immediate use.

Tip: *Store the packed column upright at 4 °C with the gel bed submerged under 1-2 mL of buffer and a top column cap securely in place. Sodium azide added to the storage buffer to a concentration of 0.02% (w/v) will help prevent microbial growth. Always remove the top cap before the bottom cap to avoid drawing air bubbles down into the gel bed. Prevent air bubbles from forming in the gel bed by using only degassed buffer and sample solutions. Degassing involves subjecting a solution to vacuum to remove excess dissolved air. Use of too high a vacuum can lead to evaporation of solvent from the solution. Check the final volume after degassing and, if necessary, add more solvent to return to original volume.*

l. Refer to Section C on page 76 for fractionation use instructions.

2. Nanosep® spin filter column format

Tip: *In this device format, the minimum amount of resin required to cover the membrane surface when spun in an angle rotor is 0.05 mL packed bed volume (0.1 mL of a 50% slurry). If smaller volumes of resin are required, a swinging bucket rotor can be used down to 0.025 mL packed bed volume (0.05 mL of a 50% slurry). The maximum amount of resin recommended for this device format is 0.2 mL packed bed volume (0.04 mL of a 50% slurry). Higher amounts are difficult to mix with sample and material will be lost from the device when spinning.*

a. Remove the spin filter from the Nanosep collection tube and place in a rubber stopper (with a suitable hole to form a tight fit to the spin fliter) on the vacuum flask. Apply a low vacuum of 5-10 mm Hg.

b. Mix the 50% Heparin HyperD® M slurry (washed in bulk to remove the 1 M NaCl, 20% ethanol preservative with buffer) and quickly pipette 0.4 mL of the slurry to the Nanosep device. If a larger volume column is reguired, a second volume of slurry can be added.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

c. Apply vacuum to remove the liquid from the resin bed, which should partially fill the Nanosep device.

d. Replace the spin filter in the collection tube for storage.

e. Refer to Section C on page 76 for fractionation use instructions.

Tip: *Because the preservative is now removed from the resin in these devices, they should be used immediately or stored at 4 ºC for no more than one week.*

3. AcroPrep™ multi-well filter plate format

a. Wash the Heparin HyperD media with 5 column volumes (CV) of buffer to remove the 1 M NaCl, 20% ethanol storage buffer. Adjust to a final 50% (v/v) slurry with the loading buffer.

b. Place an AcroPrep 96 filter plate (350 µL or 1 mL well volume) on a suitable vacuum manifold (PN 5017) with a collection plate underneath.

c. Mix the 50% slurry and quickly pipette 0.35 mL to the 350 µL multi-well plate. Rapidly follow with a second and third volume of slurry for the 1 mL filter plate.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

d. After final addition, allow the vacuum to remove the liquid from the resin bed to partially fill the well of the AcroPrep 96 filter plate.

e. The plate is ready for immediate use. To store the plate, add approximately 200 µL of buffer per well, then cover. Excess buffer will need to be removed (by vacuum or centrifuge) immediately before use.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 °C for no more than one week.*

f. Refer to Section C on page 76 for fractionation use instructions.

C. Heparin Affinity Based Pre-fractionation

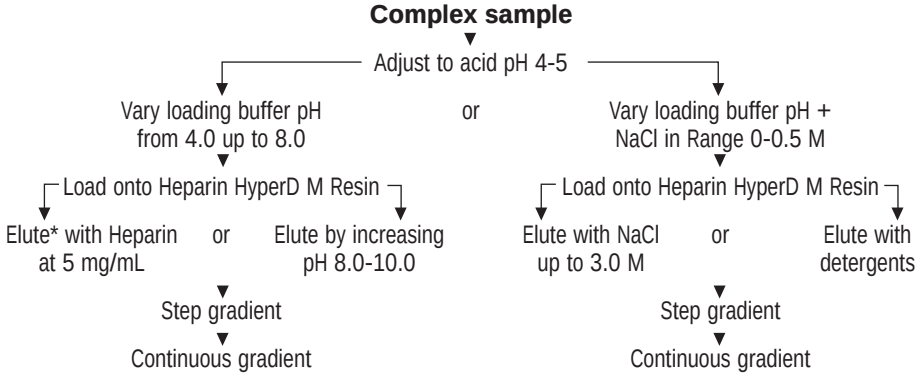
The following approaches can be used:

- Varying the binding conditions, such as pH and salt concentration can impact adsorption to the polyanionic heparin surface.
- Investigating the impact of divalent cations, such as Ca++ or Mg++ on the adsorption of lipoproteins.
- Elution options are specific elution with a heparin solution, use of a NaCl gradient up to 2-3 M, and use of a detergent, such as sodium deoxycholate, Triton* X-100, or sodium dodecyl sulfate (SDS) as a dissociating agent.

Combinations of the above approaches are useful in developing an optimal pre-fractionation strategy and are summarized in Table 2.17.

Table 2.17

Summary of Pre-Fractionation Options for Heparin HyperD® M Resin



**Elution may require several steps, such as pH, salt, and detergents linked together to achieve efficient recovery of bound material.*

Either strategy or a combination of both can be applied to pre-fractionate a complex sample in one of the following formats.

1. Gravity flow column format

- **a.** Prepare a 1-2 mL column as described above.
- **b.** Wash the media with 5 CV of buffer to remove the storage buffer.
- **c.** Allow the liquid in the column to drain to waste.
- **d.** Load the sample up to a 2 mL volume onto the column.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.17.*

- **e.** Collect the column effluent as 1 mL fractions. Measure absorbance at 280 nm to locate the protein peak.

Tip: *Unretained protein rapidly elutes from the column and should be found in the first 3 fractions. Some slight dilution of the sample will occur during elution. If necessary, the sample can be concentrated in a centrifugal UF spin filter, such as a Nanosep® or Microsep™ centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).*

f. After unretained protein has been eluted, the column should be washed with 5 CV of loading buffer before elution is attempted.

g. Retained fractions can then be eluted by biospecific elution with heparin (5 mg/mL), serial steps up to 3.0 M salt, or detergent elution with 1% (w/v) sodium deoxycholate, SDS, or 1% (v/v) Triton* X-100. The volume of each elution should be at least 2 CV to avoid "carry over" between steps in the elution. Too large a volume should be avoided as it will generate dilute samples.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF spin filter with a 10K MWCO membrane (see Section 2.4, page 152).*

h. After the heparin or NaCl elution steps, tightly bound material can be eluted with 1% (w/v) SDS in water and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

2. Nanosep spin filter column format

a. Prepare the spin column as described above.

b. Centrifuge the spin column in a swinging bucket rotor at 500 xg for 2 minutes to remove excess fluid from the packed bed.

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.1-0.2 mL) onto the top of the dry packed bed in the Nanosep device. Replace in the collection tube. The sample and resin should be mixed and allowed to remain in contact for 5-10 minutes at room temperature to improve binding.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.17.*

e. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to pass the sample through the heparin resin bed.

f. Filtrate in the collection tube will be the unretained flowthrough fraction.

g. After unretained protein has been eluted, the column should be washed with 5 CV of loading buffer before elution is attempted.

h. Retained fractions can then be eluted by biospecific elution with heparin (5 mg/mL), serial steps up to 3.0 M salt, or detergent elution with 1% (w/v) sodium deoxycholate, SDS or 1% (v/v) Triton X-100. The volume of each elution should be at least 2 CV to avoid "carry over" between steps in the elution. Too large a volume should be avoided and will generate dilute samples.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF spin filter with a 10K MWCO membrane (see Section 2.4, page 152).*

i. After the heparin or NaCl step, tightly bound material can be eluted from the support with 1% (w/v) SDS in water and recovered by acetone precipitation or detergent removal using SDR HyperD resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

3. AcroPrep™ multi-well filter plate format

Tip: *For prolonged incubations or incubations using solutions that reduce surface tension (e.g., detergents, alcohols, and acetonitrile) in 96-well plates, it may be necessary to seal the bottom of the plate to prevent leakage. This can be tested in advance with just the solutions in the chosen plates.*

An adhesive plate sealer can be used on the top of the wells to prevent cross contamination during vigorous shaking or evaporation during prolonged or warm incubations. For sealing, you can use a plastic, self-adhesive plate sealer (e.g., Sigma EASYseal) or a cap-mat (PN 5230).*

a. Prepare the packed multi-well filter plate.

b. Centrifuge the multi-well plate and collection plate in a swinging bucket rotor at 400 x g for 2 minutes to remove excess fluid from the packed bed.

Tip: *As an alternative to centrifugation, a vacuum manifold can be used. See Section 6.3 of the Appendix for more detail.*

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.125-0.25 mL for the 350 µL plate or 0.375-0.85 mL for the 1 mL plate) onto the top of the dry packed bed in the AcroPrep 96 multi-well filter plate.

Tip: *At this stage, sample-loading conditions should be optimized following recommendations in Table 2.17. The amount of sample loaded should be < 50% of the static binding capacity of the resin.*

e. The sample and well contents should be thoroughly mixed and kept in contact for 30 minutes at room temperature to improve binding.

Tip: *To minimize buffer leakage and loss due to evaporation, use an adhesive plate sealer or cap-mat (PN 5230) on the top of the plate and incubate in a moisture-resistant bag or container.*

The shaker table used should be able to agitate the plate to keep the chromatography media and sample suspended within the well on the plate. Control the mixing so sample and resin do not come in contact with the top sealing sheet.

f. Place the sealed plate on top of the collection plate to catch any filtrate. Tap the plate to dislodge any liquid near the top of the plate and then carefully and slowly remove the top sheet.

g. Centrifuge the multi-well plate and collection plate in a swinging bucket rotor at 400 x g for 2 minutes to pass the sample through the Heparin affinity bed, or apply vacuum sufficient to pass the sample through the bed in 2 mm.

h. Filtrate recovered from the collection plate will be the unretained fraction.

i. After unretained protein has been eluted, the wells should be washed with 5 CV of loading buffer before elution begins.

Tip: *At this stage, additional steps can be added to the process to increase the degree of washing stringency. Raise salt concentration or add a chaotropic agent, such as urea, to challenge non-specific binding interaction. These conditions should be controlled carefully to minimize elution of weak binding specific interactions with the chromatography support.*

j. Retained fractions can then be eluted by biospecific elution with heparin (5 mg/mL), serial steps up to 3.0 M salt, or detergent elution with 1% (w/v) sodium deoxycholate, SDS, or 1% (v/v) Triton* X-100. The volume of each elution should be at least 2 CV to avoid "carry over" between steps in the elution. Too large a volume should be avoided and will generate dilute samples.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF filter plate with a 10K MWCO membrane (see Section 2.4, page 152).*

k. After the last heparin or NaCl step, tightly bound material can be eluted with 1% (w/v) SDS in water and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

Ordering Information for Heparin HyperD M Resin

Part Number	Description	Pkg
20029-062	Heparin HyperD M	5 mL
20029-039	Heparin HyperD M	25 mL
20029-021	Heparin HyperD M	100 mL
20029-013	Heparin HyperD M	1000 mL

References for Heparin HyperD M Resin

1. Hata, A., et al. (1993). Binding of lipoprotein lipase to heparin. *J. Biol. Chem.*, (268), 8447.
2. Zhang, W., et al. (1991). Purification of a novel 55 kDa HeLa cell nuclear DNA-binding protein. *Biochem. Biophys. Res. Commun.*, (174), 542.
3. Moore, M.S., & Blobel, G. (1993). The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature*, (365), 661.
4. Bhikhabhai, R., et al. (1992). Purification, characterisation and crystallisation of recombinant HIV-1 reverse transcriptase. *J. Chromatography*, (604), 157.

2.2.3.2 Lysine HyperD® Resin

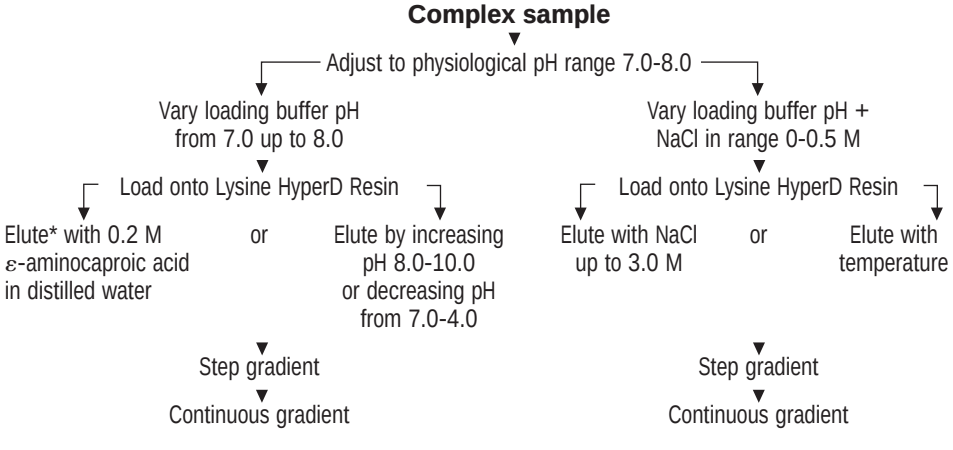
Coupling L-Lysine to an activated bead support creates a useful group-specific affinity ligand for purification of proteins associated with blood clotting, such as plasminogen and plasminogen activator protein. In addition, other proteins have been reported to bind to this ligand. A partial list is provided in Table 2.18.

Table 2.18
Macromolecules Purified or Show Affinity for a Lysine Affinity Ligand

Macromolecule	Reference
Cytochrome c oxidase	*Protein Expr Purif.* 1992 Feb;3(1):36–40.
Fibrinogen from human plasma	*Prep Biochem Biotechnol.* 2003 Nov;33(4):239–52.
Escherichia coli-derived endotoxins	*J Chromatogr A.* 1995 Sep 8;711(1):81–92.
105 kDa protein kinase from rat liver nuclei	*Biochim Biophys Acta.* 1982 May 3;703(2):171–9.
Heparin	*Thromb Res.* 1980 Dec 1–15;20(5-6):599–609.

L-Lysine is coupled via its a-amino group, leaving both the ε-amino and α-carboxyl groups free to interact with sample substances during chromatography. The mechanism by which separation occurs appears to be both electrostatic and stereospecific, depending upon the application. Such a surface, while having specific affinity for the above proteins can also be used to pre-fractionate a complex sample such as plasma and serum. Recommendations for how the binding and elution conditions can be varied to bring about pre-fractionation are summarized in Table 2.19.

Table 2.19
Summary of Pre-Fractionation Options for Lysine HyperD Resin



**Elution may require several steps, such as pH, salt, and temperature linked together to achieve efficient recovery of bound material.*

Lysine HyperD® resin provides high binding capacity and high flow rates, ideal for small- and large-scale applications. A summary of the properties of the lysine affinity resin based on HyperD particles is presented in Table 2.20. In this section, the application focus will be on small-scale pre-fractionation in single and multi-well devices with scale up into gravity flow columns. Larger scale protocols employing a liquid chromatography workstation in glass columns are described in purification applications Section 4.2.8, page 365.

Table 2.20
Properties of Lysine HyperD Resin

Specification	Parameter
Particle Size	80 µm (average)
Ligand	L-Lysine
Ligand Loading	–
Functional Capacity	–
pH Stability	3-13
Pressure Stability	Up to 70 bar (7,000 kPa, 1,015 psi)

Protocol for Lysine HyperD Resin

A. Materials Required

1. Container/column for Lysine HyperD resin, one of the following:
   - **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922)
   - **b.** Nanosep® MF centrifugal device with 0.45 µm GHP membrane (PN ODGHPC34)
   - **c.** Choose either:
     - **(1)** AcroPrep™ 96 filter plate, 350 µL well, 0.45 µm GHP membrane (PN 5030); 1.2 µm Supor® membrane (PN 5039); or
     - **(2)** AcroPrep 96 filter plate, 1 mL well, 0.45 µm GHP membrane (PN 5054)
2. Needed if filter plate is used:
   - **a.** Collection plates [e.g., 96 well polypropylene V bottom, 0.5 mL (Axygen PN P96450V) or 1.64 mL round bottom (Axygen PN PDW20)]
   - **b.** Adhesive plate sealing film
   - **c.** AcroPrep 96-well plate sealing cap mat (PN 5230)
3. Separation apparatus (if filtration is used):
   - **a.** Source of vacuum 25.4-50.8 cm Hg (10-20 in. Hg) (PN 5017); or
   - **b.** Centrifuge fitted with a swinging bucket rotor
4. Degassed 50% (v/v) slurry of the Lysine HyperD resin
5. Degassed water or suitable buffer, such as 50 mM phosphate buffer, pH 7.5

Tips on Handling Lysine HyperD® Resin:
For packed columns, use only degassed liquids. Degassing is not necessary for batch mode methods.

Some BioSepra® media are supplied as concentrated slurries and may be difficult to resuspend. Do not use magnetic stir bars with BioSepra media as they can damage the beads. Also, these resins are quite dense and settle quickly. When adding slurry to any device, mix well between additions. If it is necessary to prepare a 50% (v/v) slurry, use a clean spatula to remove some of the packed media and transfer to a graduated glass cylinder containing degassed water or buffer. DO NOT add any media back to the storage bottle to avoid contamination of the bulk media. Thoroughly mix and allow settling. Note the volume of the packing. Decant the supernatant and add back an equal volume of water or buffer to make a 50% (v/v) slurry.

For packed columns, removal of fines may be necessary. Prepare the slurry in desired buffer, mix, and allow to settle for approximately 5 minutes or for enough time that the beads have settled but small particles are still in the solution.

B. Packing Lysine HyperD Resin

1. Gravity flow column format

- **a.** Equilibrate column, degassed 50% gel slurry, and degassed buffer solution (or high purity water) to room temperature.
- **b.** Secure a bottom cap on the column tip and clamp the column (1-5 mL bed volume column, e.g., Pierce PN 29922) upright in a laboratory stand.
- **c.** Add a sufficient volume of degassed buffer/water to the column to fill it up to the reservoir (wide-mouth) portion, and then gently tap the end and side of the column to dislodge any air bubbles.
- **d.** Float a porous disc on top of the liquid within the column.
- **e.** Using the reverse end of a Pasteur pipette or reverse end of a serum separator (e.g., Pierce PN 69710), push the disc evenly to the bottom of the column.
- **f.** Decant most of the liquid from the empty column, being sure to avoid getting air bubbles in the tip region of the column below the inserted disc. Place the column back in its stand with bottom cap still in place.
- **g.** Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 1-2 mL.
- **h.** Allow gel to settle in the column for at least 5 minutes.
- **i.** Position a second porous disc on top of the settled gel bed by floating it on the liquid within the column and pushing it down to just above the settled gel. Leave 1-2 mm of space between the top of the gel bed and the top disc. Do not compress the gel bed.
- **j.** Wash the inside top part of the column with buffer/water to remove residual gel that may have remained along the sides during packing.
- **k.** Packed column is now ready for storage at 4 °C or immediate use.

Tip: *Store the packed column upright at 4 °C with the gel bed submerged under 1-2 mL of buffer and a top column cap securely in place. Sodium azide added to the storage buffer to a concentration of 0.02% (w/v) will help prevent microbial growth. Always remove the top cap before the bottom cap to avoid drawing air bubbles down into the gel bed. Prevent air bubbles from forming in the gel bed by using only degassed buffer and sample solutions. Degassing involves subjecting a solution to vacuum to remove excess dissolved air. Use of too high a vacuum can lead to evaporation of solvent from the solution. Check the final volume after degassing and, if necessary, add more solvent to return to original volume.*

l. Refer to Section C on page 84 for fractionation use instructions.

2. Nanosep® spin filter column format

Tip: *In this device format, the minimum amount of resin required to cover the membrane surface when spun in an angle rotor is 0.05 mL packed bed volume (0.1 mL of a 50% slurry). If smaller volumes of resin are required, a swinging bucket rotor can be used down to 0.025 mL packed bed volume (0.05 mL of a 50% slurry). The maximum amount of resin recommended for this device format is 0.2 mL packed bed volume (0.04 mL of a 50% slurry). Higher amounts are difficult to mix with sample and material will be lost from the device when spinning.*

a. Remove the spin filter from the Nanosep centrifugal device collection tube and place in a rubber stopper (with a suitable hole to form a tight fit to the spin filter) on the vacuum flask. Apply a low vacuum of 5-10 mm Hg.

b. Mix the 50% Lysine HyperD® M slurry (washed in bulk to remove the 1 M NaCl, 20% ethanol preservative with high purity water), and quickly pipette 0.4 mL of the slurry to the Nanosep device. If a larger volume column is required, a second volume of slurry can be added.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

c. Apply vacuum to remove the liquid from the resin bed, which should partially fill the Nanosep device.

d. Replace the spin filter in the collection tube for storage.

Tip: *Because all the preservative is now removed from the resin in these devices, they should be used immediately or stored at 4 °C for no more than one week.*

e. Refer to Section C on page 84 for fractionation use instructions.

3. AcroPrep™ multi-well filter plate format

Tip: *In this device format, the minimum amount of resin required to cover the membrane surface when spun in a swinging bucket rotor or used on a vacuum manifold is 0.025 mL packed bed volume (0.05 mL of a 50% slurry). The maximum amount of resin recommended for the 350 µL plate is 0.175 mL packed bed volume (0.35 mL of a 50% slurry) and up to 0.525 mL packed bed volume (3x 0.35 mL of a 50% slurry) for the 1 mL plate. Higher amounts are difficult to mix with sample and material will be lost from the device when spinning.*

a. Wash the Lysine HyperD® resin with 5 column volumes (CV) of water or buffer to remove the 1 M NaCl, 20% ethanol storage buffer. Adjust to a final 50% (v/v) slurry.

b. Place an AcroPrep™ 96 filter plate (350 µL or 1 mL well volume) on a suitable vacuum manifold (PN 5017) with a collection plate underneath.

c. Mix the 50% slurry and quickly pipette 0.35 mL to the 350 µL multi-well plate. Rapidly follow with a second and third volume of slurry for the 1 mL volume plate.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

d. After final addition, allow the vacuum to remove the liquid from the resin bed, which should partially fill the well of the AcroPrep 96 filter plate.

e. Cover the multi-well plate for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 °C for no more than one week.*

C. Lysine Affinity-based Pre-fractionation

The following approaches can be used:

- Varying the binding conditions around physiological values, such as pH and salt concentration, can impact adsorption to the zwitterionic lysine surface.
- Addition of NaCl up to 0.5 M can elute loosely or non-specifically bound proteins.
- Elution options are specific elution with 0.2 M ε-aminocaproic acid in distilled water for plasma plasminogen, use of a NaCl gradient up to 2-3 M, and use of a temperature gradient.

The affinity of e.g. rRNA to a Lysine agarose support is affected by temperature. As the temperature is reduced, a higher concentration of salt is required to elute each RNA species. Combinations of the above approaches are useful in developing an optimal pre-fractionation strategy, and are summarized in Table 2.18. Either strategy or a combination of both can be applied to pre-fractionate a complex sample in one of the following formats.

1. Gravity flow column format

a. Prepare a 1-2 mL column.

b. Allow the liquid to drain from the column and load the sample up to a 2 mL volume onto the column.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.19.*

c. Collect the column effluent in 1 mL fractions. Measure absorbance at 280 nm to locate the protein peak.

Tip: *Unretained protein rapidly elutes from the column and should be found in the first three fractions. Some slight dilution of the sample will occur during elution. If necessary the sample can be concentrated in a centrifugal UF spin filter, such as a Nanosep® or Microsep™ centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).*

d. After protein has eluted, the column should be washed with 5 CV of loading buffer before elution is attempted.

e. Retained fractions can then be eluted by specific elution with 0.2 M ε-aminocaproic acid in distilled water for plasma plasminogen, pH gradient up and down away from physiological values, use of a NaCl gradient up to 2-3 M, and use of a temperature gradient. The volume of each elution should be at least 2 CV to avoid "carry over" between steps in the elution. Too large a volume should be avoided as it will generate dilute samples.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF spin filter with a 10K MWCO UF membrane (see Section 2.4, page 152).*

f. After the ligand-specific or NaCl elution steps, tightly bound material can be eluted with 1% (v/v) Triton* X-100 in water warmed to 37 °C and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After a sodium dodecyl sulfate (SDS) detergent elution, the column should be discarded.

2. Nanosep® spin filter column format

a. Prepare the spin column as described above.

b. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to remove excess fluid from the packed bed.

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.1-0.2 mL) onto the top of the dry packed bed in the Nanosep device. Replace in the collection tube. The sample and resin should be mixed and allowed to remain in contact for 5-10 minutes at room temperature to improve binding.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.19.*

e. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to pass the sample through the resin bed.

f. Filtrate in the collection tube will be the unretained flowthrough fraction.

g. After unretained protein has been eluted, the column should be washed with 5 CV of loading buffer before elution is attempted.

h. Retained fractions can then be eluted by specific elution with 0.2 M ε-aminocaproic acid in distilled water, or plasma plasminogen, pH gradient up and down away from physiological values, use of a NaCl gradient up to 2-3 M, and use of a temperature gradient. The volume of each elution should be at least 2 CV to avoid "carry over" between steps in the elution. Too large a volume should be avoided and will generate dilute samples.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF spin filter with a 10K MWCO UF membrane (see Section 2.4, page 152).*

i. After the ligand specific or NaCl elution steps, tightly bound material can be eluted with 1% (v/v) Triton X-100 in water warmed to 37 °C and recovered by acetone precipitation or SDR detergent removal (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

3. AcroPrep™ multi-well filter plate format

Tip: *For prolonged incubations or incubations using solutions that reduce surface tension (e.g., detergents, alcohols, and acetonitrile) in 96-well plates, it may be necessary to seal the bottom of the plate to prevent leakage. This can be tested in advance with just the solutions in the chosen plates.*

An adhesive plate sealer can be used on the top of the wells to prevent cross contamination during vigorous shaking or evaporation during prolonged or warm incubations. For sealing, you can use a plastic, self-adhesive plate sealer (e.g., Sigma EASYseal) or a cap-mat (PN 5230).*

a. Prepare the packed multi-well filter plate as described above.

b. Centrifuge the multi-well plate and collection plate in a suitable swinging bucket rotor at 400 x g for 2 minutes to remove excess fluid from the packed bed.

Tip: *As an alternative to centrifugation, a vacuum manifold can be used. See Section 6.3 of the Appendix for more detail.*

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.125-0.25 mL for the 350 µL plate or 0.375-0.85 mL for the 1 mL plate) on to the top of the dry packed bed in the AcroPrep 96 multi-well filter plate.

Tip: *At this stage, sample-loading conditions should be optimized following recommendations in Table 2.19. The amount of sample loaded should be < 50% of the static binding capacity of the resin.*

e. The sample and well contents should be thoroughly mixed and kept in contact for 30 minutes at room temperature to improve binding.

Tip: *To minimize buffer leakage and loss due to evaporation, use an adhesive plate sealer or cap-mat (PN 5230) on the top of the plate and incubate in a moisture-resistant bag or container.*

The shaker table used should be able to agitate the plate to keep the chromatography media and sample suspended within the well. Control the mixing so sample and resin do not come in contact with the top sealing sheet.

f. Place the sealed plate on top of the collection plate to catch any filtrate. Tap the plate to dislodge any liquid near the top of the plate and then carefully and slowly remove the top sheet.

g. Centrifuge the multi-well plate and collection plate in a swinging bucket rotor at 400 x g for 2 minutes to pass the sample through the affinity bed, or apply vacuum sufficient to pass the sample through the bed in 2 mm.

h. Filtrate recovered from the collection plate will be the unretained fraction.

i. After unretained protein has been eluted, the wells should be washed with 5 CV of loading buffer before elution begins.

Tip: *At this stage, additional steps can be added to the process to increase the degree of washing stringency. Increase salt concentration or add a chaotropic agent, such as urea, to challenge non-specific binding interaction. These conditions should be controlled carefully to minimize elution of weak binding-specific interactions with the chromatography support.*

j. Retained fractions can then be eluted by specific elution with 0.2 M ε-aminocaproic acid in distilled water for plasma plasminogen, pH gradient up and down away from physiological values, use of a NaCl gradient up to 2-3 M, and use of a temperature gradient. The volume of each elution should be at least 2 CV to avoid "carry over" between steps in the elution. Too large a volume should be avoided and will generate dilute samples.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF filter plate with a 10K MWCO UF membrane (see Section 2.4, page 152).*

k. After the ligand specific or NaCl elution steps, tightly bound material can be eluted with 1% (v/v) Triton* X-100 in water warmed to 37 °C and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

Ordering Information for Lysine HyperD Resin

Part Number	Description	Pkg
20059-058	Lysine HyperD	5 mL
20059-036	Lysine HyperD	25 mL
20059-028	Lysine HyperD	100 mL
20059-010	Lysine HyperD	1000 mL

References for Lysine HyperD Resin

1. Karadi, I., et al. (1988). Lipoprotein (a) and plasminogen are immunochemically related. *Biochim. Biophys. Acta*, (960), 91.
2. Cleary, S., et al. (1989). Purification and characterisation of tissue plasminogen activator kringle-2 domain expressed in *E. coli*. *Biochemistry*, (28), 1884.
3. Gonzales-Gronow, M., et al. (1990). The role of carbohydrate in the function of human plasminogen: a comparison of the protein obtained from molecular cloning and expression in *E. coli* and COS cells. *Biochim. Biophys. Acta*, (1039), 269.

2.2.3.3 IMAC HyperCel™ Resin for Immobilized Metal Ion Affinity Chromatography

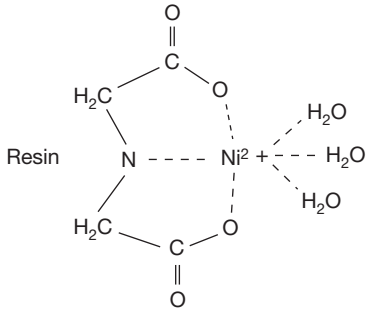
Immobilized metal affinity chromatography (IMAC) is a powerful protein fractionation method used to enrich metal-associated proteins and peptides. In proteomics, IMAC has been widely employed as a pre-fractionation method to increase the resolution in protein separation[1-3]. The combination of IMAC with other protein analytical technologies has been successfully utilized to characterize metallo-proteome and post-translational modifications, such as phosphorylation[4-5]. In the near future, newly developed IMAC integrated with other proteomic methods will greatly contribute to the evolution of expression, cell-mapping, and structural proteomics.

Resins suitable for IMAC are modified to provide strong cation chelation chemistry with a high affinity for transition metals, such as Cu (II), Ni (II), Zn (II), and Fe (III). An example of the tridentate iminodiacetic acid (IDA) chemistry on IMAC HyperCel resin is shown in Figure 2.23, for a Ni (II) complex. This chelated metal complex has a strong affinity for:

- Exposed histidine residues of a protein.
- Some interaction with cysteine or tryptophan.
- In the case of Fe (III), Ga (II), and Zn (III), strong interactions with phosphate groups added by post translation modification[3-4].

Proteins displaying single histidine exposed residues on their surface have been resolved by IMAC[6]. The strength of the affinity interaction increases with the number of histidine residues[7-8]. Histidine (6x His) tagged fusion proteins are routinely purified from native and denatured cell lysates in the presence of urea and guanidine hydrochloride denaturants.

Figure 2.23
Structure of Nickel-IDA Metal Ion Chelation Chemistry



Nickel-IDA (Imino-DiAcetic acid) resin

A summary of the properties of the IMAC HyperCel metal ion chelation affinity resin is presented in Table 2.21. In this section the application focus will be on small-scale pre-fractionation in single and multi-well devices with scale up into gravity flow columns. Larger-scale protocols employing a liquid chromatography workstation in glass columns are described in purification applications Section 4.2.4, page 332.

Table 2.21
Properties of IMAC HyperCel™ Resin

Specification	Parameter
Particle Size	80-100 µm (average)
Particle Composition	Cross-linked cellulose
Ligand	Tridentate IDA (iminodiacetic acid)
Ionic Capacity	90-140 µeq/mL
Metal Ion Loading Capacity	40-70 µmol Cu++ per mL of resin
Regeneration	0.5-1.0 M NaOH
Pressure Stability	< 3 bar (300 kPa, 44 psi)
Working Pressure	< 1 bar (100 kPa, 14 psi)
Thermal Stability	4-121 °C

Protocol for IMAC HyperCel Resin for Immobilized Metal Ion Affinity Chromatography

A. Materials Required

1. Container/column for resin, one of the following:
- **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922)
- **b.** Nanosep® MF centrifugal device with 0.45 µm GHP membrane (PN ODGHPC34)
- **c.** Choose either:
  - **(1)** AcroPrep™ 96 filter plate, 350 µL well, 0.45 µm GHP membrane (PN 5030); 1.2 µm Supor® membrane (PN 5039); or
  - **(2)** AcroPrep 96 filter plate, 1 mL well, 0.45 µm GHP membrane (PN 5054)

2. Needed if filter plate is used:
- **a.** Collection plates (e.g., 96 well polypropylene V bottom, 0.5 mL (Axygen PN P96450V) or 1.64 mL round bottom (Axygen PN PDW20)
- **b.** Adhesive plate sealing film
- **c.** AcroPrep 96-well plate sealing cap mat (PN 5230)

3. Separation apparatus
- **a.** Source of vacuum 25.4-50.8 cm Hg (10-20 in. Hg) (Pall vacuum manifold PN 5017).
- **b.** Centrifuge fitted with a swinging bucket rotor with multi-well plate adapters and able to spin up to 3000 x g.

4. Degassed 50% (v/v) slurry of the IMAC HyperCel resin.

5. Degassed suitable buffer, such as 20 mM sodium phosphate, pH 7.4, containing 0.14 M NaCl or phosphate buffered saline (PBS).

Tip: *For packed columns, use only degassed liquids. Degassing is not necessary for batch mode processing.*

EDTA or EGTA should be avoided in the buffers.

Tips on Handling IMAC HyperCel™ Resin:
Some BioSepra® media are supplied as concentrated slurries and may be difficult to resuspend. DO NOT use magnetic stir bars with BioSepra media as they can damage the beads. When adding slurry to any device mix well between additions.

If it is necessary to prepare a 50% (v/v) slurry, use a clean spatula to remove some of the packed media and transfer to a graduated glass cylinder containing buffer. DO NOT add any media back to the storage bottle to avoid contamination of the bulk media. Thoroughly mix and allow settling. Note the volume of settled resin. Decant the supernatant and add back an equal volume of buffer to make 50% (v/v) slurry.

For packed columns, removal of fines may be necessary. Prepare the slurry in desired buffer, mix, and allow settling for approximately 5 minutes or for enough time that the beads have settled but that small particles are still in solution. Decant off the suspension of fine particles and add fresh buffer and re-mix. Repeat the process until particles settle within approximately 5 minutes and leave a clear supernatant.

B. Packing IMAC HyperCel Resin

1. Gravity flow column format

- **a.** Equilibrate column, degassed 50% gel slurry, and degassed buffer solution to room temperature.
- **b.** Secure a bottom cap on the column tip and clamp the column (1-5 mL bed volume column, e.g., Pierce PN 29922) upright in a laboratory stand.
- **c.** Add a sufficient volume of degassed buffer to the column to fill it up to the reservoir (wide-mouth) portion, and then gently tap the end and side of the column to dislodge any air bubbles.
- **d.** Float a porous disc on top of the liquid within the column.
- **e.** Using the reverse end of a Pasteur pipette or reverse end of a serum separator (e.g., Pierce PN 69710), push the disc evenly to the bottom of the column.
- **f.** Decant most of the liquid from the empty column, being sure to avoid getting air bubbles in the tip region of the column below the inserted disc. Place the column back in its stand with bottom cap still in place.
- **g.** Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 1-2 mL.
- **h.** Allow gel to settle in the column for at least 5 minutes.
- **i.** Position a second porous disc on top of the settled gel bed by floating it on the liquid within the column and pushing it down to just above the settled gel. Leave 1-2 mm of space between the top of the gel bed and the top disc. Do not compress the gel bed.

Tip: *Use cut tips to transfer gel slurry if using a micropipette.*

j. Wash the inside top part of the column with buffer to remove residual gel that may have remained along the sides during packing.

k. Packed column is now ready for immediate use.

l. For storage beyond a working day, add 0.02% sodium azide to the last water wash and place at 4 °C.

m. Refer to Section C on page 92 for metal loading instructions.

Tip: *Store the packed column upright at 4 °C with the gel bed submerged under 1-2 mL of buffer and a top column cap securely in place. Sodium azide added to the storage buffer to a concentration of 0.02% (w/v) will help prevent microbial growth. Always remove the top cap before the bottom cap to avoid drawing air bubbles down into the gel bed. Prevent air bubbles from forming in the gel bed by using only degassed buffer and sample solutions. Degassing involves subjecting a solution to vacuum to remove excess dissolved air. Use of too high a vacuum can lead to evaporation of solvent from the solution. Check the final volume after degassing and, if necessary, add more solvent to return to original volume.*

2. Nanosep® spin filter column format

a. Remove the spin filter from the Nanosep collection tube and place in a rubber stopper (with a suitable hole to form a tight fit to the spin filter) on the vacuum flask. Apply a low vacuum of 5-10 mm Hg.

b. Mix the 50% (v/v) IMAC HyperCel™ slurry (washed in bulk to remove the 1 M NaCl, 20% (v/v) ethanol preservative with buffer) and quickly pipette 0.4 mL of the slurry to the Nanosep device.

c. Apply vacuum to remove the liquid from the resin bed which should partially fill the Nanosep device.

d. Replace the spin filter in the collection tube for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately.*

e. For storage beyond a working day add 0.02% (w/v) sodium azide to the last water wash and place at 4 °C.

f. Refer to Section C on page 92 for metal loading use instructions.

3. AcroPrep™ multi-well filter plate format

a. Wash the media with 5 column volumes (CV) of buffer to remove the 1 M NaCl, 20% ethanol storage buffer. Adjust to a final 50% (v/v) slurry.

b. Place an AcroPrep 96 filter plate (350 µL or 1 mL well volume) on a suitable vacuum manifold with a collection plate underneath.

c. Mix the 50% slurry and quickly pipette 0.08 mL to the small volume multi-well plate, and up to 0.1 mL for the 1.0 mL volume plate.

d. Place the AcroPrep 96 filter plate on top of a receiver plate sized to collect the volume of the filter plate.

e. Apply vacuum to manifold to initiate liquid flow. Recommended vacuum is 25.4 cm Hg (10 in. Hg) for 96-well plates; DO NOT exceed 38.1 cm Hg (15 in. Hg); most house vacuum and aspirators do not exceed 38.1 cm Hg (15 in. Hg).

f. Release vacuum and discard the first filtrate.

Tip: *Do not release the vacuum by pulling the corner of plate as it will degrade the manifold gasket.*

g. Gently tap the plate to remove any hanging droplets.

h. Release vacuum from the manifold and discard the filtrate from the collection plate.

i. As an alternative to vacuum, centrifugation can be used.

(1) Insert the two plates into a standard multi-well plate swinging bucket rotor assembly.

(2) Place a duplicate pair of plates matching the weight of the test plate (add water to the receiver plate and match weight of the test plate).

Tip: *An imbalance can result with a single test plate if no counter balancing plate is used. If different volumes of sample are used in multiple plates, they will need to be balanced in pairs by addition of water to empty wells.*

(3) Centrifuge at 500-3,000 x g for 1-2 minutes.

Tip: *The centrifugal force and time parameters can be varied to optimize the filtration rate of fluids in contact in the well of the plate.*

j. After final addition, allow the vacuum to remove the liquid from the resin bed, which should partially fill the well of the AcroPrep™ 96 filter plate.

k. Cover the multi-well plate for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately.*

l. For storage beyond a working day, add 0.02% sodium azide to the last water wash and placed at 4 °C.

m. Refer to Section C below for metal loading instructions.

C. Loading Metal Ion on to IMAC HyperCel™ Resin

1. Bulk binding (volumes up to 25 mL of resin)

a. Place up to 50 mL of the 50% (v/v) IMAC HyperCel slurry into a 50 mL screw cap centrifuge tube and allow the resin to settle or spin at 1000 x g in a swinging bucket rotor (preferred but an angle rotor will substitute) for 2 minutes to pellet.

b. Remove the supernatant and replace with high purity water. Mix thoroughly and repeat process until all the 1 M NaCl and 20% (v/v) ethanol preservative have been removed.

Tip: *Use a conductivity meter to confirm removal of salt.*

c. Remove the final supernatant and replace with 25 mL of the 0.15 M metal ion solution (see Table 2.22 for a list of solutions for a range of metal ions).

Tip: *Metal ion solution should be made up and filtered using an Acrodisc® MF syringe filter (see Section 2.5, page 198) to remove any insoluble material.*

Table 2.22
Examples of Reagents To Be Used for Metal Ion Immobilization

Metal Ion	Recommended Reagent*	MWt. (g/mol)	g/L*
Cu++	$CuSO_4$, 5 H_20	250	37.5
Ni++	Ni $(CH_3CO_2)_2$,4 H_20	249	37.4
Zn++	Zn $(CH_3CO_2)_2$, 2 H_20	219	32.9
Co++	Co $(CH_3CO_2)_2$, 4 H_20	249	37.4
Ag+	$AgNO_3$	170	25.5
Fe+++	$FeCl_3$, 6 H_20	270	40.5
Ga+++	$Ga(NO_3)_3$, X H_20	255.7	38.4
Zr++++	Zr $(CH_3CO_2)_2$	151.3	22.7

**Recommended use at 150 mM concentration*

d. Thoroughly mix by inversion and continue mixing on a suitable shaker for 30 minutes to ensure saturation of the metal ion binding sites on the resin. At this point the resin will acquire the color of the metal ion solution and the supernatant will become lighter compared to the starting material.

e. After metal ion loading, the colored resin is then washed with high purity water until the supernatant is no longer colored.

Tip: *This can be confirmed visually or can be measured at a suitable wavelength for the colored metal ion (measure absorption spectrum of starting metal ion solution).*

f. Final 2 x wash with 1 M NaCl followed by high purity water to remove salt (confirm by conductivity measurement). Ready for use at this step.

g. For storage beyond a working day add 0.02% sodium azide to the last water wash and place at 4 °C.

2. Binding in gravity flow column format

a. Prepare a 1-2 mL column.

b. Allow the liquid in the column to drain to waste.

c. Wash the column with 5-10 CV of high purity water to remove any remaining 1 M NaCl, 20% (v/v) ethanol or 0.02% (w/v) sodium azide.

d. Load 3-4 CV of the 0.15 M metal ion solution onto the column (see Table 2.22 for a list of solutions for a range of metal ions).

Tip: *Metal ion solution should be made up and filtered using an Acrodisc® MF syringe filter (see Section 4.5, page 421) to remove any insoluble material.*

e. Thoroughly mix the contents of the column by inversion.

f. Remix frequently over a 30 minute period. Allow the resin to settle and drain the column.

g. Wash the packed resin bed with 10-20 CV of high purity water till the supernatant is no longer colored.

Tip: *This can be confirmed visually or can be measured at a suitable wavelength for the colored metal ion (measure absorption spectrum of starting metal ion solution).*

h. Final 2 x wash with 1 M NaCl followed by high purity water to remove salt (confirm by conductivity measurement). Column is now ready for use.

i. For storage beyond a working day, add 0.02% sodium azide to the last water wash and place at 4 °C.

j. Refer to Section D on page 95 for use instructions.

3. Nanosep® spin filter column format

a. Prepare the Nanosep spin filter as described above.

b. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to remove excess fluid from the packed bed.

c. Remove the filtrate from the collection tube.

d. Load the 0.15 M metal ion solution onto the column (see Table 2.22 for a list of solutions for a range of metal ions). Replace in the collection tube. The sample and resin should be mixed and allowed to remain in contact for 5-10 minutes at room temperature to improve binding.

Tip: *Metal ion solution should be made up and filtered using an Acrodisc® MF syringe filter to remove any insoluble material.*

e. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes.

f. Wash the resin with high purity water four times.

g. Wash with 1 M NaCl two times.

h. The packed spin filter is now ready for use.

Tip: *Because all preservative has been removed from the resin in these devices, they should be used immediately.*

i. Refer to Section D on page 95 for use instructions.

4. Binding in an AcroPrep™ multi-well filter plate format

a. Prepare a multi-well packed filter plate.

Tip: *0.04 mL and 0.1 mL are the recommended packed volume of IMAC HyperCel™ resin for the 350 µL and 1.0 mL volume AcroPrep 96 filter plates.*

b. Wash the resin with 0.3 or 0.9 mL of high purity water depending on the plate volume. Mix on a vibrator for 2 minutes, and remove the liquid by vacuum or centrifugation. Repeat 4 times.

c. Apply an adhesive sealing sheet on the bottom of the multi-well plate and load each well with 0.2 mL or 0.8 mL of a 0.15 M metal ion solution (see Table 2.22 for a list of solutions for a range of metal ions).

Tip: *Metal ion solution should be filtered using an Acrodisc MF syringe filter (see Section 4.5, page 421) to remove any insoluble material.*

d. Place a plate top sealing sheet (PN 5230) in place to seal the wells and place on a shaker table and agitate (280 rpm) to mix the resin and liquid in a vertical position for 15 minutes. Reverse the plate and shake for another 15 minutes.

e. Remove the outlet sealing sheet first followed by the top sheet and pack the resin and remove the supernatant by vacuum or centrifugation.

f. Wash the resin by addition of 0.3 mL or 0.8 mL of high purity water and mix on the shaker table. Repeat 4 times.

g. Wash the beads by addition of 0.3 mL or 0.8 mL of a 1 M NaCl solution and mix on the shaker table. Repeat 2 times.

h. The plate can be used directly after this step.

i. Cover the multi-well filter plate for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately.*

j. For storage beyond a working day, add 0.02% sodium azide to the last water wash and place at 4 °C.

k. Refer to Section D below for use instructions.

D. Immobilized Metal Ion-based (IMAC) Pre-fractionation

One of the following approaches can be used:

- Investigating the impact of metal ions, such as Fe (III), Cu (II), Zn (II), Co (II), Ni (II), Ag (II), Ga (II), and Zn (III) loaded onto the IMAC resin on the adsorption of proteins or peptides.
- Varying the binding conditions, such as Phosphate buffer pH and salt concentration, which can impact adsorption to the IMAC surface.
- Use of denaturing conditions, such as 8 M urea and 6 M guanidine HCl.

Elution options are: 1) use of imidazole as a competitive ligand up to 0.1 M; 2) use of imidazole in the presence of up to 0.5 M NaCl; 3) use of elution with 1 M sodium phosphate, pH 3.5, containing 0.14 M NaCl; 4) use of EDTA or EGTA up to 50 mM; and 5) use of detergents, such as sodium deoxycholate, Triton* X-100, or sodium dodecyl sulfate (SDS) as a dissociating agent. Combinations of the above approaches are useful in developing an optimal pre-fractionation strategy, and are summarized in Table 2.23. Either strategy or a combination of both can be applied to pre-fractionate a complex sample in one of the following formats.

1. Gravity flow column format

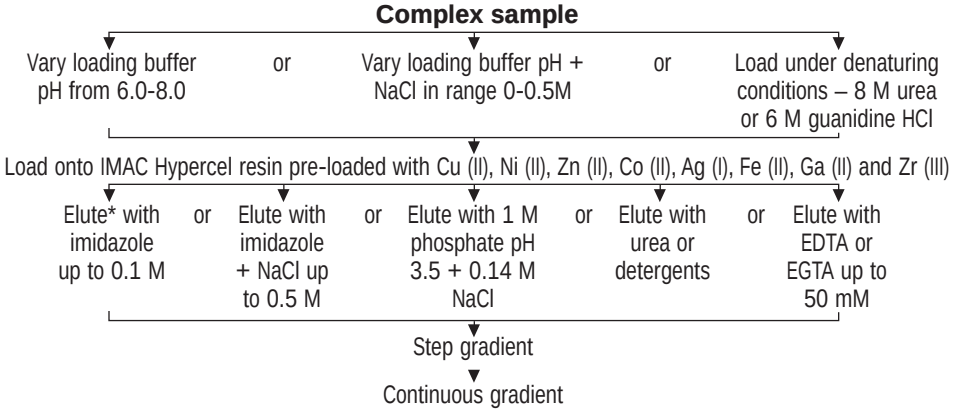
a. Prepare a 1-2 mL column as described above and load with the appropriate metal ion.

b. Allow the liquid in the column to drain to waste.

c. Load the sample up to a 2 mL volume onto the column.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.23.*

Table 2.23
Summary of Pre-Fractionation Options for IMAC HyperCel™ Resin



**Elution may require several steps, such as imidazole, pH, salt, EDTA or EGTA, and detergents linked together to achieve efficient recovery of bound material.*

d. Collect the column effluent in 1 mL fractions. Measure absorbance at 280 nm to locate the protein peak.

Tip: *Unretained protein rapidly elutes from the column and should be found in the first three fractions. Some slight dilution of the sample will occur during elution. If necessary the sample can be concentrated in a centrifugal UF spin filter, such as a Pall Nanosep® or Microsep™ centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152)*

e. After unretained protein has been eluted, the column should be washed with 5 CV of loading buffer before elution of retained fractions is attempted.

f. Retained fractions can then be eluted by imidazole steps or gradient up to 0.1 M; imidazole + NaCl up to 0.5 M; elution with 1 M sodium phosphate, pH 3.5, containing 0.14 M NaCl; use of EDTA or EGTA up to 50 mM; and detergent elution with 1% (w/v) sodium deoxycholate, SDS, or 1% (v/v) Triton* X-100. The volume of each elution should be at least 2 CV to avoid “carry over” between steps in the elution. Too large a volume should be avoided and will generate dilute samples.

Tip: *Sample re-concentration at this stage is best carried out in a centrifugal UF spin filter with a 10K MWCO membrane (see Section 2.4, page 152).*

g. After the last step, tightly bound material can be eluted from the support with 1% (w/v) SDS in water and recovered by acetone precipitation or SDR detergent removal (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

2. Nanosep® spin filter column format

a. Prepare the spin column as described above.

b. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to remove excess fluid from the packed bed.

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.1-0.2 mL) onto the top of the dry packed bed in the Nanosep centrifugal device. Replace in the collection tube. The sample and resin should be mixed and allowed to remain in contact for 5-10 minutes at room temperature to improve binding.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.23.*

e. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to pass the sample through the IMAC affinity bed.

f. Filtrate in the collection tube will be the unretained flowthrough fraction.

g. After unretained protein has been eluted, the column should be washed with 5 CV of loading buffer before elution of retained fractions is attempted.

h. Retained fractions can then be eluted using imidazole in discrete steps or a gradient up to 0.1 M; imidazole + NaCl up to 0.5 M; elution with 1 M sodium phosphate, pH 3.5, containing 0.14 M NaCl; use of EDTA or EGTA up to 50 mM; and detergent elution with 1% (w/v) sodium deoxycholate, SDS, or 1% (v/v) Triton* X-100. The volume of each elution should be at least 2 CV to avoid "carry over" between steps in the elution. Too large a volume should be avoided and will generate dilute samples.

Note: *Sample concentration at this stage is best carried out in a centrifugal UF spin filter with a 10K MWCO membrane (see Section 2.4, page 152).*

i. After the last step, tightly bound material can be eluted from the support with 1% (w/v) SDS in water and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

3. AcroPrep™ multi-well filter plate format

Tip: *For prolonged incubations or incubations using solutions that reduce surface tension (e.g., detergents, alcohols, and acetonitrile) in 96-well plates, it may be necessary to seal the bottom of the plate to prevent leakage. This can be tested in advance with just the solutions in the chosen plates.*

An adhesive plate sealer can be used on the top of the wells to prevent cross contamination during vigorous shaking or evaporation during prolonged or warm incubations. For sealing, you can use a plastic, self-adhesive plate sealer (e.g., Sigma EASYseal) or a cap-mat (PN 5230).*

a. Prepare the packed multi-well filter plate as described above.

b. Centrifuge the multi-well filter plate and collection plate in a suitable swinging bucket rotor at 400 x g for 2 minutes to remove excess fluid from the packed bed.

Tip: *As an alternative to centrifugation, a vacuum manifold can be used. See Section 6.3 of the Appendix for more detail.*

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.125-0.25 mL for the 350 µL plate or 0.375-0.85 mL for the 1 mL plate) on top of the dry packed bed in the AcroPrep 96 multi-well filter plate.

Tip: *At this stage, sample-loading conditions should be optimized following recommendations in Table 2.23. The amount of sample loaded should be < 50% of the static binding capacity of the resin.*

e. The sample and well contents should be thoroughly mixed and kept in contact for 30 minutes at room temperature to improve binding.

Tip: *To minimize buffer leakage and loss due to evaporation, use an adhesive plate sealer or cap-mat (PN 5230) on the top of the plate and incubate in a moisture-resistant bag or container.*

The shaker table used should be able to agitate the plate to keep the chromatography media and sample suspended within the well on the plate. Control the mixing so sample and resin do not come in contact with the top sealing sheet.

f. Place the sealed plate on top of the collection plate to catch any filtrate. Tap the plate to dislodge any liquid near the top of the plate and then carefully and slowly remove the top sheet.

g. Centrifuge the multi-well plate and collection plate in a swinging bucket rotor at 400 x g for 2 minutes to pass the sample through the Heparin affinity bed, or apply vacuum sufficient to pass the sample through the bed in 2 mm.

h. Filtrate recovered from the collection plate will be the unretained fraction.

i. After unretained protein has been eluted, the wells should be washed with 5 CV of loading buffer before elution begins.

Tip: *At this stage, additional steps can be added to the process to increase the degree of washing stringency. Raise salt concentration or add a chaotropic agent, such as urea, to challenge non-specific binding interaction. These conditions should be controlled carefully to minimize elution of weak binding specific interactions with the chromatography support.*

j. Retained fractions can then be eluted by imidazole steps or gradient up to 0.1 M; imidazole + NaCl up to 0.5 M; elution with 1 M sodium phosphate, pH 3.5, containing 0.14 M NaCl; use of EDTA or EGTA up to 50 mM; and detergent elution with 1% (w/v) sodium deoxycholate, SDS, or 1% (v/v) Triton* X-100. The volume of each elution should be at least 2 CV to avoid “carry over” between steps in the elution. Too large a volume should be avoided and will generate dilute samples.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF filter plate with a 10K MWCO membrane (see Section 2.4, page 152).*

k. After the last step, tightly bound material can be eluted from the support with 1% (w/v) SDS in water and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

Ordering Information for IMAC HyperCel™ Resin for Immobilized Metal Ion Affinity Chromatography

Part Number	Description	Pkg
20093-069	IMAC HyperCel	5 mL
20093-010	IMAC HyperCel	25 mL
20093-028	IMAC HyperCel	100 mL
20093-036	IMAC HyperCel	1 L

References for IMAC HyperCel™ Resin for Immobilized Metal Ion Affinity Chromatography

1. Turkova, J. (1999). Oriented immobilization of biologically active proteins as a tool for revealing protein interactions and function. *J. Chromatography B Biomed Sci Appl.*, (722), 11–31.
2. Hage, D.S. (1999). Affinity chromatography: a review of clinical applications. *Clin Chem*, (45), 593–615.
3. Wu H., & Bruley D.F. (1999, September/October). Homologous human blood protein separation using immobilized metal affinity chromatography: protein C separation from prothrombin with application to the separation of factor IX and prothrombin. *Biotechnol Prog.*, 15(5), 928–931.
4. Nuhse, T.S., Stensballe, A., Jensen, O.N., & Peck, S.C. (2003). Large-scale analysis of *in vivo* phosphorylated membrane proteins by immobilized metal ion affinity chromatography and mass spectrometry. *Mol Cell Proteomics*, (11), 1234–1243.
5. Imam-Sghiouar, N., Joubert-Caron, R., & Caron, M. (2005). Application of metal-chelate affinity chromatography to the study of the phosphoproteome. *Amino Acids*, (1), 105–109.
6. Hemdan, E.S., Zhao, Y.J., Sulkowski, E., & Porath, J. (1989). Surface topography of histidine residues: a facile probe by immobilized metal ion affinity chromatography. *Proc. Natl. Acad. Sci. USA*, (86), 1811–1815.
7. Scully, M.F., & Kakkar, V.V. (1982). Structural features of fibrinogen associated with binding to chelated zinc. *Biochim. Biophys. Acta*., (700), 130–135.
8. Hutchens, T.W., & Yip, T.T. (1990). Differential interaction of peptides and protein surface structures with free metal ions and surface-immobilized metal ions. *J. Chromatography*, (500), 531–542.

2.2.3.4 Protein A Ceramic HyperD® F Resin

Protein A is a bacterial cell wall protein that has high binding specificity to the Fc portion of certain immunoglobulins. Although Protein A has a strong affinity for IgG antibodies, it exhibits varying affinities to other immunoglobulin classes and isotypes. It is a 43,000 dalton protein produced by the bacteria *Staphylcoccus aureus*[1] and contains four binding sites to the Fc regions of IgG. It is commercially available cloned and expressed in *Escherichia coli*[2] and has been modified to reduce non-specific albumin binding and enhanced alkali stability for CIP applications in process[3].

Protein A Ceramic HyperD F resin available from Pall is a high capacity affinity sorbent designed for efficient lab scale pre-fractionation and larger process-scale purification of immunoglobulin G. The resin offers ease of use, rigid bead structure, and high capacity at fast flow rates. Protein A Ceramic HyperD F resin is prepared using a rigid proprietary ceramic bead. Recombinant Protein A is then immobilized to a specially formulated hydrogel within the porous ceramic bead. The properties of Protein A Ceramic HyperD F resin are summarized in Table 2.24.

Table 2.24
Properties of Protein A Ceramic HyperD F Resin

Specification	Parameter
Particle Size	50 µm (average)
Ligand	Recombinant Protein A
Ligand Loading	4-5 mg/mL
Human IgG Capacity (10% Breakthrough at 100 cm/h Linear Velocity)*	> 30 mg/mL
Working pH	2-11
Cleaning pH	2-13
Volume Changes Due to pH and Ionic Strength	Non-compressible
Pressure Stability	Up to 70 bar (7,000 kPa, 1,015 psi)

**Determined using 10 mg/mL human IgG in phosphate buffered saline (PBS), pH 7.4; elution with 0.1 M sodium citrate, pH 2.5 column 4.6 ID x 100 mm.*

Binding of IgG to Protein A can be influenced by:

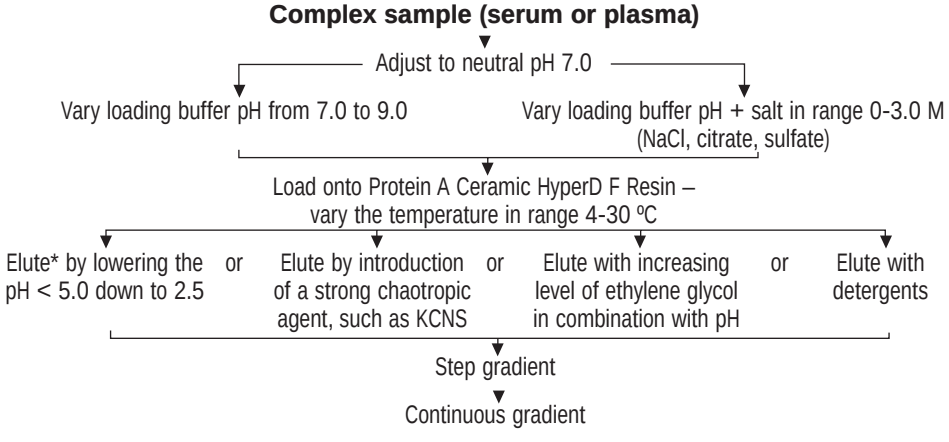
- pH in the range 7- 9.
- Ionic strength and salt type, such as citrate or sodium sulfate to influence IgG partitioning onto the Protein A support.

This has lead to successful optimized binding of many immunoglobulin and isotype classes[4]. Since the binding interaction is of high affinity, it is possible to use stringent wash buffer conditions to reduce non-specific binding. Elution is usually carried out by lowering the pH down to < 5.0 or introduction of strong chaotropic agents, such as potassium thiocyanate (KCNS) to reversibly denature the Protein A and release the IgG.

Use of milder elution conditions, such as ethylene glycol[5] at low pH, leads to disruption of hydrophobic interactions. Temperature has been reported to impact binding conditions for some classes of immunoglobulins[6]. A summary of the pre-fractionation method development options for using a Protein A resin are shown in Table 2.25.

Table 2.25

Summary of Pre-Fractionation Options for Protein A Ceramic HyperD® F Resin



**Elution may require several steps, such as pH gradient, chaotropic agents, and detergents combined together to achieve efficient recovery of bound material.*

In this section, the emphasis will be on methodology development and screening using the Protein A Ceramic HyperD F resin.

Tip: *For larger scale purification of immunoglobulins, refer to Section 4.2.6, page 350 for protocols using packed glass columns on a liquid chromatography system or Section 2.1, page 7 for information and protocols on full kits offered by Pall for the purification or removal of IgG. This later section builds on the method development data generated in the single and multi-well format devices employed in this section.*

Protocol for Protein A Ceramic HyperD® F Resin

A. Materials Required

1. Container/column for Protein A resin, one of the following:
   - **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922)
   - **b.** Nanosep® MF centrifugal device with 0.45 µm GHP membrane (PN ODGHPC34)
   - **c.** Choose either:
     - **(1)** AcroPrep™ 96 filter plate, 350 µL well, 0.45 µm GHP membrane (PN 5030); 0.45 µm Supor® membrane (PN 5029); 1.2 µm Supor membrane (PN 5039); or
     - **(2)** AcroPrep 96 filter plate, 1 mL well, 0.45 µm GHP membrane (PN 5054)
2. Needed if filter plate is used:
   - **a.** Collection plates (e.g., 96 well polypropylene V bottom, 0.5 mL (Axygen PN P96450V) or 1.64 mL round bottom (Axygen PN PDW20)
   - **b.** Adhesive plate sealing film
   - **c.** AcroPrep 96-well plate sealing cap mat (PN 5230)
3. Separation apparatus
   - **a.** Source of vacuum 25.4-50.8 cm Hg (10-20 in. Hg) (PN 5017); or
   - **b.** Centrifuge fitted with a swinging bucket rotor
4. Degassed 50% (v/v) slurry of the Protein A Ceramic HyperD F resin
5. Degassed buffer, such as PBS, pH 7.4

Tips on Handling Protein A Ceramic HyperD F Resin:
For packed columns, use only degassed liquids. Degassing is not necessary for batch mode methods.

Some BioSepra® media are supplied as concentrated slurries and may be difficult to resuspend. Do not use magnetic stir bars with BioSepra media as they can damage the beads. Also, these resins are quite dense and settle quickly. When adding slurry to any device, mix well between additions. If it is necessary to prepare a 50% (v/v) slurry, use a clean spatula to remove some of the packed media and transfer to a graduated glass cylinder containing degassed water or buffer. DO NOT add any media back to the storage bottle to avoid contamination of the bulk media. Thoroughly mix and allow settling. Note the volume of settled resin. Decant the supernatant and add back an equal volume of water or buffer to make a 50% (v/v) slurry.

For packed columns, removal of fines may be necessary. Prepare the slurry in desired buffer, mix, and allow to settle for approximately 5 minutes or for enough time that the beads have settled but small particles are still in the solution.

B. Packing Protein A Ceramic HyperD F Resin

1. Gravity flow column format
   - **a.** Equilibrate column, degassed 50% gel slurry, and degassed buffer solution to room temperature.
   - **b.** Secure a bottom cap on the column tip and clamp the column (1-5 mL bed volume column, e.g., Pierce PN 29922) upright in a laboratory stand.

c. Add sufficient volume of degassed buffer to the column to fill it up to the reservoir (wide-mouth) portion, and then gently tap the end and side of the column to dislodge any air bubbles.

d. Float a porous disc on top of the liquid within the column.

e. Using the reverse end of a Pasteur pipette or reverse end of a serum separator (e.g., Pierce PN 69710), push the disc evenly to the bottom of the column.

f. Decant most of the liquid from the empty column, being sure to avoid getting air bubbles in the tip region of the column below the inserted disc. Place the column back in its stand with bottom cap still in place.

g. Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 1-2 mL.

h. Allow gel to settle in the column for at least 5 minutes.

i. Position a second porous disc on top of the settled gel bed by floating it on the liquid within the column and pushing it down to just above the settled gel. Leave 1-2 mm of space between the top of the gel bed and the top disc. Do not compress the gel bed.

j. Wash the inside top part of the column with buffer to remove residual gel that may have remained along the sides during packing.

k. Packed column is now ready for storage at 4 °C or immediate use.

Tip: *Store the packed column upright at 4 °C with the gel bed submerged under 1-2 mL of buffer and a top column cap securely in place. Sodium azide added to the storage buffer to a concentration of 0.02% (w/v) will help prevent microbial growth. Always remove the top cap before the bottom cap to avoid drawing air bubbles down into the gel bed. Prevent air bubbles from forming in the gel bed by using only degassed buffer and sample solutions. Degassing involves subjecting a solution to vacuum to remove excess dissolved air. Use of too high a vacuum can lead to evaporation of any solvent from the solution. Check the final volume after degassing and, if necessary, add more solvent to return to original volume.*

l. Refer to Section C on page 104 for fractionation use instructions.

2. Nanosep® spin filter column format

Tip: *In this device format, the minimum amount of resin required to cover the membrane surface when spun in an angle rotor is 0.05 mL packed bed volume [0.1 mL of a 50% (v/v) slurry]. If smaller volumes of resin are required, a swinging bucket rotor can be used down to 0.025 mL packed bed volume [0.05 mL of a 50% (v/v) slurry]. The maximum amount of resin recommended for this device format is 0.2 mL packed bed volume [0.04 mL of a 50% (v/v) slurry]. When higher amounts of resin are used, it becomes difficult to mix resin with the sample and material will be lost from the device while spinning.*

a. Remove the spin filter from the Nanosep collection tube and place in a rubber stopper (with a suitable hole to form a tight fit to the spin filter) on the vacuum flask. Apply a low vacuum of 5-10 mm Hg.

b. Mix the 50% Protein A Ceramic HyperD® F slurry (washed in bulk to remove the 1 M NaCl, 20% ethanol preservative with PBS) and quickly pipette 0.4 mL of the slurry to the Nanosep device. If a larger volume column is required, a second volume of slurry can be added.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

c. After final addition, allow the vacuum to remove the liquid from the resin bed which should partially fill the Nanosep® device.

d. Replace the spin filter in the collection tube for storage.

Tip: *Because the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 °C for no more than one week.*

e. Refer to Section C below for fractionation use instructions.

3. AcroPrep™ multi-well filter plate format

Tip: *In this device format, the minimum amount of resin required to cover the membrane surface when spun in a swinging bucket rotor or used on a vacuum manifold is 0.025 mL packed bed volume [0.05 mL of a 50% (v/v) slurry]. The maximum amount of resin recommended for the 350 µL well plate is 0.175 mL packed bed volume [0.35 mL of a 50% (v/v) slurry], and up to 0.525 mL packed bed volume for the 1 mL plate [3x 0.35 mL of a 50% (v/v) slurry]. When higher amounts of resin are used, it becomes difficult to mix resin with the sample and material will be lost from the device while spinning.*

a. Wash the Protein A Ceramic HyperD® F resin with 5 column volumes (CV) of water or buffer to remove the 1 M NaCl, 20% ethanol storage buffer. Adjust to a final 50% (v/v) slurry.

b. Place an AcroPrep 96 filter plate (350 µL or 1 mL well volume) on a suitable vacuum manifold (PN 5017) with a collection plate underneath.

c. Mix the 50% (v/v) slurry and quickly pipette 0.05-0.35 mL to the 350 µL multi-well plate. Rapidly follow with a second and third volume of slurry for the 1.0 mL volume plate. The resin loaded in the 1 mL plate can be in the range of 0.05-0.525 mL packed bed volume.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

d. After final addition, allow the vacuum to remove the liquid from the resin bed, which should partially fill the well of the AcroPrep 96 filter plate.

e. Cover the multi-well filter plate for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 ºC for no more than one week.*

f. Refer to Section C below for fractionation use instructions.

C. Protein A Affinity-based Pre-fractionation

The following approaches can be used:

- Varying the binding conditions, such as pH, salt concentration, and temperature can impact adsorption to the Protein A surface.
- Investigating the impact of divalent anions, such as (SO4)2- or citrate, on the adsorption of immunoglobulins.
- Elution options are lower pH < 5.0, use of a strong chaotropic agent, such as KCNS, or milder conditions using ethylene glycol.

Combinations of the above approaches are useful in developing an optimal pre-fractionation strategy and are summarized in Table 2.25. Either strategy or a combination of both can be applied to pre-fractionate a complex sample in one of the following formats.

1. Gravity flow column format
   a. Prepare a 1-2 mL column.
   b. Wash the Protein A Ceramic HyperD® F resin with 5 column volumes (CV) of buffer to remove the 0.02% sodium azide storage buffer.
   c. Allow the liquid to drain from the column and load the sample up to a 2 mL volume onto the column.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.25. The amount of sample loaded should be < 50% (v/v) of the static binding capacity of the resin.*

   d. Collect the column effluent in 1 mL fractions. Measure absorbance at 280 nm to locate the protein peak.

Tip: *Unretained protein rapidly elutes from the column and should be found in the first three fractions. Slight dilution of the sample will occur during elution. If necessary the sample can be concentrated in a centrifugal UF spin filter, such as a Nanosep® or Microsep™ centirfugal device, with a 10K MWCO membrane.*

   e. After unretained protein has been eluted, the column should be washed with 5 CV of loading buffer before elution is attempted.
   f. Retained fractions can then be eluted by lowering pH < 5.0, addition of up to 3.0 M KCNS, and detergent elution with 1% (w/v) sodium deoxycholate, or 1% (w/v) Triton* X-100. The volume of each elution should be at least 2 CV to avoid “carry over” between steps in the elution. Too large a volume should be avoided and will generate dilute samples.

Tip: *If necessary, the sample can be concentrated in a centrifugal UF spin filter with a 10K MWCO membrane (see Section 2.4, page 152).*

   g. After elution the column should be washed with 10-20 CV of equilibration buffer to remove any residual elution agents. Monitor effluent pH and conductivity to confirm return to equilibration conditions.
   h. If it is to be re-used within 24 hours, store at 4 ºC. If longer, add 0.02% (w/v) sodium azide to the last 5 CV of equilibration buffer and store at 4 ºC.
   i. If after the above elution steps recovery of remaining tightly bound material is required, a final wash with 1% (w/v) sodium dodecyl sulfate (SDS) in water followed by recovery using acetone precipitation or detergent removal using SDR HyperD resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

2. Nanosep spin filter column format
   a. Prepare the spin column as described above.
   b. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to remove excess fluid from the packed bed.
   c. Remove the filtrate from the collection tube.
   d. Very carefully pipette the sample (0.1-0.2 mL) onto the top of the dry packed bed in the Nanosep device. Replace in the collection tube. The sample and resin should be mixed and allowed to remain in contact for 5-10 minutes at room temperature to improve binding.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.25. The amount of samples loaded should be < 50% of the static binding capacity of the resin.*

e. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to pass the sample through the Protein A affinity bed.

f. Filtrate in the collection tube will be the unretained flowthrough fraction.

g. After unretained protein has been eluted, the column should be washed with 5 CV of loading buffer before elution is attempted.

h. Retained fractions can then be eluted by lowering pH < 5.0, addition of up to 3.0 M KCNS, and detergent elution with 1% (w/v) sodium deoxycholate, or 1% (v/v) Triton* X-100. The volume of each elution should be at least 2 CV to avoid "carry over" between steps in the elution. Too large a volume should be avoided as it will generate dilute samples.

Tip: *Sample concentration at this stage can be carried out in a centrifugal UF spin filter, such as a Nanosep® centrifugal device, with a 10K MWCO membrane (see Section 2.4, page 152).*

i. After elution the spin column should then be washed with 10-20 CV of equilibration buffer to remove any residual elution agents. Monitor effluent pH and conductivity to confirm return to equilibration conditions.

j. If the column will be re-used within 24 hours, store at 4 ºC. If longer, add 0.02% (w/v) sodium azide to the last 5 CV of equilibration buffer and store at 4 ºC.

k. If recovery of remaining tightly bound material is required, do a final wash with 1% (w/v) SDS in water followed by recovery by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

3. AcroPrep™ multi-well filter plate format

Tip: *For prolonged incubations or incubations using solutions that reduce surface tension (e.g., detergents, alcohols, and acetonitrile) in 96-well plates, it may be necessary to seal the bottom of the plate to prevent leakage. This can be tested in advance with just the solutions in the chosen plates.*

An adhesive plate sealer can be used on the top of the wells to prevent cross contamination during vigorous shaking or evaporation during prolonged or warm incubations. For sealing, you can use a plastic, self-adhesive plate sealer (e.g., Sigma EASYseal) or a cap-mat (PN 5230).*

a. Prepare the packed multi-well filter plate as described above.

b. Centrifuge the multi-well filter plate and collection plate in a suitable swinging bucket rotor at 400 x g for 2 minutes to remove excess fluid from the packed bed.

Tip: *As an alternative to centrifuging, a vacuum manifold can be used. See Section 6.3 of the Appendix for more detail.*

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.125-0.25 mL for the 350 µL plate or 0.375-0.85 mL for the 1 mL plate) on to the top of the dry packed bed in the AcroPrep 96 multi-well filter plate.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.25. The amount of sample loaded should be < 50% of the static binding capacity of the resin.*

e. The sample and well contents should be thoroughly mixed and kept in contact for 30 minutes at room temperature to improve binding.

Tip: *To minimize buffer leakage and loss due to evaporation, use an adhesive plate sealer or cap-mat (PN 5230) on the top of the plate and incubate in a moisture-resistant bag or container.*

The shaker table used should be able to agitate the plate to keep the chromatography media and sample suspended within the well on the plate. Control the mixing so the sample and resin do not come in contact with the top sealing sheet.

f. Place the sealed plate on top of the collection plate to catch any filtrate. Tap the plate to dislodge any liquid near the top of the plate and then carefully and slowly remove the top sheet.

g. Centrifuge the multi-well plate and collection plate in a swinging bucket rotor at 400 x g for 2 minutes to pass the sample through the Protein A affinity bed, or apply vacuum sufficient to pass the sample through the bed in 2 mm.

h. Filtrate recovered from the collection plate will be the unretained fraction.

i. After unretained protein has been eluted, the wells should be washed with 5 CV of loading buffer before elution begins.

Tip: *At this stage, additional steps can be added to the process to increase the degree of washing stringency. Raise salt concentration or add a chaotropic agent, such as urea, to challenge non-specific binding interaction. These conditions should be controlled carefully to minimize elution of weak binding specific interactions with the chromatography support.*

j. Retained fractions can then be eluted by lowering pH < 5.0, addition of up to 3.0 M KCNS, and detergent elution with 1% (w/v) sodium deoxycholate, or 1% (v/v) Triton* X-100. The volume of each elution should be at least 2 CV to avoid “carry over” between steps in the elution. Minimal volume is recommended to minimize sample dilution.

Tip: *If necessary, the sample can be concentrated in a centrifugal UF spin filter with a 10K MWCO membrane (see Section 2.4, page 152).*

k. After elution, the plate should then be washed with 2-5 mL of equilibration buffer per well to remove any residual elution agents. Monitor effluent pH and conductivity to confirm return to equilibration conditions. Apply a plate sealer and place in a moisture resistant container to control loss of liquid from the wells by evaporation.

l. If recovery of remaining tightly bound material is required, a final wash with 0.5 mL of 1% (w/v) SDS in water followed by recovery using acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141) can be employed. After an SDS detergent elution, the plate should be discarded.

m. If the plate will be re-used within 24 hours, store at 4 °C. If longer, add 0.02% (w/v) sodium azide to the last 1 mL of equilibration buffer and store at 4 °C.

Application Data for Protein A Ceramic HyperD® F Resin

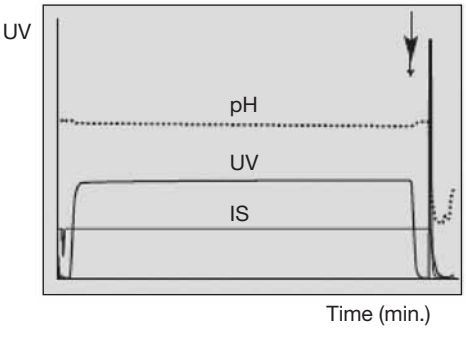
Preparative Purification of Monoclonal IgG on Protein A Ceramic HyperD F Resin
During studies with various IgG subclasses and sample matrices, Protein A Ceramic HyperD F resin provided high purity product, even at high column loading. Results are summarized in Table 2.26. In all cases, the purity was greater than 98% as determined by SDS-PAGE. No albumin was detected in IgG1 isolated from ascites fluid. Isolation of antibody from serum-containing cell culture supernatant is illustrated in Figure 2.24. Analysis by SDS-PAGE of selected fractions is shown in Figure 2.25.

Table 2.26
High Purity, High Capacity IgG Purification Using Protein A Ceramic HyperD Resin

Source	Origin	IgG Subclass	Initial IgG Conc. (mg/mL)	Eluted IgG (mg)	Purity SDS-PAGE
Ascites Fluid	Murine	IgG, kappa	6.34	37	> 98%
Ascites Fluid	Murine	IgG1*	2.26	20	> 98%
Ascites Fluid	Murine	IgG1*	3.86	31	> 98%
Ascites Fluid	Murine	IgG2b**	2.31	6	> 98%
Ascites Fluid	Murine	IgG2a**	2.43	10	> 98%
Ascites Fluid	Murine	IgG2a*	2.86	28	> 98%
Cell Culture Supernatant	Murine	IgG1*	0.05	15	> 98%
Cell Culture Supernatant	Humanized	IgG1*	0.20	30	> 98%

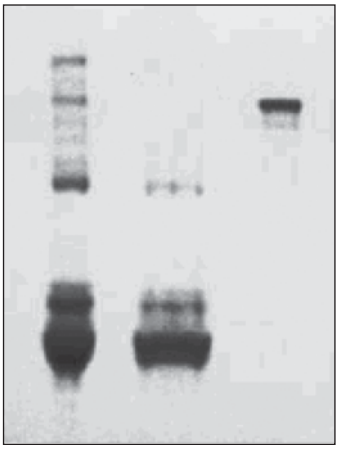
Column: 6.6 mm ID x 120 mm; Volume: 4 mL, 10-30 mg IgG per run; Loading: 1 M Glycine/2 M NaCl (*), pH 8.9 or PBS (**); Elution: 0.1 M acetic acid.

Figure 2.24
Isolation of IgG1 from Serum-Containing Cell Culture Supernatant on Protein A Ceramic HyperD F Resin



Column: 3 mm ID x 100 mm. Loading and wash: 1 M glycine/2 M NaCl, pH 8.9. Elution: 100 mM acetate buffer, pH 4.6. Linear velocity: 300 cm/h. Sample: 50 mL cell culture supernatant. (The arrow indicates introduction of elution and buffer.)

Figure 2.25
SDS-PAGE Analysis of Tissue Culture Fluid, Flowthrough and Eluted IgG1 Fractions



Analysis of tissue culture fluid (Lane 1), flowthrough (Lane 2), and eluted IgG1 (Lane 3).

FAQ and Troubleshooting for Protein A Ceramic HyperD® F Resin

1. How do I determine if a Protein A affinity column will be optimal for purifying my antibody of interest?

   There are differences in the affinity that Protein A has for antibodies from different species and for the different isotypes within a species. The following chart will provide a guideline for determining the effectiveness of Protein A for binding the antibody and isotype being purified.

Species	Antibody Class	Protein A
Human	Total IgG	+++
Human	IgG1	+++
Human	IgG2	+++
Human	IgG3	+
Human	IgG4	+++
Human	IgM	+
Human	IgD	None
Human	IgE	++
Human	IgA	+
Human	IgA1	+
Human	IgA2	+
Human	Fab	+
Human	ScFv	+

(Continued) Species	Antibody Class	Protein A
Mouse	Total IgG	+++
Mouse	IgG1	+
Mouse	IgG2a	+++
Mouse	IgG2b	+++
Mouse	IgG3	+++
Mouse	IgM	None
Rat Total	IgG	+
Rat	IgG1	+
Rat	IgG2a	None
Rat	IgG2b	None
Rat	IgG2c	+++
Horse	Total IgG	+
Horse	IgG(ab)	+
Horse	IgG(c)	+
Horse	IgG(T)	None
Cow	Total IgG	+
Cow	IgG1	+
Cow	IgG2	+++
Goat	Total IgG	+
Goat	IgG1	+
Goat	IgG2	+++
Sheep	Total IgG	+
Sheep	IgG1	+
Sheep	IgG2	+++
Cat	Total IgG	+++
Chicken	Total IgY	None
Dog	Total IgG	+++
Donkey	Total IgG	++
Guinea Pig	Total IgG	+++
Hamster	Total IgG	++
Pig	Total IgG	+++
Rabbit	Total IgG	+++
Rhesus Monkey	Total IgG	+++

Key: Strong Binding = +++, Medium Binding = ++, Weak Binding = +, No Binding = None

2. Can I neutralize my Protein A purified fractions?

   Yes. To neutralize the purified 1 mL fractions, add either 0.1 mL of binding buffer or 0.05 mL of 1 M Tris, pH 9.5. The advantage of using the 1 M Tris solution is to allow you to keep your samples in smaller volumes.

Ordering Information for Protein A Ceramic HyperD® F Resin

Part Number	Description	Pkg
20078-036	Protein A Ceramic HyperD F	5 mL
20078-028	Protein A Ceramic HyperD F	25 mL
20078-010	Protein A Ceramic HyperD F	100 mL
20078-044	Protein A Ceramic HyperD F	1000 mL

References for Protein A Ceramic HyperD F Resin

1. Hjelm, H., Hjelm, K., & Sjoquist, J. (1972, November 15). Protein A from *Staphylococcus aureus*. Its isolation by affinity chromatography and its use as an immunosorbent for isolation of immunoglobulins. *FEBS Lett.*, 28(1), 73–76.
2. Hammond, P.M., Philip, K.A., Hinton, R.J., & Jack, G.W. (1990). Recombinant Protein A from *Escherichia coli* JM83. *Ann N Y Acad Sci.*, (613), 863–867.
3. Linhult, M., Gulich, S., Graslund, T., Simon, A., Karlsson, M., Sjoberg, A., et al. (2004, May 1). Improving the tolerance of a Protein A analogue to repeated alkaline exposures using a bypass mutagenesis approach. *Proteins*, 55(2), 407–416.
4. Van Sommeren, A.P., Machielsen, P.A., & Gribnau, T.C. (1992, June). Effects of temperature, flow rate and composition of binding buffer on adsorption of mouse monoclonal IgG1 antibodies to protein A Sepharose 4 Fast Flow. *Prep Biochem*, 22(2), 135–149.
5. Bywater, R., Eriksson, G.B., & Ottosson, T. (1983, November 11). Desorption of immunoglobulins from Protein A-Sepharose CL-4B under mild conditions. *J Immunol Methods*, 64(1–2), 1–6.
6. Tu, Y.Y., Primus, F.J., & Goldenberg, D.M. (1988, April 22). Temperature affects binding of murine monoclonal IgG antibodies to protein A. *J Immunol Methods*, 109(1), 43–47.

2.2.4 Mixed Mode

2.2.4.1 MEP HyperCel™ Mixed Mode Resin

The mixed mode synthetic affinity ligand 4-mercapto-ethyl-pyridine (MEP) on a HyperCel resin support a range of pre-fractionation options for complex samples such as serum or plasma. This resin shows a strong affinity for IgG under some defined conditions and is a potential replacement for Protein A and Protein G. A summary of its chromatographic properties is shown in Table 2.27.

In this application section, protocols will be described for pre-fractionation of complex samples employing single and multi-well small volume devices and gravity flow columns for initial methods development studies. For use of these resins in a glass chromatography column format, refer to Section 4.2.6, page 350 where methods for packing resins for purification procedures are more thoroughly described.

Table 2.27
Pre-Fractionation Properties of MEP HyperCel Resin

Specification	Parameter
Particle Size	80-100 µm
Bead Composition	Cellulose
Ligand	4-mercapto ethyl pyridine sulfonic acid
Ligand Density	70-125 µmoles/mL media
Working pH Range Binding pH Range Elution pH Range	3-12 7.0-9.0 5.0-6.0
Cleaning pH Range	3-14
Pressure Range	< 3 bar (300 kPa, 44 psi)
Dynamic Binding Capacity for Human IgG	pH 8.0 > 30 mg/mL media pH 7.0 25 pH 6.0 15 pH 5.0 5
Salt Impact on Dynamic Binding Capacity for Human IgG	> 30 mg/mL media 0.2 M > 30 0.6 M > 30 1.0 M > 30
Salt Impact on Dynamic Binding Capacity at Optimal Binding pH	None
Species Affinity Human Polyclonal IgG Murine IgG Murine IgG2a	 32 mg/mL media 37 34

Protocol for MEP HyperCel™ Mixed Mode Resin

A. Materials Required

1. Container/column for MEP HyperCel resin, one of the following:
- **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922)
- **b.** Nanosep® MF centrifugal device with 0.45 µm GHP membrane (PN ODGHPC34)
- **c.** Choose either:
  - **(1)** AcroPrep™ filter plate, 100 µL well, 0.45 µm GHP membrane (PN 5070); or
  - **(2)** AcroPrep 96 filter plate, 350 µL well, 0.45 µm GHP membrane (PN 5029); 1.2 µm Supor® membrane (PN 5039); or
  - **(3)** AcroPrep 96 filter plate, 1 mL well, 0.45 µm GHP membrane (PN 5054)

2. Needed if filter plate is used:
- **a.** Collection plates (e.g., 96 well polypropylene V bottom, 0.5 mL (Axygen PN P96450V) or 1.64 mL round bottom (Axygen PN PDW20)
- **b.** Adhesive plate sealing film
- **c.** AcroPrep 96-well plate sealing cap mat (PN 5230)

3. Separation apparatus
- **a.** Source of vacuum 25.4-50.8 cm Hg (10-20 in. Hg) (PN 5017); or
- **b.** Centrifuge fitted with a swinging bucket rotor

4. Degassed 50% (v/v) slurry of the MEP HyperCel resin

5. Degassed water or suitable loading buffer, such as 50 mM Tris HCL pH 8.0

Tips on Handling MEP HyperCel Mixed Mode Resin:
For packed columns, use only degassed liquids. Degassing is not necessary for batch mode methods.

Some BioSepra® media are supplied as concentrated slurries and may be difficult to resuspend. Do not use magnetic stir bars with BioSepra media as they can damage the beads. Also, these resins are quite dense and settle quickly. When adding slurry to any device, mix well between additions.If it is necessary to prepare a 50% (v/v) slurry, use a clean spatula to remove some of the packed media and transfer to a graduated glass cylinder containing degassed water or buffer. DO NOT add any media back to the storage bottle to avoid contamination of the bulk media. Thoroughly mix and allow settling. Note the volume of the packing. Decant the supernatant and add back an equal volume of water or buffer to make a 50% (v/v) slurry.

For packed columns, removal of fines may be necessary. Prepare the slurry in desired buffer, mix, and allow to settle for approximately 5 minutes or for enough time that the beads have settled but small particles are still in the solution.

B. Packing MEP HyperCel Resin

1. Gravity flow column format
- **a.** Equilibrate column, degassed 50% gel slurry, and degassed buffer solution (or high purity water) to room temperature.

b. Secure a bottom cap on the column tip and clamp the column (1-5 mL bed volume column, e.g., Pierce PN 29922) upright in a laboratory stand.

c. Add sufficient volume of degassed buffer/water to the column to fill it up to the reservoir (wide-mouth) portion, and then gently tap the end and side of the column to dislodge any air bubbles.

d. Float a porous disc on top of the liquid within the column.

e. Using the reverse end of a Pasteur pipette or reverse end of a serum separator (e.g., Pierce PN 69710), push the disc evenly to the bottom of the column.

f. Decant most of the liquid from the empty column, being sure to avoid getting air bubbles in the tip region of the column below the inserted disc. Place the column back in its stand with bottom cap still in place.

g. Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 1-2 mL.

h. Allow gel to settle in the column for at least 5 minutes.

i. Position a second porous disc on top of the settled gel bed by floating it on the liquid within the column and pushing it down to just above the settled gel. Leave 1-2 mm of space between the top of the gel bed and the top disc; do not compress the gel bed.

j. Wash the inside top part of the column with buffer/water to remove residual gel that may have remained along the sides during packing.

k. Packed column is now ready for storage at 4 °C or immediate use.

l. Refer to Section C on page 115 for use instructions.

Tip: *Store the packed column upright at 4 °C with the gel bed submerged under 1-2 mL of buffer and a top column cap securely in place. Sodium azide added to the storage buffer to a concentration of 0.02% (w/v) will help prevent microbial growth. Always remove the top cap before the bottom cap to avoid drawing air bubbles down into the gel bed. Prevent air bubbles from forming in the gel bed by using only degassed buffer and sample solutions. Degassing involves subjecting a solution to vacuum to remove excess dissolved air. Use of too high a vacuum can lead to evaporation of solvent from the solution. Check the final volume after degassing and, if necessary, add more solvent to return to original volume.*

2. Nanosep® spin filter column format

Tip: *In this device format, the minimum amount of resin required to cover the membrane surface when spun in an angle rotor is 0.05 mL packed bed volume [0.1 mL of a 50% (w/v) slurry]. If smaller volumes of resin are required, a swinging bucket rotor can be used down to 0.025 mL packed bed volume [0.05 mL of a 50% (w/v) slurry]. The maximum amount of resin recommended for this device format is 0.2 mL packed bed volume [0.04 mL of a 50% (w/v) slurry]. Higher amounts are difficult to mix with sample and material will be lost from the device when spinning*

a. Remove the spin filter from the Nanosep collection tube and place in a rubber stopper (with a suitable hole to form a tight fit to the spin filter) on the vacuum flask. Apply a low vacuum of 5-10 mm Hg.

b. Mix the 50% MEP HyperCel™ slurry (washed in bulk to remove the 1.0 M NaCl, 20% (v/v) ethanol preservative with high purity water) and quickly pipette 0.4 mL of the slurry to the Nanosep device.

c. Apply vacuum to remove the liquid from the resin bed which should partially fill the Nanosep® device.

d. Replace the spin filter in the collection tube for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 °C for no more than one week.*

e. Refer to Section C below for use instructions.

3. AcroPrep™ multi-well filter plate format

Tip: *In this device format, the minimum amount of resin required to cover the membrane surface when spun in a swinging bucket rotor or used on a vacuum manifold is 0.025 mL packed bed volume (0.05 mL of a 50% slurry). The maximum amount of resin recommended for the 350 µL plate is 0.175 mL packed bed vlume (0.35 mL of a 50% slurry) and up to 0.525 mL packed bed volume (3x 0.35 mL of a 50% slurry) for the 1 mL plate. Higher amounts are difficult to mix with sample and material will be lost from the device when spinning.*

a. Wash the MEP HyperCel™ resin with 5 column volumes (CV) of water to remove the 1.0 M NaCl, 20% ethanol storage buffer.

b. Place an AcroPrep 96 filter plate (350 µL or 1 mL well volume) on a suitable vacuum manifold (PN 5017) with a collection plate underneath.

c. Mix the 50% HyperCel resin and quickly pipette 0.4 mL of the slurry to the multi-well plate. For the 1.2 mL volume plate followed with a second and third volume of slurry.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

d. After final addition, allow the vacuum to remove the liquid from the resin bed which should partially fill the well of the AcroPrep 96 filter plate.

e. Cover the multi-well plate for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 °C for no more than one week.*

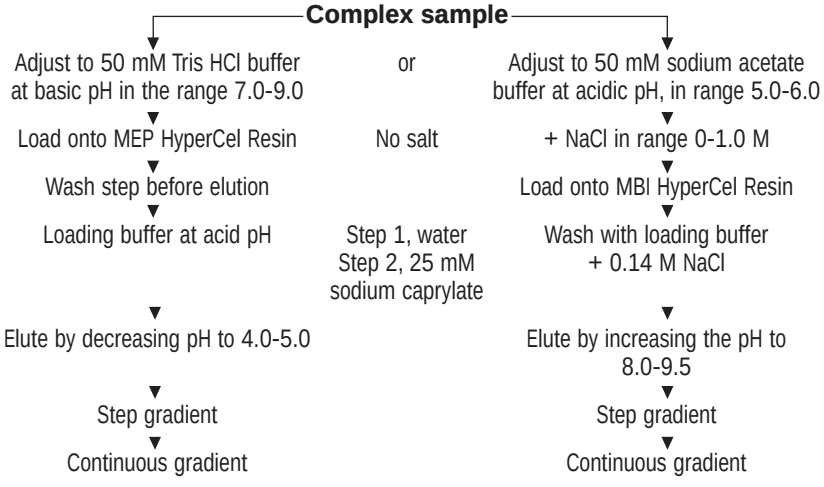
f. Refer to Section C below for use instructions.

Tip: *For use of these resins in a glass chromatography column format, refer to Section 4.2.6, page 350 where methods for packing resins for purification procedures is more thoroughly described.*

C. Mixed Mode Chromatography Pre-fractionation

A summary of the binding and elution conditions of MEP are shown in Table 2.28. The optimal elution conditions for MEP are at acidic pH. Clearly, it should be possible to develop a matrix of binding and elution conditions to pre-fractionate a complex sample such as serum or plasma.

Table 2.28
Summary of Pre-Fractionation Options for MEP HyperCel™ Mixed Mode Resin



This methods development process can be carried out in a range of device formats, such as single and multi-well small volume devices and then scaled up into gravity flow columns. Protocols are described below for operation of the above device formats in a pre-fractionation experiment.

1. Gravity flow column format
   - **a.** Prepare a 1-2 mL column as described above.
   - **b.** Wash the MEP HyperCel resin with 5 CV of water to remove the 1 M NaCl, 20% (v/v) ethanol storage buffer.
   - **c.** Allow the water to drain.
   - **d.** Add 5 CV of loading buffer to the drained resin.

Tip: *At this point binding condition variables such as pH and ionic strength can be tested with replicate devices.*

   - **e.** Allow the liquid to drain from the column and load the sample onto the column.

Tip: *The sample should be prefiltered with an Acrodisc® Syringe Filter or a VacuCap® and VacuCap PF Vacuum Filtration system to ensure that the MEP HyperCel column will not be blocked by particulate material.*

   - **f.** Wash with 5-10 CV of loading buffer, loading buffer + 0.14 M NaCl, water, or 25 mM sodium cacodylate.
   - **g.** Elute the retained material with a 1-2 CV of sodium acetate buffers in the pH range 4.0-5.0.
   - **h.** Collect the column effluent in 0.5 mL fractions and measure A_{280} to locate the protein peaks. Slight dilution of the sample will occur during elution.

Tip: *If necessary the sample can be re-concentrated with a centrifugal UF device, such as a Nanosep® or Microsep™ device, with a 10K MWCO UF membrane (see Section 2.4, page 152).*

2. Nanosep® spin filter column format

a. Prepare the spin column as described above.

b. Centrifuge the spin column in a swinging bucket rotor at 1000 x g for 2 minutes to remove excess fluid from the packed bed.

c. Remove the filtrate from the collection tube.

d. Add 0.4 mL of loading buffer to the dry resin and mix.

Tip: *At this point binding condition variables such as pH and ionic strength can be tested with replicate devices*

e. Centrifuge the spin column in a swinging bucket rotor at 1000 x g for 2 minutes to remove excess fluid from the packed bed.

f. Remove the filtrate from the collection tube.

g. Very carefully pipette the sample (0.1-0.2 mL) onto the top of the dry packed bed in the Nanosep device. Replace in the collection tube.

Tip: *The sample should be prefiltered with a Nanosep GHP spin filter or an Acrodisc® syringe filter to ensure that the MEP HyperCel™ column will not be blocked by particulate material.*

h. Centrifuge the spin column in a swinging bucket rotor at 1000 x g for 4 minutes to pass the sample through the MEP Hypercel resin bed.

i. Filtrate in the collection tube will be labeled the flowthrough fraction (FT).

j. Wash with loading buffer; loading buffer + 0.14 M NaCl; water; or 25 mM sodium caprylate.

k. Filtrate in the collection tube will labeled wash (W).

l. Elute the retained material with a series of sodium acetate buffers in the pH range 4.0-5.0 for MEP.

m. Filtrates in the collection tube will be labeled elution(s) (EL1, 2,…, etc.).

3. AcroPrep™ multi-well filter plate format

Tip: *For prolonged incubations or incubations using solutions that reduce surface tension (e.g., detergents, alcohols, and acetonitrile) in 96-well plates, it may be necessary to seal the bottom of the plate to prevent leakage. This can be tested in advance with just the solutions in the chosen plates.*

An adhesive plate sealer can be used on the top of the wells to prevent cross contamination during vigorous shaking or evaporation during prolonged or warm incubations. For sealing, you can use a plastic, self-adhesive plate sealer (e.g., Sigma EASYseal) or a cap-mat (PN 5230).*

a. Prepare the packed multi-well filter plate.

b. Centrifuge the filter plate and collection plate in a suitable swinging bucket rotor at 1000 x g for 2 minutes to remove excess fluid from the packed bed. Or place on a suitable device manifold and operate at 10-15 mm Hg vacuum to flow liquid down through the plate. Apply vacuum until the wells appear “dry” or when the liquid above the resin is gone. Do not over dry the resin at this stage by applying too high a vacuum or prolonged times.

c. Discard the filtrate from the collection plate.

d. Add 0.4 mL of loading buffer to the dry resin and mix.

Tip: *At this point binding condition variables such as pH and ionic strength can be tested with replicate devices.*

e. Centrifuge the multi-well plate and collection plate in a suitable swinging bucket rotor at 1000 x g for 2 minutes to remove excess fluid from the packed bed; or place on the vacuum manifold.

f. Remove the filtrate from the collection plate.

g. Very carefully pipette the sample (0.125-0.25 mL for the 350 µL plate or 0.375-0.85 mL for the 1 mL plate) onto the top of the dry packed bed in the AcroPrep™ 96 multi-well filter plate.

Tip: *The sample should be prefiltered with an Acrodisc® syringe filter or a VacuCap® and VacuCap PF Vacuum Filtration system to ensure that the MEP HyperCel™ column will not be blocked by particulate material.*

h. Centrifuge the filter plate and collection plate in a swinging bucket rotor at 1000 x g for 4 minutes to pass the sample through the MEP HyperCel resin bed; or place on the vacuum manifold.

i. Follow the procedures described earlier in this section to pre-fractionate the sample.

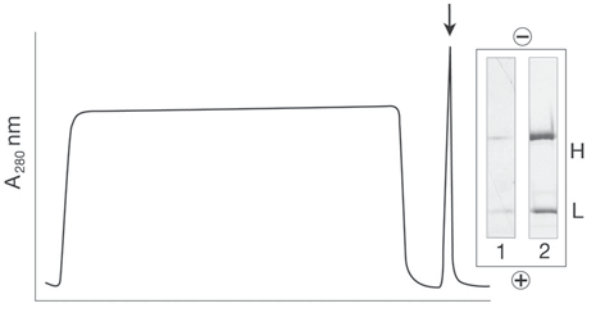
Tip: *For use of these resins in a glass chromatography column format, refer to Section 4.2.6, page 350 where methods for packing resins for purification procedures is more thoroughly described.*

Application Data for MEP HyperCel Mixed Mode Resin

Monoclonal Antibody Purification from Cell Culture Supernatants Using MEP HyperCel Resin
The capture of antibody from both serum-free and serum-supplemented [5% (v/v) fetal bovine serum] cell culture supernatant (CCS) is described. In both cases, clarified samples were loaded without concentration or adjustment of composition. The IgG concentration in the protein-free CCS was 114 µg/mL. Chromatograms are shown in Figure 2.26 and results are summarized in Table 2.29. In both applications, the column was equilibrated with 50 mM Tris-buffer, pH 8.0. After loading, the column was washed with the same buffer. Phosphate buffered saline (PBS), pH 7.4 may also be used. During chromatography of serum-supplemented CCS, two additional wash steps (Figure 2.26, Panel B) were added to promote desorption of albumin. In both cases, the IgG fraction was eluted under the influence of 50 mM sodium acetate, pH 4.0. Product isolated from protein-free CCS was 99% pure (SDS-PAGE). Despite the presence of abundant albumin in the feedstock, product isolated from serum supplemented CCS was 69% pure. The latter could be brought to 99% purity by application of a simple anion exchange procedure.

Figure 2.26
Monoclonal Antibodies Purification on MEP HyperCel™ Resin from Cell Culture Supernatants

Panel A, from serum-free cell culture media



Panel B, from 5% serum supplemented cell culture media

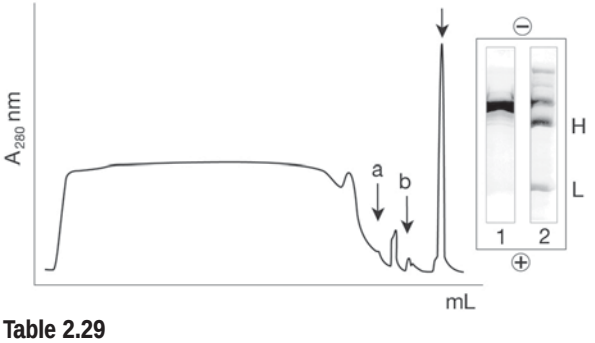
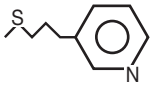


Table 2.29
Examples of Purifications on MEP HyperCel Resin

Feedstock	(mg)	(mg)	Purity (%)	Purity (%)	IgG (mg)	Yield (%)	Factor
CCS with FBS	1740	30	1.7	69	23	76	40
Protein-free CCS	330**	17	5**	99	17	99	19**
Bovine serum	440	121	28	75	105	87	3
Ascites fluid	55	9	16	83	7	79	5

Figure 2.27
MEP Hypercel Mimetic Affinity Ligand

Panel A, MEP affinty ligand



4-Mercapto-Ethyl-Pyridine
(4-MEP)
pka-4.8

Ordering Information for MEP HyperCel™ Mixed Mode Resin

Part Number	Description	Pkg
12035-069	MEP HyperCel	5 mL
12035-010	MEP HyperCel	25 mL
12035-028	MEP HyperCel	100 mL
12035-036	MEP HyperCel	1000 mL

References for MEP HyperCel Mixed Mode Resin

1. Boschetti, E., et al. (2000). *Sep. Sci. & Tech.*, 2(15), 535.
2. Manzke, O., et al. (1997). *J. Immunol Methods*, (208), 65.
3. Burton, S.C., & Hardling, D.R.K. (1998). *J. Chromatography*, (814), 71.
4. Scholz, G.H., et al. (1998). *J. Chromatography*, (709), 189.
5. Scholz, G.H., et al. (1998). *J. Immunol. Meth.*, (219), 109.
6. Schwartz, W., et al. (2001). *J. Chromatography*, A908, 251.
7. Guerrier, L., et al. (2000). *Bioseparation*, (9), 211.
8. Guerrier, L., et al. (2000). *J. Chromatography*, B755, 37.
9. Boschetti, E. (2001). *J. Biochem. Biophys. Methods*, (49) 361.

2.2.4.2 HA Ultrogel® Resin

Hydroxyapatite chromatography is considered to be a "pseudo-affinity," or "mixed-mode" ion exchange mechanism. It has proven to be an effective purification mechanism in a variety of processes, providing biomolecule selectivity complimentary to more traditional ion exchange or hydrophobic interaction techniques. Its retention mechanism can employ:

- Anion ion exchange via Ca++
- Metal ion affinity with Ca++
- Cation ion exchange with phosphate groups
- Hydrogen bonding with crystal hydroxyl groups1

These all can play synergistic roles in molecular adsorption to the crystal hydroxyapatite surface. Desorption can be achieved with increased phosphate buffer with and without salt, such as NaCl or NaF. In the case of metal ion chelation,[2,3] elution with EDTA or imidazole up to 0.3 M can be used. In contrast to IMAC, the latter is not as effective as elution with phosphate + NaCl.

HA Ultrogel hydroxyapatite resin is composed of cross-linked agarose beads with microcrystals entrapped within 60-180 µm agarose particles. The agarose particles are cross-linked with epichlorohydrin under strongly alkaline conditions. This creates glycerol bridges between the polysaccharide chains and gives the resin beads an excellent rigidity and stability to pH and ionic strength changes, as well as to high temperature. HA Ultrogel resin can be regularly treated with 0.1-1.0 M NaOH for regeneration and sanitization. The resin porosity is comparable to an agarose gel, with an exclusion limit for globular proteins of 5,000,000 daltons thus eliminating any molecular sieving effects during the separation. HA Ultrogel resin is easily scaleable and is currently used in research scale to multi-liter column applications.[4-12]

A summary of the properties of the HA Ultrogel mixed mode affinity resin is presented in Table 2.30. In this section the application focus will be on small-scale pre-fractionation in single and multi-well devices with scale up into gravity flow columns. Larger scale protocols employing a liquid chromatography workstation in glass columns are described in purification applications Section 4.2.5, page 341.

Table 2.30
Properties of HA Ultrogel® Resin

Specification	Parameter
Particle Size	60-80 µm (average)
Particle Composition	4% (w/v) Agarose
Ligand	Microcrystalline hydroxyapatite
Ligand Loading	40% (w/v) hydroxyapatite content
Protein Binding Capacity Cytochrome C* BSA**	 > 7 mg/mL > 7 mg/mL
Exclusion Limit	> 5,000,000 daltons
pH Stability	5-13 **Note:** DO NOT use < pH 4.0
Thermal Stability	4-121 °C

**Determined using 5 mg/mL cytochrome C diluted 50/50 in 1 mM phosphate buffer, pH 6.8 at 30 cm/h.*
***Determined using 1 mg/mL BSA diluted 50/50 in 1 mM phosphate buffer, pH 6.8 at 12.5 cm/h.*

Protocol for HA Ultrogel Resin

A. Materials Required

1. Container/column for resin, one of the following:
- **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922)
- **b.** Nanosep® MF centrifugal device with 0.45 µm GHP membrane (PN ODGHPC34)
- **c.** Choose either:
  - **(1)** AcroPrep™ 96 filter plate, 350 µL well, 0.45 µm GHP membrane (PN 5030); 1.2 µm Supor® membrane (PN 5039); or
  - **(2)** AcroPrep 96 filter plate, 1 mL well, 0.45 µm GHP membrane (PN 5054)

2. Needed if filter plate is used:
- **a.** Collection plates [e.g., 96 well polypropylene V bottom, 0.5 mL (Axygen PN P96450V) or 1.64 mL round bottom (Axygen PN PDW20)]
- **b.** Adhesive plate sealing film
- **c.** AcroPrep 96-well plate sealing cap mat (PN 5230)

3. Separation apparatus (if filtration is used)
- **a.** Source of vacuum 25.4-50.8 cm Hg (10-20 in. Hg) (PN 5017); or
- **b.** Centrifuge fitted with a swinging bucket rotor

4. Degassed 50% (v/v) slurry of the HA Ultrogel resin

5. Degassed suitable buffer, such as 1 mM phosphate buffer pH 6.8

Tips on Handling HA Ultrogel® Resin:
For packed columns, use only degassed liquids. For batch mode processing, degassing is not necessary.

Some BioSepra® media are supplied as concentrated slurries and may be difficult to resuspend. DO NOT use magnetic stir bars with BioSepra media as they can damage the beads. When adding slurry to any device, mix well between additions.

If it is necessary to prepare a 50% (v/v) slurry, use a clean spatula to remove some of the packed media and transfer to a graduated glass cylinder containing buffer. DO NOT add any media back to the storage bottle to avoid contamination of the bulk media. Thoroughly mix and allow settling. Note the volume of settled resin. Decant the supernatant and add back an equal volume of buffer to make 50% (v/v) slurry.

For packed columns, removal of fines may be necessary. Prepare the slurry in desired buffer, mix, and allow settling for approximately 5 minutes or enough time that the beads have settled but that small particles are still in solution. Decant off the suspension of fine particles and add fresh buffer and re-mix. Repeat the process until particles settle within approximately 5 minutes and leave a clear supernatant.

B. Packing HA Ultrogel Resin

1. Gravity flow column format

- **a.** Equilibrate column, degassed 50% (v/v) gel slurry, and degassed buffer solution to room temperature.
- **b.** Secure a bottom cap on the column tip and clamp the column (1-5 mL bed volume column, e.g., Pierce PN 29922) upright in a laboratory stand.
- **c.** Add a sufficient volume of degassed buffer to the column to fill it up to the reservoir (wide-mouth) portion, and then gently tap the end and side of the column to dislodge any air bubbles.
- **d.** Float a porous disc on top of the liquid within the column.
- **e.** Using the reverse end of a Pasteur pipette or reverse end of a serum separator (e.g., Pierce PN 69710), push the disc evenly to the bottom of the column.
- **f.** Decant most of the liquid from the empty column, being sure to avoid getting air bubbles in the tip region of the column below the inserted disc. Place the column back in its stand with bottom cap still in place.
- **g.** Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 1-2 mL.
- **h.** Allow gel to settle in the column for at least 5 minutes.
- **i.** Position a second porous disc on top of the settled gel bed by floating it on the liquid within the column and pushing it down to just above the settled gel. Leave 1-2 mm of space between the top of the gel bed and the top disc. Do not compress the gel bed.
- **j.** Wash the inside top part of the column with buffer to remove residual gel that may have remained along the sides during packing.
- **k.** Packed column is now ready for use immediately or store at 4 °C for no more than one week.

l. Refer to Section C on page 125 for use instructions.

Tip: *Store the packed column upright at 4 °C with the gel bed submerged under 1-2 mL of buffer and a top column cap securely in place. Sodium azide added to the storage buffer to a concentration of 0.02% (w/v) will help prevent microbial growth. Always remove the top cap before the bottom cap to avoid drawing air bubbles down into the gel bed. Prevent air bubbles from forming in the gel bed by using only degassed buffer and sample solutions. Degassing involves subjecting a solution to vacuum to remove excess dissolved air. Use of too high a vacuum can lead to evaporation of solvent from the solution. Check the final volume after degassing and, if necessary, add more solvent to return to original volume.*

2. Nanosep® spin filter column format

Tip: *In this device format, the minimum amount of resin required to cover the membrane surface when spun in an angle rotor is 0.05 mL packed bed volume [0.1 mL of a 50% (v/v) slurry]. If smaller volumes of resin are required, a swinging bucket rotor can be used down to 0.025 mL packed bed volume [0.05 mL of a 50% (v/v) slurry]. The maximum amount of resin recommended for this device format is 0.2 mL packed bed volume [0.04 mL of a 50% (v/v) slurry]. Higher amounts are difficult to mix with sample and material will be lost from the device when spinning.*

a. Remove the spin filter from the Nanosep collection tube and place in a rubber stopper (with a suitable hole to form a tight fit to the spin filter) on the vacuum flask. Apply a low vacuum of 5-10 mm Hg.

b. Mix the 50% (v/v) HA Ultrogel® slurry (washed in bulk to remove the 1 M NaCl, 20% (v/v) ethanol preservative with buffer), and quickly pipette 0.4 mL of the slurry to the Nanosep device.

c. Apply vacuum to remove the liquid from the resin bed which should partially fill the Nanosep device.

d. Replace the spin filter in the collection tube for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 °C for no more than one week.*

e. Refer to Section C on page 125 for use instructions.

3. AcroPrep™ multi-well filter plate format

Tip: *In this device format, the minimum amount of resin required to cover the membrane surface when spun in a swinging bucket rotor or used on a vacuum manifold is 0.025 mL packed bed volume (0.05 mL of a 50% slurry). The maximum amount of resin recommended for the 350 µL plate is 0.175 mL packed bed vlume (0.35 mL of a 50% slurry) and up to 0.525 mL packed bed volume (3x 0.35 mL of a 50% slurry) for the 1 mL plate. Higher amounts are difficult to mix with sample and material will be lost from the device when spinning.*

a. Wash the media with 5 column volumes (CV) of buffer to remove the 1 M NaCl, 20% (v/v) ethanol storage buffer. Adjust to a final 50% (v/v) slurry.

b. Place an AcroPrep 96 filter plate (350 µL or 1 mL well volume) on a suitable vacuum manifold (PN 5017) with a collection plate underneath.

c. Mix the 50% (v/v) slurry and quickly pipette 0.35 mL to the 350 µL multi-well plate. Rapidly follow with a second and third volume of slurry for the 1.2 mL volume plate.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

d. After final addition, allow the vacuum to remove the liquid from the resin bed, which should partially fill the well of the AcroPrep™ 96 filter plate.

e. Cover the multi-well plate for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 °C for no more than one week.*

f. Refer to Section C below for use instructions.

C. Hydroxyapatite Pseudo-affinity-based Pre-fractionation

The following approaches can be used:

- Varying the binding conditions, such as Phosphate buffer pH and salt concentration, can impact adsorption to the HA surface.
- Investigating the impact of metal ions, such as Fe (III), Cu (II), Zn (II), or Co (II) on the adsorption of proteins.
- Elution options are use of a phosphate buffer gradient up to 0.5 M, addition of NaCl to the above phosphate buffer gradient up to 1.5 M, use of chaotropic agents such as urea up to 8 M, and use of a detergents, such as sodium deoxycholate, Triton* X-100, or SDS as a dissociating agent.

In the case of metal ion chelation studies, elution can be evaluated with imidazole up to 0.3 M and EDTA up to 50 mM. Combinations of the above approaches are useful in developing an optimal pre-fractionation strategy, and are summarized in Table 2.31. Either strategy or a combination of both can be applied to pre-fractionate a complex sample in one of the following formats.

1. Gravity flow column format

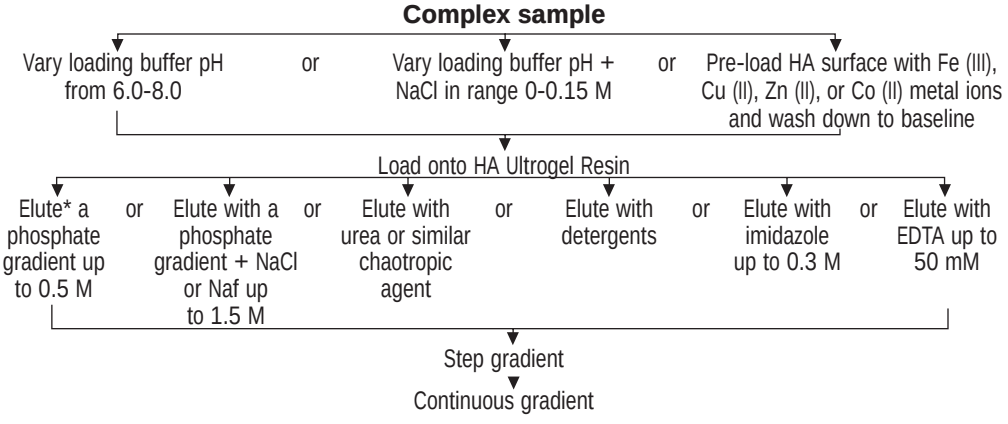
a. Prepare a 1-2 mL column.

b. Allow the liquid in the column to drain to waste.

c. Load the sample up to a 2 mL volume onto the column.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.31.*

Table 2.31
Summary of Pre-Fractionation Options for HA Ultrogel® Resin



**Elution may require several steps, such as pH, salt and detergents linked together to achieve efficient recovery of bound material.*

d. Collect the column effluent in 1 mL fractions. Measure A_{280} to locate the protein peak.

Tip: *Unretained protein rapidly elutes from the column and should be found in the first three fractions. Some slight dilution of the sample will occur during elution. If necessary the sample can be concentrated in a centrifugal UF spin filter, such as a Nanosep® or Microsep™ centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).*

e. After unretained protein has been eluted, the column should be washed with 5 CV of loading buffer before elution of retained fractions is attempted.

f. Retained fractions can then be eluted by phosphate buffer gradient up to 0.5 M; addition of NaCl or NaF to the phosphate buffer up to 1.5 M; elution with urea up to 8.0 M; or detergent elution with 1% (w/v) sodium deoxycholate, sodium dodecyl sulfate (SDS), or 1% (v/v) Triton* X-100. For metal ion chelation studies elution can be evaluated with imidazole up to 0.3 M and EDTA up to 50 mM. The volume of each elution should be at least 2 CV to avoid “carry over” between steps in the elution. Too large a volume should be avoided and will generate diluted samples.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF spin filter with a 10K MWCO membrane (see Section 2.4, page 152).*

g. After the last step, tightly bound material can be eluted from the support with 1% (w/v) SDS in water and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

2. Nanosep spin filter column format

a. Prepare the spin column as described above.

b. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to remove excess fluid from the packed bed.

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.1-0.2 mL) onto the top of the dry packed bed in the Nanosep® device. Replace in the collection tube. The sample and resin should be mixed and allowed to remain in contact for 5-10 minutes at room temperature to improve binding.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 2.31.*

e. Centrifuge the spin column in a swinging bucket rotor at 500 x g for 2 minutes to pass the sample through the Heparin affinity bed.

f. Filtrate in the collection tube will be the unretained flowthrough fraction.

g. After unretained protein has been eluted, the column should be washed with 5 CV of loading buffer before elution of retained fractions is attempted.

h. Retained fractions can then be eluted by phosphate buffer gradient up to 0.5 M; addition of NaCl or NaF to the phosphate buffer up to 1.5 M; elution with urea up to 8.0 M; or detergent elution with 1% (w/v) sodium deoxycholate, SDS, or 1% (v/v) Triton* X-100. For metal ion chelation studies, elution can be evaluated with imidazole up to 0.3 M and EDTA up to 50 mM. The volume of each elution should be at least 2 CV to avoid “carry over” between steps in the elution. Too large a volume should be avoided and will generate dilute samples.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF spin filter with a 10K MWCO membrane (see Section 2.4, page 152).*

i. After the last step, tightly bound material can be eluted from the support with 1% (w/v) SDS in water and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

3. AcroPrep™ multi-well filter plate format

Tip: *For prolonged incubations or incubations using solutions that reduce surface tension (e.g., detergents, alcohols, and acetonitrile) in 96-well plates, it may be necessary to seal the bottom of the plate to prevent leakage. This can be tested in advance with just the solutions in the chosen plates.*

An adhesive plate sealer can be used on the top of the wells to prevent cross contamination during vigorous shaking or evaporation during prolonged or warm incubations. For sealing, you can use a plastic, self-adhesive plate sealer (e.g., Sigma EASYseal) or a cap-mat (PN 5230).*

a. Prepare the packed multi-well filter plate as described above.

b. Centrifuge the multi-well filter plate and collection plate in a suitable swinging bucket rotor at 400 x g for 2 minutes to remove excess fluid from the packed bed.

Tip: *As an alternative to centrifuging, a vacuum manifold can be used. See Section 6.3 of the Appendix for more detail.*

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.125-0.25 mL for the 350 µL plate or 0.375-0.85 mL for the 1 mL plate) onto the top of the dry packed bed in the AcroPrep™ 96 multi-well filter plate.

Tip: *At this stage, sample-loading conditions should be optimized following recommendations in Table 2.31. The amount of sample loaded should be < 50% of the static binding capacity of the resin.*

e. The sample and well contents should be thoroughly mixed and kept in contact for 30 minutes at room temperature to improve binding.

Tip: *To minimize buffer leakage and loss due to evaporation, use an adhesive plate sealer or cap-mat (PN 5230) on the top of the plate and incubate in a moisture-resistant bag or container.*

The shaker table used should be able to agitate the plate to keep the chromatography media and sample suspended within the well on the plate. Control the mixing so sample and resin do not come in contact with the top sealing sheet.

f. Place the sealed plate on top of the collection plate to catch any filtrate. Tap the plate to dislodge any liquid near the top of the plate and then carefully and slowly remove the top sheet.

g. Centrifuge the multi-well plate and collection plate in a swinging bucket rotor at 400 x g for 2 minutes to pass the sample through the Heparin affinity bed or apply vacuum sufficient to pass the sample through the bed, in 2 mm.

h. Filtrate recovered from the collection plate will be the unretained fraction.

i. After unretained protein has been eluted, the wells should be washed with 5 CV of loading buffer before elution begins.

j. Retained fractions can then be eluted by phosphate buffer gradient up to 0.5 M; addition of NaCl or NaF to the phosphate buffer up to 1.5 M; elution with urea up to 8.0 M; or detergent elution with 1% (w/v) sodium deoxycholate, SDS, or 1% (v/v) Triton* X-100. For metal ion chelation studies, elution can be evaluated with imidazole up to 0.3 M and EDTA up to 50 mM. The volume of each elution should be at least 2 CV to avoid “carry over” between steps in the elution. Too large a volume should be avoided and will generate diluted samples.

Tip: *Sample concentration at this stage is best carried out in a centrifugal UF filter plate with a 10K MWCO membrane (see Section 2.4, page 152).*

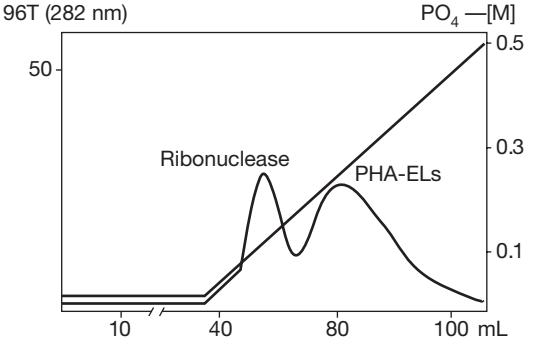
k. After the last step, tightly bound material can be eluted from the support with 1% (w/v) SDS in water and recovered by acetone precipitation or detergent removal using SDR HyperD® resin (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

Application Data for HA Ultrogel® Resin

Hydroxyapatite adsorption chromatography can be used in a variety of applications, including the separation of proteins, peptides and nucleic acids, from pilot to production scale (see Figures 2.28 and 2.29). For proteins, the most well-known application of hydroxyapatite is the separation of basic proteins (cytochrome c, lysozyme, etc.) and phosphoproteins. HA Ultrogel resin can be used for the separation of human serum proteins and plant proteins such as lectins, glycoproteins, glycosidases, phospholipidases, sulfohydrolases, sphingomyelinases, transferases, trehalases, and kinases. As a phosphate-containing sorbent, HA Ultrogel resin can be used for the separation of phosphate dependent proteins and enzymes as well as DNA-dependent enzymes. HA Ultrogel resin provides an efficient tool for IgG purification in a one step chromatographic purification by separation with phosphate buffer. This approach is very mild (neutral pH, physiological conditions) compared to traditional elution in acidic solutions, and preserves the biological activity of the antibody. In addition, HA Ultrogel resin can be used to remove DNA and endotoxin from a protein sample in a single step.[1]

Figure 2.28

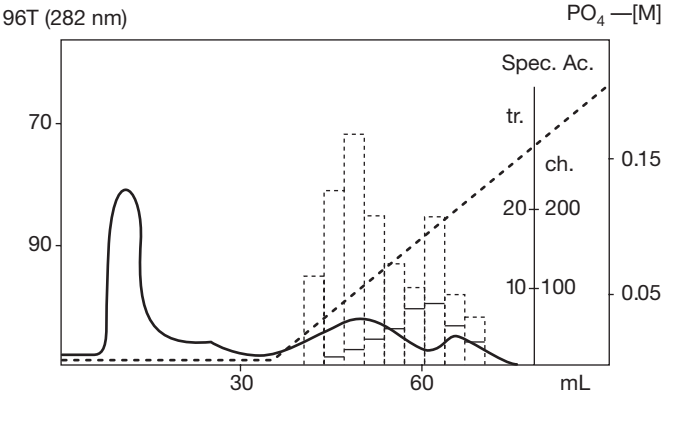
Separation of a Mixture of Ribonuclease and Phytohemagglutinins (PHA-EL).



Column: 1.6 x 6.5 cm; Sample: 1 mg of protein mixture composed of ribonuclease (MW 14,700) and PHA-ELs (Erythroagglutinating and lymphostimulating Phytohemagglutinin) (MW 128,000) from Phaseolus vulgaris, in 1 mL of 5 mM potassium phophate, pH 6.8; Elution gradient: 5 mM to 500 mM potassium phosphate, pH 6.8; Flow rate: 14.4 cm/h.

Figure 2.29

Separation of Trypsin and Chymotrypsin from a Porcine Pancreatic Enzyme Extract.



Column: 1.6 x 5 cm; Sample: 30 mg protein in 1 mL of 5 mM phosphate buffer, pH 6.8; Gradient: 5 to 200 mM sodium phosphate, pH 6.8; Flow rate: 10 cm/h; Temperature: 10 °C; Histogram with broken line: trypsin activity; Histogram with solid line: chymotrypsin activity. Spec. Ac.: specific activity in U/mg. tr: trypsin, ch: chymostrypsin. Trypsin activity was primarily found in the peak eluted by 50 mM phosphate where the chymotrypsin was eluted by 100 mM phosphate. The final yield was approximately 50%.

Ordering Information for HA Ultrogel® Resin

Part Number	Description	Pkg
24775-075	HA Ultrogel Hydroxyapatite	5 mL
24775-082	HA Ultrogel Hydroxyapatite	25 mL
24775-025	HA Ultrogel Hydroxyapatite	100 mL
24775-017	HA Ultrogel Hydroxyapatite	500 mL
24775-041	HA Ultrogel Hydroxyapatite	1000 mL

References for HA Ultrogel Resin

1. Gagnon, P., Ng, P., Zhen, J., Aberin, C., He, J., Mekosh, H., et al. (2006, February). A ceramic hydroxyapatite based purification platform; simultaneous removal of leached Protein A, aggregates, DNA and endotoxins from MAb's. *BioProcess International*.
2. Nordstrom, T., Senkas, A., Eriksson, S., Pontynen, N., Nordstrom, E., & Lindqvist, C. (1999). Generation of a new protein purification matrix by loading ceramic hydroxyapatite with metal ions; demonstration with poly histidine tagged green fluorescent protein. *J. Biotechnology*, (69), 125–133.
3. Suen, R.B., Lin, S.C., & Hsu, W.H. (2004). Hydroxyapatite based immobilized metal affinity adsorbents for protein purification. *J. Chromatography*, A1048, 31–39.
4. Achazi, R.K., Flenner, C., Livingstone, D.R., Peters, L.D., Schaub, K., & Scheiwe, E. (1998). Cytochrome P-450 and dependent activities in unexposed and PAH-exposed terrestrial annelids. *Comp. Biochem. Physiol C Pharmacol. Toxicol. Endocrinol.*, (121), 339–350.
5. Lomo, P.O., Coaetzer, T.H., & Lonsdale-Eccles, J.D. (1997). Characterization of a multicatalytic proteinase complex (20s proteasome) from Trypanosoma brucei brucei. *Immunopharmacology*, (36), 285–293.
6. Decaro, J., Ferrato, F., Verger, R., & Decaro, A. (1995). Purification and molecular characterization of lamb pregastric lipase. *Biochim. Biophys. Acta*, (1252), 321–329.
7. Ripamonti, U., Cunningham, N.S., Yeates, L., & Reddi, A.H. (1993). Reconstruction of the bone-bone marrow organ by osteogenin, a bone morphogenic protein, and demineralized bone matrix in a calvarial defects of adult primates. *Plastic Reconstructive Surgery*, (91), 27–36.
8. Barone, E., Van Boxel, T., Borney, F., Dal Monte, M., Chiavicci, L., D'Urso, M., et al. (1992). Partial purification of a high molecular weight hepatocyte growth stimulating factor from normal calk serum. *J. Biol. Regul. Homeost. Agents*, (6), 121–131.

9. Husain, A., & Branton, D. (1986). Purification of erythrocyte band 4.1 and other cytoskeletal components using hydroxyapatite Ultrogel®. *Analytical Biochemistry*, (155), 206–211.

10. Moore, R.B., Plishker, G.A., & Shriver, S.K. (1990). Purification and measurement of calpromotin, the cytoplasmic protein which activates calcium-dependent potassium transport. *Biochem. Biophys. Res. Communication*, (166), 146–153.

11. Smith, G.J., McFarland, R.D., Reisner, H.M., & Hudson, G.S. (1984). Lymphoblastoid cell produced immunoglobulins: preparative purification from culture medium by hydroxylapatite chromatography. *Analytical Biochemistry*, (141), 432–436.

12. Galand, G. (1984). Purification and characterization of kidney and intestinal brush-border membrane trehalases from the rabbit. *Biochim. Biophys. Acta*, (789), 10–19.

2.2.5 Size Fractionation

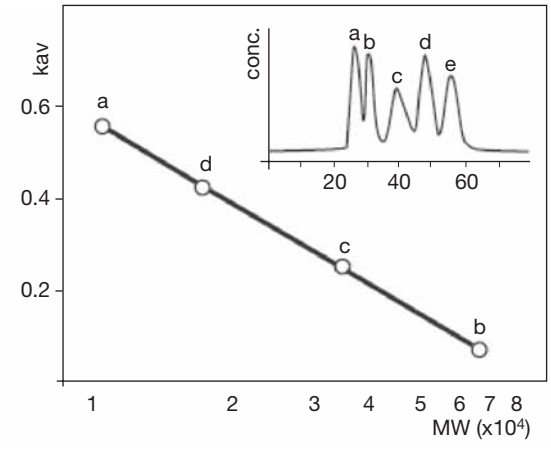
2.2.5.1 Ultrogel® AcA Size Exclusion Chromatography Resin

Separation of complex mixtures, such as plasma or serum, on the basis of size or molecular shape can be achieved by size exclusion or gel filtration chromatography. The molecular sieving process takes place as a solute passes through a packed bed stationary phase. The separation depends on the different abilities of the various sample molecules to enter the pores of the bead-based stationary phase. Large molecules, which cannot enter the pores, are excluded and pass through the column in the interstitial phase eluting early in the void volume (Vo). Smaller molecules that can enter the pores are retarded and move through the column more slowly. Molecules are, therefore, eluted in a volume (Ve), in order of decreasing molecular size. Very small molecules, such as salt, are able to fully permeate and elute last. These elution volumes can be used to calibrate the size exclusion column with purified molecules of known molecular weight so that the elution of an unknown can be used to estimate its molecular weight. The term Kav, which represents the fraction of the stationary gel volume which is available for diffusion of a given solute species, can be calculated from the following equation:

$$Kav = \frac{Ve - Vo}{Vt - Vo}$$

In this case Vt = Vz (interstitial volume) + Vp (pore volume) + Vm (matrix volume). An example of a plot of Kav vs. molecular weight (plotted on a log scale) is shown in Figure 2.30 and clearly illustrates the linear relationship between Kav and molecular size. Molecular size of an unknown can be estimated from the elution volume by extrapolation from the selectivity curve.

Figure 2.30
Determination of a Selectivity Curve for Ultrogel AcA 54 Resin Using a Mixture of Known Molecular Weight Proteins



Column: 1.6 x 40 cm; Buffer: 0.05 M Tris-HCl, pH 7.4 containing 0.17 M sodium chloride: sample constituted of Blue-Dextran 2000 (a) for the determination of the void volume(Vo); Bovine serum albumin (b) (MW 68,000); ß-lactoglobulin (c) (MW 35,000); Myoglobin (d) (MW 17,800); And cytochrome c (e) (MW 12,400) ; Sample volume: 0.6 mL; Flow rate: 4.8 cm/h. The insert represents the elution profile of the above protein standards from the above column.

Ultrogel® AcA resins are based on semi-rigid particles composed of a polyacrylamide and agarose composite gel matrix which possess good mechanical properties. They can be used at a high flow rate while maintaining a good resolution. These resins offer a choice between maximum resolution at recommended flow rates or rapid separations at higher flow rates with minimal loss of resolution. Resolution by molecular size by size exclusion chromatography is influenced by a number of factors:

- Exclusion limit of the media, defining the molecular weight of the smallest molecule that cannot penetrate the pores of the gel matrix.
- Fractionation range corresponding to the linear region of the selectivity curve.
- Particle shape, ideally being spherical and able to pack in a column to form a highly uniform bed volume.
- Particle size and particle size distribution can have a significant impact on volumetric flow and resolution in the packed column bed.

Presence of very small particles or "fines" can lead to reduced eluant flow rates and should be removed during manufacture. The Ultrogel AcA family of pre-swollen gel media have been developed to meet the above critical success factors. Five different resin types are available for fractionation of molecules with molecular weights ranging from 1,000 to 1,200,000. A summary of the properties of the Ultrogel AcA size exclusion resins are summarized in Table 2.32.

Table 2.32
Properties of Ultrogel AcA Size Exclusion Resin

Specification	AcA 22	AcA 34	AcA 44	AcA 54	AcA 202
Particle Size	60-140 µm				
Acrylamide (%)	2	3	4	5	20
Agarose (%)	2	4	4	4	2
Exclusion Limit (kda)	3,000	750	200	90	22
Linear Fractionation Range (kda)	100-1,200	20-350	10-30	5-70	1-15
HETP (mm)*	0.15	0.15	0.15	0.15	3.0
Resolution Power (plates/m)	1,500	1,500	1,500	1,500	3,000
Desalting Capacity	–	–	–	–	45% gel.vol.
Working pH	3-10	3-10	3-10	3-10	3-10
Detergent Limits	SDS-ND; Triton* X-100-ND; Deoxycholate-ND				
Denaturant Limits	Guanidine HCl < 2.0 M; Urea < 2.0 M				

**Height Equivalent to a Theoretical Plate (HETP).*

ND – detergent limits have not been determined. Preliminary experiments should be conducted at the concentration required to ensure that the Ultrogel AcA beads are compatible. Beads should be examined for swelling or fragmentation which will impact the flow characteristics of this medium in a packed bed configuration.

In this section, the application focus will be on small-scale size-based pre-fractionation (0.25-1.0 mL volume) in packed glass columns gravity fed or pumped with a liquid chromatography workstation or peristaltic pump. Larger scale protocols (volumes > 10 mL) for size exclusion and desalting applications employing Ultrogel® resins on a liquid chromatography workstation in glass columns are described in purification applications Section 4.2.9, page 371.

Protocol for Ultrogel AcA Size Exclusion Chromatography Resin

A. Materials Required

1. Glass column where the column diameter/length ratio is > 20 to provide the optimal geometry for size exclusion chromatography. Two examples are described in this Protocol section to cover the 0.25-10 mL sample volume range. Other column configurations are possible as long as the above diameter/length ratio is met.
   - **a.** 0.66 cm x 25 cm, 7.5 mL max bed volume (e.g., Omnifit* PN 006-CC-06-25-AF) for a 0.25-0.75 mL sample volume load.
   - **b.** 1.5 cm ID x 50 cm length, max 83 mL bed volume (e.g., Omnifit PN 006-CC-15-50-AF) for a 2-10 mL sample volume load.
2. Column packing reservoir for 0.66 cm x 25 cm column (e.g. Omnifit PN 006-PS-06 plus empty glass column Omnifit PN 006-RG-06-25).
3. Column packing reservoir for 1.5 cm x 50 cm column (e.g. Omnifit PN 006-PS-15 plus empty glass column Omnifit PN 006-RG-15-50).
4. Suitable gravity flow system of adjustable height to achieve flow in the above columns.
5. Peristaltic pump or LC workstation able to provide buffer linear velocities with the above columns at up to 4.8 cm/h.
6. Degassed 50% (v/v) slurry of the Ultrogel AcA resin.
7. Degassed suitable buffer, such as 0.05 M Tris-HCl, pH 7.4 containing 0.17 M NaCl.

Tips on Handling Ultrogel AcA Resin:

Some BioSepra® media are supplied as concentrated slurries and may be difficult to resuspend. DO NOT use magnetic stir bars with BioSepra media as they can damage the beads. When adding slurry to any device, mix well between additions.

If it is necessary to prepare 50% (v/v) slurry, use a clean spatula to remove some of the packed media and transfer to a graduated glass cylinder containing buffer. DO NOT add any media back to the storage bottle to avoid contamination of the bulk media. Thoroughly mix and allow settling. Note the volume of settled resin. Decant the supernatant and add back an equal volume of buffer to make 50% (v/v) slurry.

For packed columns, removal of fines may be necessary. Prepare the slurry in desired buffer, mix, and allow settling for approximately 5 minutes or for enough time that the beads have settled but small particles are still in the solution. Decant off the suspension of fine particles and add fresh buffer and re-mix. Repeat the process until particles settle within approximately 5 minutes and leave a clear supernatant.

B. Packing Ultrogel® AcA Resin

1. Packed glass column format
For successful size exclusion chromatography, it is very important to prepare a properly packed Ultrogel AcA resin bed. The final column should present a low and uniform (with respect to cross sectional area) resistance to eluant flow and needs to be free of air bubbles and channels that may lead to uneven flow. Flow can be from a hydrostatic head in a gravity system or via pumping with a peristaltic pump or a liquid chromatography workstation.

- **a.** Equilibrate column, packing accessories, degassed 50% gel slurry, and degassed buffer solution to room temperature. This is very important to prevent a volume change, as the media or column adjusts to temperature.
- **b.** Install the fixed bottom fitting onto the glass column and attach the column packing adapter to the top of the column followed by the second length of glass column. Clamp the column and packing adapter upright in a stable laboratory stand. It may be necessary to use more than one clamp to hold the column. Check that the column is vertical in two dimensions with a suitable leveling device, such as a carpenter's level.
- **c.** Add a sufficient volume of degassed buffer to the column to fill it up to the reservoir (wide-mouth) portion, and then gently tap the end and side of the column to dislodge any air bubbles.
- **d.** Open up the bottom fitting and allow flow to displace air from underneath the bottom support frit. Drain down to about 20% of the expected bed height. Stop flow.
- **e.** Add sufficient volume of 50% degassed gel slurry to obtain the desired settled gel volume of 69-83 mL (15 x 50 cm column) or 4.8-7.5 mL (6.6 x 25 cm column). The slurry should fill the combined column and packing adapter. Initial brief gentle stirring with a long plastic rod will ensure uniform settling of the media.
- **f.** Allow gel to settle in the column for at least 15-30 minutes with the outlet closed.
- **g.** Open the column outlet and allow the supernatant eluant to drain to waste, until approximately 1 cm of buffer remains above the gel bed. If the bed height is low, add more of the 50% gel slurry after resuspending the top 2 cm of the packed bed with the plastic rod. If the bed height is too high, excess media can be removed by suction. At this stage it is important to allow the media to settle to a constant height and measure the final gel bed volume accurately.
- **h.** Drain and leave 1 cm of buffer on top of the packed bed.
- **i.** Remove the column adapter and set aside.
- **j.** Attach the top adjustable length fitting onto the column and screw down the top fitting until the top of the packed gel bed is reached. The top fitting should not be screwed down too far, compressing the packed bed which can lead to reduced flow rates.

k. The bottom outlet should then be connected to a gravity flow system or pump and buffer flowed up through the column at 0.5 mL/min to displace air from the top fittings.

l. Packed column is now ready for storage at 4 °C or immediate use.

Tip: *Size exclusion chromatography can be carried out at a range of temperatures, including 4 °C. The column must reach the desired temperature and equilibrate for 4-6 hours to ensure the column packing is uniform. Some slight bed height adjustment may be required adjusting to the different temperatures. When a column is moved to a new location, for example a cold room, it should be supported with a stable clamp stand and maintained in an upright orientation. Check with a level for storage.*

Store the packed column upright at 4 °C with the gel bed submerged under 1-2 mL of buffer and a top column cap securely in place. Sodium azide added to the storage buffer to a concentration of 0.02% (w/v) will help prevent microbial growth. Always remove the top cap before the bottom cap to avoid drawing air bubbles down into the gel bed. Prevent air bubbles from forming in the gel bed by using only degassed buffer and sample solutions. Degassing involves subjecting a solution to vacuum to remove excess dissolved air. Use of too high a vacuum can lead to evaporation of solvent from the solution. Check the final volume after degassing and, if necessary, add more solvent to return to original volume.

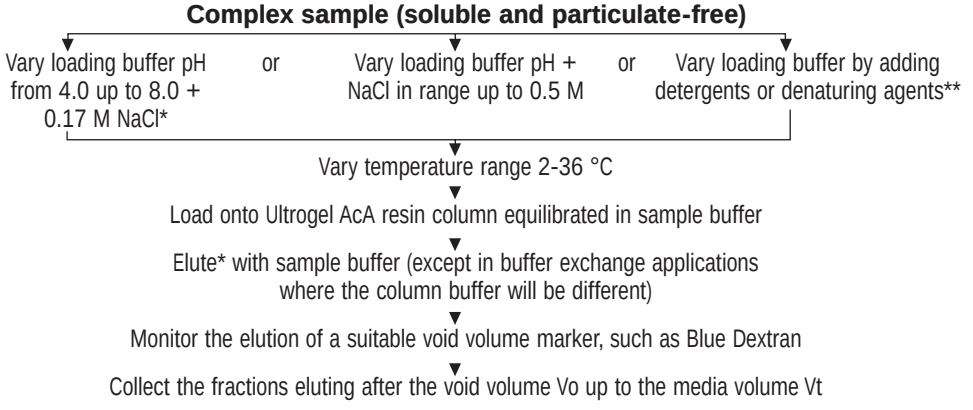
C. Size-based Pre-fractionation Using Ultrogel® AcA Resin

One of the following approaches can be used:

- Varying the binding conditions of the sample, such as pH and salt concentration, to influence the size or shape of the proteins in the complex sample. In addition, size exclusion can be carried out in the presence of limited amounts of detergents, such as sodium dodecyl sulfate (SDS), Triton* X-100, and sodium deoxycholate, and denaturants such as urea or guanidine hydrochloride. These conditions may be used where sample solubility is an issue, such as in the case of bacterial exclusion bodies.
- Varying the size exclusion limit and fractionation range of the Ultrogel AcA resin.
- Temperature can have a significant impact on eluant flow, peak shape, and molecular shape.

Combinations of the above approaches are useful in developing an optimal pre-fractionation strategy and are summarized in Table 2.33.

Table 2.33
Summary of Pre-fractionation Options for Ultrogel® AcA Size Exclusion Media



**Initial conditions recommended; 0.05 M Tris-HCl, pH 7.4 containing 0.17 M sodium chloride. The presence of salt helps to minimize non-specific adsorption to the size exclusion resin. Increasing salt concentration during loading can be employed to alter the shape or aggregation state of molecules in the complex sample which may impact the size-based pre-fractionation.*

***Presence of detergents may be necessary for sample solubilization. Proteins in the presence of detergents such as SDS exist in micellar structures when used at concentrations above the critical micellar concentration (CMC) of the detergent. These structures in solution are much larger than the true size of the molecule of interest and will require a higher size fractionation range of Ultrogel AcA resin. It may be necessary to use two Ultrogel AcA size fractionation range beads for native and denatured forms of the same protein sample. For removal of detergents from the final samples, SDR HyperD® resin is recommended (see Section 2.3.1, page 141).*

Either strategy or a combination of both can be applied to pre-fractionate a complex sample in packed glass column formats. This process is usually not sufficient to resolve individual components from complex mixtures and is frequently used sequentially with other techniques such as ion exchange or affinity chromatography (see Sections 2.2.2, page 49 and 2.2.3, page 72) to achieve the necessary degree of sample complexity reduction. Moving samples between two different chromatographic processes may require buffer exchange or concentration to achieve efficient pre-fractionation. If necessary the sample can be concentrated with a centrifugal UF spin filter, such as a Microsep™ or Macrosep® centrifugal UF device, or AcroPrep™ multi-well 1.0 mL plates with a 10K MWCO UF membrane (see Section 2.4, page 152). Rapid buffer exchange can also be carried out with pre-packed gravity flow columns using Ultrogel AcA 202 and GF-05M desalting resin (see Section 2.4, page 152).

1. Packed glass column format
Sample introduction onto the packed bed of the size exclusion column can have a significant impact on the resulting peak shape and resolution. The volume of sample loaded for most analytical separations should be 5-10% of the media volume to achieve optimal resolution and acceptable dilution. Too high a volume sample leads to reduced resolution and too small a volume leads to high sample dilution and poor recovery.

a. Prepare a size exclusion column as described above and equilibrate with suitable buffer of composition the same as the sample. The exception being in the case of a buffer exchange application (see Section 2.4, page 152) with AcA 202 and GF-05M resins, where the column equilibration buffer will be different.

b. Prepare a sample in the appropriate buffer and prefilter with a suitable volume 0.45 µm pore size MF filtration device (see Section 2.5, page 198). The sample should not contain any particulate material that could get trapped on the head of the packed bed column. If a large amount of material is lost during MF filtration, it may be necessary to evaluate different sample buffer conditions or homogenization conditions to render the sample more soluble.

c. Load the sample onto the column using one of the following methods:

(1) Conventional method: remove the top fixture (try to keep filled with buffer and keep air out) and make sure the resin bed is covered with 1-2 cm of buffer. Gently stir the top 1 cm of the packed bed and allow it to settle to form a new flat surface. Open the column outlet and drain the eluant until the bed surface appears slightly dry.

Tip: *At no time should the bed be allowed to "run dry" > 1-2 mm.*

Close the column outlet and carefully introduce the sample onto the bed surface without causing too much disturbance of the packed bed. Open the column outlet and allow the sample to run into the packed bed. Stop flow and add buffer to the top of the column. Open outlet and allow sample to enter 1-2 cm into the column. Stop flow and carefully replace the top fixture without disturbing the top of the packed bed. Reconnect the bottom to the fluidic system and reverse flow (0.5 mL/min) in the column to displace any air that might be in the top fitting. Reconnect the fluidic system to the top fitting and commence elution at a constant flow rate.

(2) Layering method: follow the procedure as described above in Step c(1) but do not drain the buffer down to a dry packed bed. Leave 1-2 cm of buffer on top of the column. The sample, which must be denser than the buffer (adjust with glucose or sucrose to about 10-15%) is then applied to the bed surface with either a long "gel loading" tip or a syringe with a long needle attached. The dense sample should form a sharp layer on top of the packed bed. Allow the sample to enter and complete the step as described above.

(3) Adjustable plunger method: this procedure requires the use of a three-way valve attached as close to the top fitting as possible. The column is set up as described in Step B on page 135 above and the top fitting is left in place at all times. To prevent sample dilution and "tailing" (peak shape asymmetrical), the tubing length between the valve and the adjustable top fitting should be as short as possible. Sample is introduced with a syringe via the three-way valve without disturbing the packed column bed. This method, when properly executed, is the most reliable and reproducible sample loading process.

d. Elute with the column buffer and collect the effluent in 1 mL fractions. Measure absorbance at 280 nm to locate the protein peaks. If a void volume marker, such as Dextran Blue, has been included in the sample, only start fraction collection when this colored marker reaches the bottom of the column. Fractions eluting after this point are being fractionated by the size exclusion resin, with the largest size fractions eluting first. As the volume approaches Vt, the media volume fraction collection should be stopped, as these fractions should be only very low molecular weight, such as the glucose or sucrose added to the sample if you used loading method c(2). Peaks can either be pooled or processed singly and some dilution of the sample will occur during elution.

Tip: *If necessary, the sample can be concentrated with a centrifugal UF spin filter, such as a Microsep™ or Macrosep® centifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).*

e. Buffer exchange, prior to loading onto a second chromatography pre-fractionation step such as ion exchange or affinity, can be carried out by diafiltration in the above centrifugal UF devices (1-2 hours). For more rapid buffer exchange (< 10 minutes) small packed gravity flow columns of Ultrogel® AcA 202 or GF-O5M desalting size exclusion resin (see Section 2.4, page 152) can be used.

f. After unretained protein has been eluted, the column can be washed with 2 CV of loading buffer before re-use is attempted.

g. Packed column is now ready for immediate use at room temperature or can be stored at 4 °C for no more than one week. Sodium azide can be added to the buffer to a concentration of 0.02% to prevent microbial growth if storage > 1 week is required. Before re-use at a different temperature after storage at 4 °C, the packed bed column should be re-equilibrated for 4-6 hours or preferably overnight. If sodium azide is present in the column before use, it should be removed by washing with 2 CV of buffer.

Ordering Information for Ultrogel® AcA Size Exclusion Chromatography Resin

Part Number	Description	Pkg
23013-025	Ultrogel AcA 22	100 mL
23013-014	Ultrogel AcA 22	1000 mL
23015-025	Ultrogel AcA 34	100 mL
23015-019	Ultrogel AcA 34	1000 mL
23022-024	Ultrogel AcA 44	100 mL
23022-015	Ultrogel AcA 44	1000 mL
23019-023	Ultrogel AcA 54	100 mL
23019-011	Ultrogel AcA 54	1000 mL
24892-022	Ultrogel AcA 202	100 mL
24892-010	Ultrogel AcA 202	1000 mL

References for Ultrogel AcA Size Exclusion Chromatography Resin

1. Gomi, K., & Jajiyama, N. (2001). *J. Biol. Chem.*, 276(39), 36508-13.
2. Sehgal, N., Goswami, S., & Indian, J. (2001). *Biochem. Biophys.*, 38(4) 263.
3. Knutson, V.P., et al. (1991). *J. Immunol. Meth.*, 145, 263.
4. Diesterhaft, M. (1984). *Biochem. Biophys. Res. Commun.*, 125(3), 888.
5. Boschetti, E., et al. (1972). *Biochimie*, (54), 439.

2.3.1 SDR HyperD® Solvent and Detergent Removal Chromatography Resin

The elimination of detergents and/or solvents from biological samples is often necessary and can be achieved by various methods including resin partitioning, size exclusion, affinity, or batch extraction with vegetable oils combined with reverse phase on C18 resin.

SDR HyperD resin is a composite sorbent that combines a silica-bead moiety filled with a three-dimensional cross-linked hydrophobic polymer. The SDR HyperD resin structure has been engineered to optimize solvent/detergent retention. Due to the degree of three-dimensional polymer cross-linking, an exclusion limit of 10 kDa allows target proteins to be "excluded" from the resin. They are found unretained in the column void volume. Conversely, the high specific surface area (200 m^2/g) of the porous silica allows a high binding capacity for detergent and solvents. The particle size distribution (40-100 µm), the small pore size of the silica beads, and the polymer have been optimized for retention of solvents and detergents used in viral inactivation processes (i.e., Tri-n-Butyl Phosphate (TnBP) and Triton* X-100)[1]. SDR HyperD resin is also very effective at removing detergents typically used in sample solubilization for other applications (e.g., ASB-14, CHAPS and SDS).

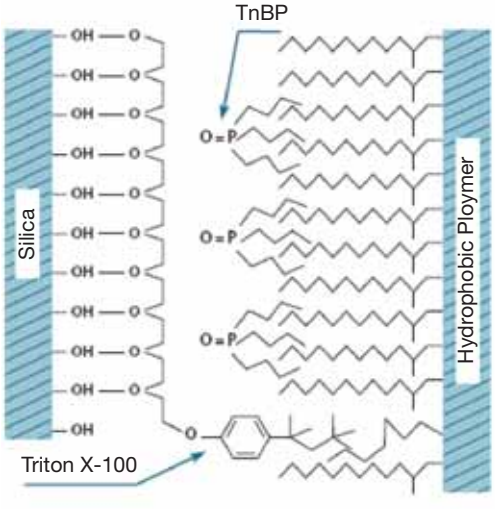
Its suggested mechanism of action for adsorption of Triton X-100 is illustrated in Figure 2.31. Triton X-100 interacts both with the silica surface (formation of hydrogen bonds between the silanols and the polyoxyethylene chain) and with the hydrophobic polymer moiety. TnBP interacts only with the hydrophobic polymer of the resin. The adsorption mechanism involves both the silica moiety and the hydrophobic polymer. The adsorption of Triton X-100 is proportional to the silica surface area, whereas the adsorption of TnBP is dependent on binding to the organic polymer moiety. In addition to the chemical interaction of the detergent with the surface, the bead itself has small pores such that only molecules < 10kD will enter the bead. This means that some peptides might also bind to this resin. SDR HyperD resin has proven useful for rapid detergent removal even when the detergent concentration is above the critical micellar concentration (CMC) and micelles are present. This is probably related to the equilibrium between micelle and free detergent molecules and/or a disruption of micelle structure on contact with the bead. The properties of SDR HyperD resin are summarized in Table 2.34 and the adsorption mechanism represented in Figure 2.31.

Table 2.34
Properties of SDR HyperD® Resin

Specification	Parameter
Resin Structure	Spherical silica beads filled with a three-dimensional hydrophobic polymer
Average Particle Size	40-100 µm
Nature of Polymer	Hydrophobic, long aliphatic chains bind solvents. 10 kDa limit prevents proteins from being retained.
Typical Sample Load	2-3 times the column volume with residence times of 5 minutes using IgG or ATIII treated solutions
Recommended Residence Time	5-15 minutes
Binding Capacity for Triton* X-100	60-80 mg/mL*
Adsorption Buffer	Phosphate buffered saline (PBS)
Solvent/Detergent Elution Buffer	PBS/Ethanol (50/50) and EtOH or/and isopropanol
Operating pH Range	2-12
Resin Pressure Resistance	70 bar (7,000 kPa, 1,015 psi)

**Determined using 5 mg/mL Triton X-100 in PBS, pH 7.4, 10% breakthrough, 300 cm/h.*

Figure 2.31
Schematic Interaction Mechanism of Triton X-100 and TnBP on SDR HyperD Resin



Protocol for SDR HyperD® Solvent and Detergent Removal Chromatography Resin

Triton* X-100 interacts both with the silica surface (formation of hydrogen bonds between the silanols and the polyoxyethylene chain) and with the hydrophobic polymer moiety. TnBP interacts only with the hydrophobic polymer of the resin.

A. Materials Required

1. Container for SDR HyperD resin, one of the following:
- **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922)
- **b.** Nanosep® MF centrifugal device with 0.45 µm GHP membrane (PN ODGHPC34)
- **c.** Choose either:
  - **(1)** AcroPrep™ 96 filter plate, 350 µL well, 0.45 µm GHP membrane (PN 5030); 0.45 µm Supor® membrane (PN 5029); 1.2 µm Supor membrane (PN 5039); or
  - **(2)** AcroPrep 96 filter plate, 1 mL well, 0.45 µm GHP membrane (PN 5054)
- **d.** Glass column 6.6 mm ID x 10 cm length, 1-2 mL volume (OmniFit PN 006CC-06-10-AF)

2. Needed if filter plate is used:
- **a.** Collection plates [e.g., 96 well polypropylene V bottom, 0.5 mL (Axygen PN P96450V) or 1.64 mL round bottom (Axygen PN PDW20)]
- **b.** Adhesive plate sealing film
- **c.** AcroPrep 96-well plate sealing cap mat (PN 5230)

3. Separation apparatus (if filtration is used)
- **a.** Source of vacuum 25.4-50.8 cm Hg (10-20 in. Hg) (PN 5017); or
- **b.** Centrifuge fitted with a swinging bucket rotor

4. Degassed 50% (v/v) slurry of the SDR HyperD resin

5. Degassed suitable buffer, such as Phosphate buffered saline

Tips on Handling SDR HyperD Resin:

For packed columns, use only degassed liquids. Degassing is not necessary for batch mode methods.

Some BioSepra® media are supplied as concentrated slurries and may be difficult to resuspend. Do not use magnetic stir bars with BioSepra media as they can damage the beads. Also, these resins are quite dense and settle quickly. When adding slurry to any device, mix well between additions. If it is necessary to prepare a 50% (v/v) slurry, use a clean spatula to remove some of the packed media and transfer to a graduated glass cylinder containing degassed water or buffer. DO NOT add any media back to the storage bottle to avoid contamination of the bulk media. Thoroughly mix and allow settling. Note the volume of settled resin. Decant the supernatant and add back an equal volume of water or buffer to make a 50% (v/v) slurry.

For packed columns, removal of fines may be necessary. Prepare the slurry in desired buffer, mix, and allow to settle for approximately 5 minutes or for enough time that the beads have settled but small particles are still in the solution.

B. Packing SDR HyperD® Resin

1. Gravity flow column format

- **a** Equilibrate column, degassed 50% gel slurry, and degassed buffer solution at room temperature.
- **b.** Secure a bottom cap on the column tip and clamp the column (1-5 mL bed volume column, e.g., Pierce PN 29922) upright in a laboratory stand.
- **c.** Add a sufficient volume of degassed buffer to the column to fill it up to the reservoir (wide-mouth) portion, and then gently tap the end and side of the column to dislodge any air bubbles.
- **d.** Float a porous disc on top of the liquid within the column.
- **e.** Using the reverse end of a Pasteur pipette or reverse end of a serum separator (e.g., Pierce PN 69710), push the disc evenly to the bottom of the column.
- **f.** Decant most of the liquid from the empty column, being sure to avoid getting air bubbles in the tip region of the column below the inserted disc. Place the column back in its stand with bottom cap still in place.
- **g.** Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 1-2 mL.
- **h.** Allow gel to settle in the column for at least 5 minutes.
- **i.** Position a second porous disc on top of the settled gel bed by floating it on the liquid within the column and pushing it down to just above the settled gel. Leave 1-2 mm of space between the top of the gel bed and the top disc. Do not compress the gel bed.
- **j.** Wash the inside top part of the column with buffer to remove residual gel that may have remained along the sides during packing.
- **k.** Packed column is now ready for storage at 4 °C or immediate use.
- **l.** Refer to Section C on page 146 for use instructions.

Tip: *Store the packed column upright at 4 °C with the gel bed submerged under 1-2 mL of buffer and a top column cap securely in place. Sodium azide added to the storage buffer to a concentration of 0.02% (w/v) will help prevent microbial growth. Always remove the top cap before the bottom cap to avoid drawing air bubbles down into the gel bed. Prevent air bubbles from forming in the gel bed by using only degassed buffer and sample solutions. Degassing involves subjecting a solution to vacuum to remove excess dissolved air. Use of too high a vacuum can lead to evaporation of solvent from the solution. Check the final volume after degassing and, if necessary, add more solvent to return to original volume.*

2. Nanosep® spin filter column format

Tip: *Adjust resin volume as needed for amount of detergent to be removed.*

- **a.** Remove the spin filter from the Nanosep collection tube and place in a rubber stop per (with a suitable hole to form a tight fit to the spin filter) on the vacuum flask. Apply a low vacuum of 5-10 mm Hg.
- **b.** Mix the 50% (w/v) SDR HyperD slurry (washed in bulk with buffer to remove the 20% (v/v) ethanol preservative) and quickly pipette 0.4 mL of the slurry into the Nanosep device. Rapidly follow with a second and third volume of slurry.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

c. After final addition, allow the vacuum to remove the liquid from the resin bed which should now fill the Nanosep® device.

d. Place the spin filter back into the collection tube to hold until sample addition.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 °C for no more than one week. If resin-filled devices will be stored, add just enough buffer so that the resin looks wet (~200 µL/well).*

e. Refer to Section C on page 146 for use instructions.

3. AcroPrep™ multi-well filter plate format

a. In bulk (e.g., a centrifuge tube) wash the SDR HyperD® media with 5 column volumes (CV) of buffer to remove the 20% (v/v) ethanol storage buffer.

b. Place an AcroPrep filter plate (350 µL or 1.0 mL well volume) on a suitable vacuum manifold (PN 5017) with a collection plate underneath.

c. Mix the 50% (v/v) SDR HyperD slurry and quickly pipette 0.4 mL of the slurry into the multi-well plate. Rapidly follow with a second and third volume of slurry if using the deep well plate. Resin volume should be adjusted according to need.

Tip: *In between each addition of the slurry, allow the resin bed to settle. Mix the slurry before each addition to the well to prevent settling.*

d. After final addition, allow the vacuum to remove the liquid from the resin bed which should partially fill the well of the AcroPrep 96 filter plate (~400 µL of resin).

Tip: *Visually inspect to ensure equivalent volume of resin in each well.*

e. The plate is ready for immediate use. To store the plate, add sufficient buffer/well to wet the resin, then cover. Excess buffer will need to be removed (by vacuum or centrifugation) immediately before use.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 °C for no more than one week.*

f. Refer to Section C on page 146 for use instructions.

4. Glass column format for packed bed chromatography applications

Tip: *Adjust bed height and resin volume to suit the specific application.*

a. Equilibrate column, degassed 50% gel slurry, and degassed buffer solution to room temperature.

b. Attach the bottom end fitting on to the column and clamp upright in a laboratory stand.

c. Add a 1 mL of degassed buffer to the column to cover the bottom frit, and then gently tap the end and side of the column to dislodge any air bubbles.

d. Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 1-2 mL.

e. Allow gel to settle in the column for at least 5 minutes.

f. Position the adjustable height top fitting on to the column. Gently screw the top fitting down on to the settled gel bed. This should displace air out of the top fitting in the column. Do not over compress the gel bed.

g. Place the column on a suitable chromatography system and pump liquid up though the column at 1 mL/min for 2-3 minutes to displace any trapped air. Reverse the flow and equilibrate the column for at least 10 column volumes at up to 10 mL/min.

h. Packed column is now ready for storage at 4 °C or immediate use.

i. Refer to Section C below for use instructions.

C. Detergent Removal

1. Gravity flow column format

a. Prepare a column as described above.

b. Wash the SDR HyperD® resin with 5 CV of buffer to remove the 20% (v/v) ethanol storage buffer.

c. Allow the liquid to drain from the column and load the sample, up to 2 mL, onto the column. (If more volume is preferred, column size can be increased. This will depend on amount of detergent to be removed.)

d. Collect the column effluent in 1 mL fractions. Measure absorbance at 280 nm to locate the protein peak.

Tip: *Protein rapidly elutes from the column and should be found in the first three fractions. Some slight dilution of the sample will occur during elution. If necessary, the sample can be concentrated with a centrifugal UF spin filter, such as a Nanosep® centrifugal device, with a 10K MWCO UF membrane.*

e. After unretained protein has been eluted, discard the resin.

2. Nanosep spin filter column format

a. Prepare the spin column as described above.

b. Centrifuge the spin column in a swinging bucket rotor at 1000 x g for 2 minutes to remove excess fluid from the packed bed.

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.05-0.2 mL, maximum volume depends on resin capacity vs detergent concentration, and well size) onto the top of the dry packed bed in the Nanosep device. Replace in the collection tube.

e. Centrifuge the spin column in a swinging bucket rotor at 1000 x g for 4 minutes to pass the sample through the SDR resin bed.

f. Filtrate in the collection tube will be detergent depleted.

3. AcroPrep™ multi-well filter plate format

Tip: *For prolonged incubations or incubations using solutions that reduce surface tension (e.g., detergents, alcohols, and acetonitrile) in 96-well plates, it may be necessary to seal the bottom of the plate to prevent leakage. This can be tested in advance using just the solutions to be evaluated loaded into the chosen plates.*

An adhesive plate sealer can be used on the top of the wells to prevent cross-contamination during vigorous shaking or evaporation during prolonged or warm incubations. For sealing, you can use a plastic, self-adhesive plate sealer (e.g., Sigma EASYseal) or a cap-mat (PN 5230).*

a. Prepare the packed multi-well filter plate as described above.

b. Centrifuge the multi-well plate and collection plate in a suitable swinging bucket rotor at 1000 x g for 2 minutes to remove excess fluid from the packed bed.

Tip: *As an alternative to centrifuging, a vacuum manifold can be used. See Section 6.3 of the Appendix for more detail.*

c. Remove the filtrate from the collection tube.

d. Very carefully pipette the sample (0.05-0.2 mL, maximum volume depends on resin capacity vs detergent concentration, and well size) onto the top of the dry packed bed in the Nanosep® device.

Tip: *At this stage, sample-loading conditions should be optimized. The amount of sample loaded should be < 50% of the static binding capacity of the resin.*

e. Centrifuge the multi-well plate and collection plate in a swinging bucket rotor at 1000 x g for 4 minutes to pass the sample through the SDR resin bed.

f. Detergent free samples are recovered from the collection plate.

4. Packed bed chromatography column format, pumped system

a. Prepare the column as described above.

b. Load the sample up to a 2 mL volume (maximum volume depends on resin capacity vs detergent concentration) onto the column at 1 mL/min flow rate. Monitor effluent at 280 nm.

c. Collect the column effluent in 1 mL fractions. Measure A_{280} to locate the protein peak.

Tip: *Protein rapidly elutes from the column and should be found in the first three fractions. Some dilution of the sample will occur during elution. If necessary, the sample can be concentrated with a centrifugal UF spin filter, such as a Nanosep centrifugal device, with a 10K MWCO membrane.*

d. After unretained protein has been eluted, the resin can be regenerated to remove retained detergent.

Tip: *If re-use is desired, the retained detergents can be eluted with 10 CV of PBS/ethanol (50/50 mix) followed by 10 CV of 95% (v/v) ethanol, followed by re-equilibration with buffer. If sanitization or depyrogenation is required, two methods are recommended:*

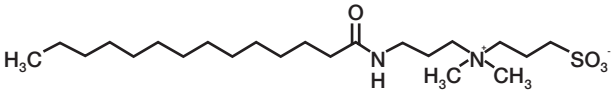
- Alcohol/acid treatment. Wash with at least 3 CV of degassed 20% (v/v) ethanol containing 1 M Acetic acid (**Note:** monitor volume during degassing). Allow the resin to soak in the presence of the solution (recycle effluent manually or using a pump) for an exposure time of 1 hour. Then re-equilibrate with buffer.
- Diethyl pyrocarbonate/ethanol treatment. Wash with 2 CV of PBS in 50% (v/v) ethanol and 5% (v/v) diethyl pyrocarbonate. Allow the resin to soak in the presence of this solution (recycle effluent manually or using a pump) for an exposure time of 1 hour. Wash the column with 3 CV of 3M pyrogen free sterilized NaCl/1 M acetic acid to remove all pyrogens. Then re-equilibrate with sterile pyrogen-free buffer.

Application Data for SDR HyperD® Solvent and Detergent Removal Chromatography Resin

Dynamic Binding Capacity Data for SDR HyperD Resin in a Gravity Flow Column Format
Efficient binding of various detergents in various formats

Removal of detergents from protein samples can be achieved in a gravity flow format for samples in the 1-2 mL volume range. To estimate the detergent removal capacity of the resin, a panel of packed columns (1-2 mL) were loaded with a 1 mL sample of 5 mg/mL human serum albumin (HSA) in 1% (w/v) detergent and allowed to drain into the bed, followed by 1% detergent solution. 1 mL fractions were collected. The protein was measured at 280 nm and then dye binding reagent was added to the effluent to visualize detergent in the fractions. When the column was saturated with detergent, a "break through" curve was recorded. The dynamic binding capacity was calculated as follows; detergent capacity (mg/mL) = [Volume to breakthrough peak (# of fractions x Vol) – volume to protein elution] x 10/Column volume A breakthrough curve and estimate of the dynamic binding capacity for ASB-14 (see Figure 2.32 for chemical structure) is illustrated in Figure 2.33 below. A range of detergents have been evaluated with SDR HyperD resin and their dynamic binding capacity data is summarized in Table 2.35 below. Under these conditions, SDR HyperD resin exhibits a high binding capacity for zwitterionic detergents such as ASB-14 and CHAPS. SDS, an anonic detergent, is influenced by the ionic strength of the sample. Addition of 0.1 M NaCl to 1% SDS (w/v) enhanced the SDR HyperD resin binding capacity by 80-100%. Protein recovery was > 95% in a volume of 1.5-2.0 mL (50-100% volume increase). This diluted sample can be rapidly concentrated with a Nanosep® centrifugal UF spin filter with a 10K MWCO UF membrane if necessary (see Section 2.4, page 152).

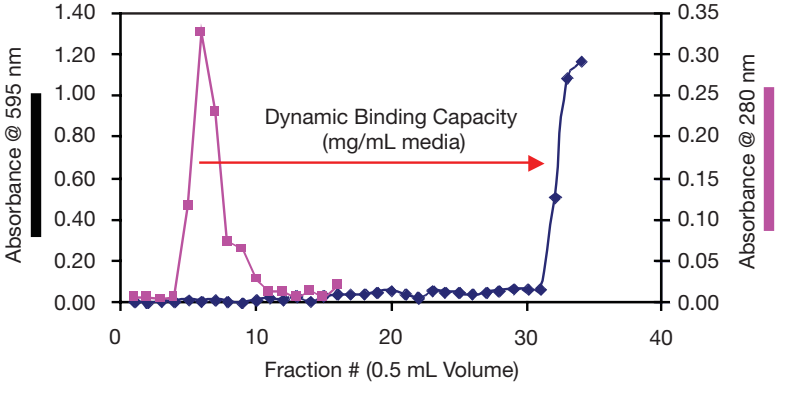
Figure 2.32
Chemical Structure and Properties of Detergent ASB-14



Molecular Weight: 434.7
Molecular Formula: C22H46N2O4S
A zwitterionic amidosulfobetaine detergent useful for solubilizing proteins to be analyzed by 2D-electrophoresis. The increased solubilization produced by this detergent enables the identification pf previously undetected membrane proteins.

Figure 2.33

Dynamic Binding Capacity (mg/mL Media) for ASB-14 Removal by SDR HyperD® Resin in a Gravity Flow Column Format



SDR HyperD resin (1 mL packed volume) packed in a gravity flow column as described in the Protocol section was loaded with 1 mL of a 5 mg/mL albumin solution in 1% ASB-14 detergent in water. The sample was allowed to enter the resin followed by 1% ASB-14 in water (no protein) to elute the protein detergent. Effluent fractions of 1 mL were collected. Protein was monitored at 280 nm and detergent detection was measured interference in the BioRad dye binding assay read at 595 nm.

Table 2.35

Dynamic Binding Capacity of SDR HyperD Resin in a Gravity Flow Format (1 mL Volume)

Detergent	Detergent Dynamic Binding Capacity (mg/mL)
ASB-14	60.0
ASB-14 + 6M Urea/2M Thiourea	70.0
CHAPS	75.0
SDS	15.0
SDS + 0.1 M NaCl	28.0

Dynamic Binding Capacity Data for SDR HyperD® Resin in a Spin Column Format
A spin column format was developed to process smaller volume samples (≤ 0.2 mL) in a shorter time frame using a modified centrifugal desalting procedure. In this study, SDR-HyperD resin was first washed with water to bring the conductivity down to < 0.045 mS/cm and then packed into empty Nanosep® GHP devices under a low vacuum facilitated flow conditions (see Protocol Section B on page 144). The packed bed was then "dewatered" by spinning at 1,000 x g in a swinging bucket rotor for 2 minutes. Samples (0.1 mL) were carefully added to the top of the dewatered resin bed and then spun at 1,000 x g in the above rotor for 4 minutes. The resulting filtrate was placed in a multi-well plate and 0.2 mL of the diluted dye binding reagent was added (BioRad). The presence of detergent correlated with development of a blue color after the addition of the dye. The results are summarized in Table 2.36 below.

Table 2.36
Detergent Binding Capacity of 1 mL SDR HyperD Resin in a Spin Column Format

Sample	# of Cycles*	Volume 1% (w/v) Detergent	SDR/Device
ASB-14	22	2.2 mL	22 mg
ASB-14 + 6 M Urea/2 M Thiourea	7	0.7 mL	7 mg
CHAPS	24	2.4 mL	24 mg
CHAPS + 6 M Urea/2M Thiourea	12	1.2 mL	12 mg
SDS	20	2.0 mL	20 mg
SDS + 0.1 M NaCl	24	2.4 mL	24 mg

**Detergent binding capacity was measured by adding 0.1 mL volumes of the detergent to challenge the device, followed by centrifugation as described in the Protocol section. This was repeated until detergent "break through" was seen with the protein assay. The volume of detergent was then calculated from volume 1% detergent = # cycles x 0.1.*

Dynamic Binding Capacity Data for SDR HyperD Resin in a Pumped Packed Bed Column Format
The high specific surface area (200 m^2/g) of the porous silica of this resin allows a high capacity for Triton* X-100 and TnBP. The dynamic binding capacities obtained are 60-80 mg/mL for Triton X-100 and 40-50 mg/mL for TnBP at 100 cm/hr (initial concentrations of Triton X-100 and TnBP in bovine plasma are respectively 10 and 5 mg/mL). Examples of removal efficiencies from various samples are summarized in Table 2.37.

Table 2.37
Solvent-Detergent Depletion Example

Solution	Surfactant	Before Depletion	After Depletion	Removal Efficiency
IgG	TnBP Triton* X-100	5,000 ppm 10,000 ppm	< 0.4 ppm < 10 ppm	99.9% 99.9%
ATIII	TnBP Triton X-100	5,000 ppm 10,000 ppm	< 0.4 ppm <10 ppm	99.9% 99.9%
Bovine Serum	TnBP Triton X-100	5,000 ppm 10,000 ppm	< 0.4 ppm < 10 ppm	99.9% 99.9%

Sample volume: 3.6 CV, flow rate: 150 cm/h; column length: 10 cm; residence time: 4 minutes.

Tip: *Removal efficiency is also dependent on flow rate and column loading: i.e. when using a 10 cm column at 150 cm/h (2 CV load of bovine serum containing Triton X-100 or TnBP), a removal of 95.5% was observed for Triton X-100. This removal efficiency was decreased to 80% when 8 CV loads were used.*

In summary, the SDR HyperD® resin is an excellent tool for removal of detergent from protein samples and can be used in a range of device formats to achieve rapid processing with high recovery of sample.

Ordering Information for SDR HyperD Solvent and Detergent Removal Chromatography Resin

Part Number	Description	Pkg
20033-065	SDR HyperD	5 mL
20033-031	SDR HyperD	25 mL
20033-023	SDR HyperD	100 mL
20033-015	SDR HyperD	1000 mL

References for SDR HyperD Solvent and Detergent Removal Chromatography Resin

1. Guerrier, L., et al. (1995). Specific sorbent to remove solvent-detergent mixtures from virus-inactivated biological fluids. *J. Chromatogr. B.*, (664), 119.

2.4.1 Introduction

Biomolecule purification involves a complex series of steps where targets are selectively separated through sequential processes. The processes by which separation is performed often creates a need for the sample to be desalted or concentrated to prepare the biomolecule sample for the next step in the purification process.

Pall offers several technologies to perform efficient sample concentration, desalting, and buffer exchange including ultrafiltration (UF) spin filters, UF multi-well filtration plates, and bottled gel filtration media. Refer to Table 2.38 for a selection of UF products available.

Compared to other methods, UF methods for concentration and desalting offer a number of advantages including:

- Reduction of processing steps – desalting and concentration take place in a single step.
- Consistency – the use of UF products eliminates problems with gel beds.
- Versatility – UF is useful in both low and high throughput modes.

Pall also offers chromatography resins for desalting applications including Ultrogel® AcA 202 and Trisacryl® GF-05 resins. Refer to Table 2.39 for general properties.

Table 2.38
UF Products for Concentration and Desalting

Device	Sample Volume
AcroPrep™ 384 filter plate	< 100 µL
AcroPrep 96 filter plate	< 350 µL
Nanosep® device	< 0.5 mL
Microsep™ device	0.5-3.5 mL
Macrosep® device	3-15 mL
Jumbosep™ device	15-60 mL

Table 2.39
Properties of Ultrogel® AcA and Trisacryl® GF-05 Resin

Specification	Ultrogel AcA 202 Resin	Trisacryl GF-05M Resin
Particle Size	60-140 µm	40-80 µm
Monomer	20% (w/v) Acrylamide	N-acryloyl-2-amino-2-hydroxymethyl-1,3-propanediol
Cross-linker	2% (w/v) Agarose	Hydroxylated acrylic bifunctional monomer
Exclusion Limit	22,000 dalton	3,000 dalton
Linear Fractionation Range	1,000-15,000 dalton	200-2,500 dalton
Resolving Power (plates/m)	3,000	2,500
Working pH Range	3-10	1-11

Figure 2.34
Pall Centrifugal Spin Filters

2.4.2 Concentration and Desalting Using Ultrafiltration

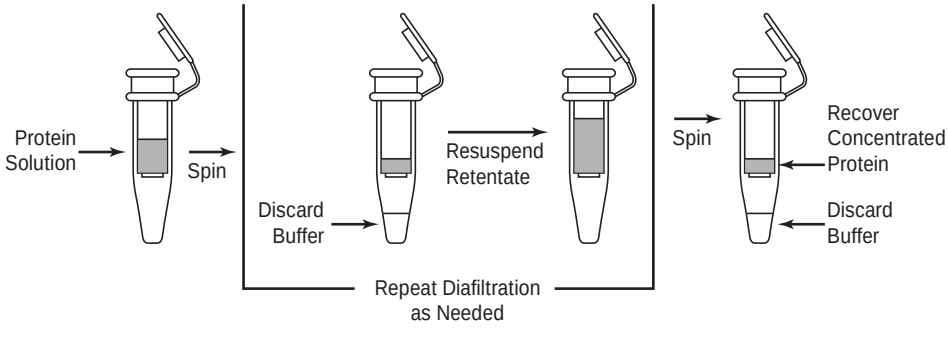
2.4.2.1 Concentration and Diafiltration of Samples (< 60 mL) in a Spin Filter

Ultrafiltration (UF) is a membrane separation technique based on selection by molecular size, although other factors such as molecular shape and charge can also play a role. Molecules larger than the membrane pores in the UF membrane will be retained at the surface of the membrane while solvent and smaller solute molecules will freely pass. This molecular exclusion at the UF membrane surface lead to concentration of the protein solute in the retained fraction termed the retentate and can be recovered from above the membrane.

There are two classic applications of UF membranes in Proteomics.

1. Concentration of dilute protein or peptide samples.

Figure 2.35
Retentate Recovery



2. Desalting and buffer exchange (diafiltration) to remove or exchange salts, separate free from bound molecules, remove low molecular weight materials, or rapidly change the ionic or pH environment.

Choosing the Correct MWCO

The retention properties of UF membranes are expressed as Molecular Weight Cut-off (MWCO), referring to the approximate molecular weight (MWt.) of a dilute globular solute (i.e., a typical protein), which is 90% retained by the membrane. An important first step in the application of UF in sample processing is the selection of the appropriate MWCO membrane able to retain the protein of interest. In Tables 2.40 and 2.43 the retention characteristics of Omega™ UF membranes for proteins is summarized for a range of UF devices. For proteins, it is recommended that an MWCO be selected that is 3 to 6 times smaller than the MWt. of the solute being retained. If flow rate is a consideration, choose a membrane with an MWCO at the lower end of this range (3x). If the main concern is retention, choose a tighter membrane (6x). The retention of a molecule by a UF membrane can be influenced by a number of factors such as MWt., molecular shape, electrical charge, and the composition of the sample matrix. In choosing UF, it is important to consider performing pilot experiments to verify membrane performance for a particular pre-analytical sample preparation application in Proteomics.

Table 2.40
Typical Protein Recovery/Passage with Omega™ UF Membranes in a Nanosep® Centrifugal UF Device

		MWCO	**3K**	**10K**	**30K**	**100K**	**300K**
Solute	Solute MW (Kd)	Spin Time (min.)	15	10	8	5	3
Vitamin B12	1,335	% Recovery	7	-	-	-	-
Aprotinin	6,200	% Recovery	99	51	11	-	-
Cytochrome C	12,400	% Recovery	100	89	77	2	-
Chymotrypsinogen A	25,000	% Recovery	–	97	94	2	-
Ovalbumin	45,000	% Recovery	–	97	92	3	-
BSA	67,000	% Recovery	–	–	100	26	1.5
Phosphorylase B	97,400	% Recovery	–	–	95	91	1
lgG	156,000	% Recovery	–	–	–	97	1.5
Thyroglobulin	677,000	% Recovery	–	–	–	100	91

Samples of 0.5 mL of a 1.0 mg/mL solution were centrifuged at 14,000 x g and were concentrated to a volume of 0.01-0.06 mL.

Application Guidelines for Concentration and Diafiltration of Samples (< 60 mL) in a Spin Filter

With the above UF membranes and devices able to process < 60 mL volume samples, there are many options available for concentration or desalting applications. Pall offers four sizes of single sample, single-use devices for concentration and desalting applications. A simple guide to choosing the appropriate sized centrifugal UF spin filter for a range of applications is summarized in Table 2.41. A similar guide for choosing the right MWCO UF membrane is summarized in Table 2.42. Full specifications of the range of Pall UF devices available are summarized in Table 2.43.

Table 2.41
Choosing the Correct Centrifugal UF Device

Application	Sample Volume			
	Nanosep® (< 1 mL)	Microsep™ (< 3.5 mL)	Macrosep® (< 15 mL)	Jumbosep™ (< 60 mL)
Concentrate/desalt – peptides, proteins, oligonucleotides, and nucleic acids	✓	✓	✓	✓
Size fractionation of complex mixtures	✓	✓	✓	✓
Clean up labeling reactions – proteins and nucleic acids	✓	✓		
Separate proteins from SDS-PAGE gels	✓			
Buffer exchange as an alternative to dialysis	✓	✓	✓	✓
Buffer exchange or salt removal of chromatography eluates, gradient fractions		✓	✓	
Concentrating dilute samples to enhance sensitivity for electrophoresis	✓	✓		
Remove protein for HPLC analysis	✓	✓		
Recovery of antibodies from cell culture	✓	✓	✓	✓
Recovery of low molecular weight compounds from fermentation broth		✓	✓	✓
Free drug monitoring in serum or plasma	✓	✓		
Natural products screening for medicinal chemistry	✓	✓	✓	
Virus concentration or removal			✓	✓
Concentrating recombinant proteins in conditioned media			✓	✓

Table 2.42
MWCO UF Membrane Selection

	MWCO UF Membrane		
Application	10K	30K	100K
Concentrate/desalt peptides, proteins, oligonucleotides, and nucleic acids	✓	✓ (PCR primers)	✓ (PCR primers)
Size fractionation of complex mixtures	✓	✓	✓
Clean up labeling reactions – proteins and nucleic acids	✓		
Separate proteins from SDS-PAGE gels	✓	✓	
Buffer exchange as an alternative to dialysis	✓	✓	✓
Buffer exchange or salt removal of chromatography eluates, gradient fractions	✓		
Concentrating dilute samples to enhance sensitivity for electrophoresis	✓		
Remove protein for HPLC analysis	✓	✓	
Recovery of antibodies from cell culture			✓ (IgM)
Recovery of low molecular weight compounds from fermentation broth	✓	✓	
Free drug monitoring in serum or plasma	✓		
Natural products screening for medicinal chemistry	✓	✓	✓
Virus concentration or removal			✓
Concentrating recombinant proteins in conditioned media		✓	✓

Table 2.43
Specifications of UF Centrifugal Devices

Specification	Nanosep®	Microsep™	Macrosep®	Jumbosep™
UF Membrane	Omega™ membrane (low protein-binding, modified polyethersulfone on polyethylene substrate)			
Materials of Construction				
Device	Polypropylene	Styrene acrylonitrile (SANS)	Polypropylene, Polyethylene	Polysulfone, Polypropylene
Collection Tube	Polypropylene	Polypropylene	Polypropylene	Polypropylene
Effective Membrane Area	0.28 cm^2	0.46 cm^2	10 cm^2	15.2 cm^2
Dimensions				
Diameter	*	1.7 cm	2.9 cm	6.0 cm
Overall Length (with Cap)	4.5 cm	9.3 cm	10.9 cm	11.3 cm
Capacities				
Maximum Sample Volume	0.5 mL	3.5 mL	15.0 mL	60.0 mL
Final Retentate Volume	0.05 mL	0.030-0.05 mL	0.5-1.5 mL	3.5-4.0 mL
Final Receiver Volume	0.5 mL	3.5 mL	15.0 mL	60.0 mL
Hold-up Volume (Membrane/Support)	< 0.005 mL	0.02 mL	0.03 mL	0.2 mL
Operating Temperature Range	0-40 °C (32-104 °F)			
pH Range	1-14			
Maximum Centrifugal Force	14,000 x g	7,500 x g	5,000 x g	3000 x g
Centrifuge	1.5 mL microcentrifuge rotors	Rotor accepting 1.7 x 10 cm tubes	Rotor accepting 50 mL volume tubes	Swinging bucket rotor accepting 250 mL bottles
Sanitization	70% ethanol			

**Fits rotors that accept 1.5 mL tubes.*

Protocol for Nanosep® UF Centrifugal Device — Working with Samples < 1 mL

A. Materials Required

1. Nanosep UF centifugal spin filte. For specifications, see Table 2.43.
2. Extra collection tubes for the Nanosep UF device
3. Degassed high purity water or buffer, such as phosphate buffered saline (PBS)

B. Diafiltration or Buffer Exchange of Samples

1. Select the Nanosep device with an MWCO three times smaller than the MWt. of the protein to be retained (see Table 2.40).
2. If the devices have been pre-treated, proceed directly to Step B4.
3. Add 0.5 mL of high purity water to the retentate cup and centrifuge at 14,000 x g for 5-10 minutes. Discard the filtrate.
4. Add 0.5 mL of the sample and centrifuge at 14,000 x g for 5-10 minutes, depending on the MWCO membrane used (see Table 2.40). At this stage, it is important to achieve concentration of the sample to < 0.05 mL to achieve efficient salt removal or buffer exchange in diafiltration. Transfer the filtrate into a clean tube and retain in case the protein of interest was not retained by the UF membrane.

Tip: *A pilot experiment is usually necessary to confirm that > 99% of the protein target is retained before using this MWCO membrane for diafiltration.*

5. Commence diafiltration adding 0.45 mL of high purity water or buffer to the retentate cup. Mix using a pipette (cycle up and down) to thoroughly mix the retentate with the diafiltration solution. Do not touch the MWCO membrane with the pipette tip. Re-centrifuge at 14,000 x g for 5-10 minutes and retain the filtrate as in Step B3.
6. Usually three cycles of dilution and concentration will remove over 99% of salts and achieve buffer exchange.

Tip: *Multiple diafiltration steps can decrease overall yields.*

7. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 0.01-0.02 mL new buffer or water.

C. Concentration of Samples

1. Select the Nanosep device with an MWCO three times smaller than the MWt. of the protein to be retained (see Table 2.40).
2. If the devices have been pre-treated, proceed directly to Step C4.
3. Add 0.5 mL of high purity water to the retentate cup and centrifuge at 14,000 x g for 5-10 minutes. Discard the filtrate.
4. Add 0.5 mL of the sample and centrifuge at 14,000 x g for 5-10 minutes depending on the MWCO membrane used (see Table 2.40). Transfer the filtrate into a clean tube and retain in case the protein of interest was not retained by the UF membrane.
5. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 0.01-0.02 mL new buffer or water.

Protocol for Microsep™ UF Centrifugal Device — Working with Samples from 1-3.5 mL

Omega™ membranes in Microsep devices contain trace amounts of glycerin and sodium azide (0.65-1.0 mg). If these chemicals interfere with an assay, they may be removed by filtering 3 mL high purity water or buffer through the membrane and repeat. If further flushing is required, start with 0.05N NaOH and repeat this procedure. Use device within 20 minutes to prevent irreversible membrane damage due to dehydration.

A. Materials Required

1. Microsep UF centifugal spin filter. For specifications, see Table 2.43.
2. Degassed high purity water or buffer, such as PBS

B. Instructions for Use

1. Attach the filtrate receiver to the bottom of the sample reservoir (see Figure 2.36).
2. Add 0.1-3.5 mL of sample to the sample reservoir. Place cap on reservoir to prevent evaporation during centrifugation.
3. Place device into fixed angle centrifuge rotor that accepts 17 x 100 mm tubes.

CAUTION: *Always counterbalance the rotor with another Microsep device containing equivalent sample volume.*

4. Spin at 3,000-7,500 x g for the required length of time, typically 30-90 minutes, to achieve desired concentrate volume. It is recommended that spin time and g-force be determined for each application. See Tables 2.44 and 2.45 to determine appropriate protocol.

Table 2.44
Typical Protein Recovery/Passage with Omega UF Membranes in a Microsep Centrifugal UF Device

Microsep Device MWCO	Recommended g-Force	MWt. Range
1K, yellow	5,000-7,000 x g	3K-10K
3K, gray	5,000-7,500 x g	10K-30K
10K, blue	5,000-7,500 x g	30K-90K
30K, red	5,000-7,500 x g	90K-180K
50K, green	1,000 x g	150K-300K
100K, clear	1,000 x g	300K-900K
300K, orange	1,000 x g	900K-1,800K
1000K, purple	1,000 x g	> 3000K

Figure 2.36
Components of the Microsep™ Centrifugal UF Device

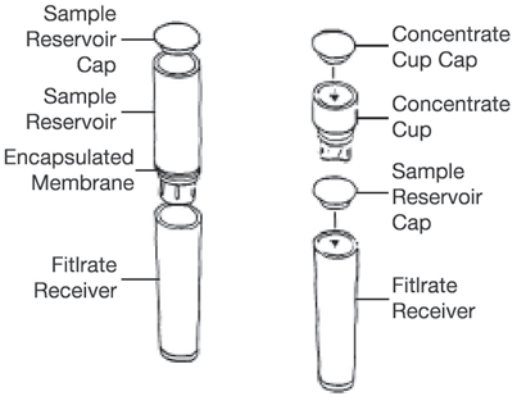
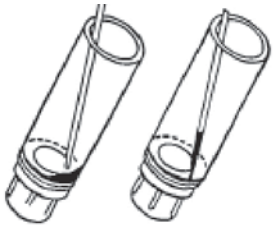


Table 2.45
Processing Times for Microsep UF Centrifugal Devices

		Time to "Dead Stop" (Minutes)		
MWCO	Solute	3,000 x g	5,000 x g	7,500 x g
1K	Cytochrome C (1 mg/mL)	200	130	100
3K	Cytochrome C (1 mg/mL)	180	120	90
10K	Albumin (1 mg/mL)	55	40	25
30K	Albumin (1 mg/mL) IgG (0.1 mg/mL)	40 60	30 30	20 30
50K	Albumin (1 mg/mL) IgG (0.1 mg/mL)	25 45	15 30	10 30
100K	Apoferritin (1 mg/mL) IgG (0.1 mg/mL)	40 45	35 30	30 30
300K	Yeast (0.5%)	20	14	—
1000K	Yeast (0.5%)	8	5	—

5. At the end of spin time, stop centrifuge and remove devices. Using a pipette, transfer concentrated sample to concentrate cup. To pipette concentrated sample, slowly siphon the concentrate by moving the pipette tip around the perimeter of the plastic ring at the bottom of the sample reservoir (Figure 2.37).

Figure 2.37
Recovery of Retentate from Microsep™ UF Centrifugal Device



6. Cap storage cup containing concentrated sample and store. Filtrate collected in filtrate receiver can also be stored for further analysis.

C. Diafiltration or Buffer Exchange of Samples

1. Select the Microsep device with an MWCO three times smaller than the MWt. of the protein to be retained (see Table 2.40).
2. If the devices have been pre-treated as described in Appendix 6.2, proceed directly to Step C4.
3. Add 3.5 mL of high purity water to the retentate cup and centrifuge at 3,000-7,500 x g for 30-90 minutes, depending on the MWCO membrane used (see Tables 2.44 and 2.45). Discard the filtrate.
4. Add up to 3.5 mL of the sample and centrifuge as described in Step C3. At this stage, it is important to achieve concentration of the sample to < 0.05 mL to achieve efficient salt removal or buffer exchange in diafiltration. Transfer the filtrate into a clean tube and retain in case the protein of interest was not retained by the UF membrane.

Note: *A pilot experiment is usually necessary to confirm that > 99% of the protein target is retained before using this MWCO membrane for diafiltration.*

5. Commence diafiltration by adding 3.5 mL of high purity water or buffer to the retentate cup. Mix using a pipette (cycle up and down) to thoroughly mix the retentate with the diafiltration solution. Re-centrifuge as described in Steps C3-C4.
6. Usually three cycles of dilution and concentration will remove over 99% of salts and achieve buffer exchange.

Tip: *Multiple diafiltration steps can decrease overall yields.*

7. Recover the retained sample with a pipette tip (see Figure 2.37). To maximize recovery, rinse the retentate cup twice with 0.01-0.02 mL new buffer or water.

D. Concentration of Samples

1. Select the Microsep device with an MWCO three times smaller than the MWt. of the protein to be retained (see Table 2.40).
2. If the devices have been pre-treated as described in Appendix 6.2, proceed directly to Step D4.
3. Add 3.5 mL of high purity water to the retentate cup and centrifuge at 3,000-7,500 x g for 30-90 minutes, depending on the MWCO membrane used (see Tables 2.44 and 2.45). Discard the filtrate.

4. Add up to 3.5 mL of the sample and centrifuge as described in Step D3. Transfer the filtrate into a clean tube and retain in case the protein of interest was not retained by the UF membrane.
5. Recover the retained sample with a pipette tip (see Figure 2.37). To maximize recovery, rinse the retentate cup twice with 0.01-0.02 mL new buffer or water.

E. Sample Preparation for SDS-PAGE Electrophoresis

1. Pipette 0.05-0.1 mL sample containing 5-60 µg of protein into Microsep™ device.
2. Dilute sample to 3.5 mL with buffer compatible with SDS-PAGE electrophoresis and centrifuge at 3,000 to 7,500 x g for 30-90 minutes depending on the MWCO membrane used (see Tables 2.44 and 2.45). Repeat twice.
3. Transfer final retentate sample to the concentrate cup. Add SDS to a final concentration of 2% (w/v). Cap and heat to 80 °C for 10 minutes or more.
4. Remove from incubator or water bath and add a reducing agent, such as dithiothreitol, to a final concentration 5-10 mM. Incubate at 56 °C for 5 minutes.
5. Remove from incubator. Cool to room temperature. Prepare to layer on gel.[3]

Protocol for Macrosep® Centrifugal Device — Working with Samples from 3.5-15 mL

Each Macrosep device (see Figure 2.38) consists of a sample reservoir, a sample reservoir cap, a membrane paddle, a concentrate cup and cap, a filtrate receiver, and a filtrate receiver cap. Centrifugation up to 5,000 x g provides the driving force for filtration, moving the sample towards the encapsulated Omega™ membrane. Biomolecules larger than the nominal MWCO of the membrane are retained in the sample reservoir. Solvent and low molecular weight molecules pass through the membrane into the filtrate receiver.

Figure 2.38
Components of a Macrosep Centrifugal UF Device

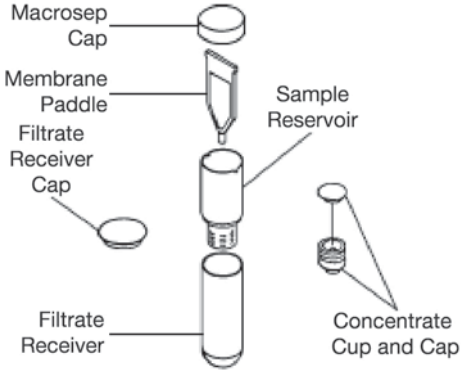


Table 2.46
Choice of Centrifuge Rotors for Operation of the Macrosep® UF Centrifugal Device

Rotor Angle	Maximum Sample Volume	Dead-Stop Volume	Concentration Factor
Swinging Bucket	15 mL 20 mL*	0.5-1.0 mL	15-30 x 20-40 x
45° Fixed Angle	15 mL	1.0-1.5 mL	10-15 x
34° Fixed Angle	12.5 mL	1.0-1.5 mL	8-12.5 x
25° Fixed Angle	9 mL	1.5-2.0 mL	4.5-6 x

**Use a 20 mL filtrate receiver for 20 mL sample volumes.*

The Macrosep sample reservoir capacity is 20 mL; however, the device is provided with a 15 mL filtrate tube to fit into most common fixed angle rotors.

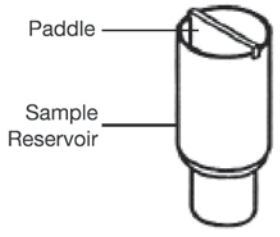
- When using a fixed angle rotor, it is important to measure the 15 mL of sample into the reservoir. Because the sample spins at an angle to the rotor axis, leakage can occur around the notches if the reservoir is overfilled. The excess sample will flow out through the notches at the top of the sample reservoir and into the rotor cavity.
- In a swinging bucket rotor, the sample spins perpendicular to the rotor axis so no sample is lost through the notches if the reservoir is filled to 20 mL. However, the optional 20 mL filtrate receiver (PN FD001X37) must be used to avoid overflow of filtrate out of the filtrate receiver.

A. Instructions for Use

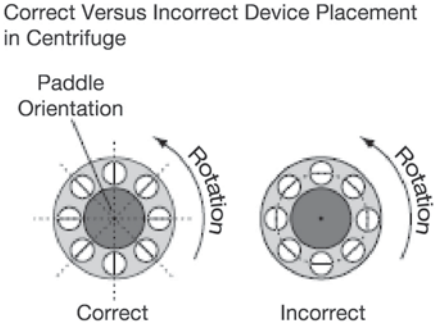
1. Insert the paddle firmly into the bottom of the sample reservoir. The “hooks” on the top part of the paddle must rest firmly in the notches on top of the sample reservoir (Figure 2.39, Panel A). For best alignment, turn the reservoir upside down on the bench top and gently press the paddle into place. Once the paddle is firmly in place, there will still be a small gap in between the hooks of the paddle and the notches of the sample reservoir.

Figure 2.39
Details on Placement of Macrosep® Membrane "Paddle" in the Device and Centrifuge

Panel A, Placement of UF "Paddle" in the Macrosep Device



Panel B, Orientation of Macrosep Device in the Centrifuge



2. Attach the filtrate receiver to the bottom of the sample reservoir.
3. Add 5-15 mL of sample to the sample reservoir. Place cap on reservoir to prevent evaporation during centrifugation.

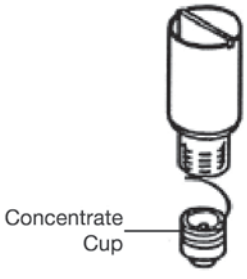
Tip: *If introducing a volume of 20 mL into the sample reservoir, make sure the optional 20 mL filtrate receiver is attached.*

4. Place the Macrosep device into a bucket or rotor which accepts standard 50 mL tubes.
5. In a fixed-angle rotor, align the Macrosep device so that one of the "hooks" faces the center of the centrifuge rotor (see Figure 2.39, Panel B). This prevents a build-up of macromolecules on the membrane paddle and allows the device's dead-stop to function properly. A swinging bucket rotor is self-aligning.
6. Spin at 1,000-5,000 x g, typically for 30-90 minutes, to achieve desired concentrate volume. It is recommended that spin time and g-force be determined for each application.
7. At the end of spin time, stop centrifuge and remove devices. Concentrate recovery can be accomplished by one of the following methods:

a. For concentrate volumes less than 1.5 mL:

(1) Remove filtrate receiver and screw on the concentrate cup (see Figure 2.38). The center pin will cause the paddle to lift up and out of the bottom of the sample reservoir, allowing concentrate to flow into concentrate cup (see Figure 2.40).

Figure 2.40
Concentrate Recovery in Macrosep® UF Centrifugal Device



(2) Place the "hooks" into the notches on the sample reservoir. Replace filtrate receiver.

(3) Place the Macrosep device back into the centrifuge and spin at 1,000-5,000 x g for 1-3 minutes. Remove the device and unscrew the concentrate cup.

b. For concentrate volumes greater than 1.5 mL:

(1) Pour off concentrate to reduce volume to 1.5 mL or less, then perform Step (1)-(3) above. An alternative method of reducing volume is described below.

(2) Attach a clean filtrate receiver to the sample reservoir.

(3) Lift the membrane paddle out and turn so that the "hooks" rest on the lip of the sample reservoir, not in the notches. This allows concentrate to flow into the filtrate receiver.

(4) Spin at 1,000-5,000 x g for 1-3 minutes for maximum concentrate recovery.

B. Diafiltration or Buffer Exchange of Samples

1. Select the Macrosep device with an MWCO three times smaller than the MWt. of the protein to be retained (see Table 2.40).

2. If the devices have been pre-treated, proceed directly to Step B4.

3. Add 15 mL of high purity water into the sample reservoir. Place cap on reservoir. Centrifuge as described in Steps A4-A7 above. Discard the filtrate.

4. Add up to 15 mL of the sample and centrifuge as described in Steps A4-A7. Transfer the filtrate into a clean tube and retain in case the protein of interest was not retained by the UF membrane. At this stage, it is important to achieve concentration of the sample to < 0.5 mL to achieve efficient salt removal or buffer exchange in diafiltration. Transfer the filtrate into a clean tube and retain in case the protein of interest was not retained by the UF membrane.

Tip: *A pilot experiment is usually necessary to confirm that > 99% of the protein target is retained before using this MWCO membrane for diafiltration.*

5. Commence diafiltration by adding 15 mL of high purity water or buffer to the sample reservoir. Mix using a pipette (cycle up and down) to thoroughly mix the retentate with the diafiltration solution. Place cap on reservoir. Re-centrifuge as described in Steps A4-A7.
6. Usually three cycles of dilution and concentration will remove over 99% of salts and achieve buffer exchange.

Note: *Multiple diafiltration steps can decrease overall yields.*

7. Recover the retained sample as described in Steps A7a-A7b.

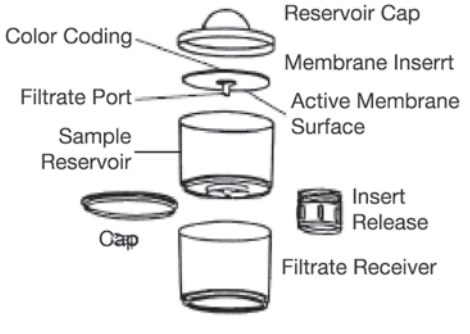
C. Concentration of Samples

1. Select the Macrosep® device with an MWCO three times smaller than the MWt. of the protein to be retained (see Table 2.40).
2. If the devices have been pre-treated as described in Appendix 6.2, proceed directly to Step 4.
3. Add 15 mL of high purity water into the non-membrane side of the sample reservoir. Place cap on reservoir. Centrifuge as described in Steps A4-A7. Discard the filtrate.
4. Add up to 15 mL of the sample and centrifuge as described in Steps A4-A7. Transfer the filtrate into a clean tube and retain in case the protein of interest was not retained by the UF membrane.
5. Recover the retained sample as described in Steps A7a-A7b.

Protocol for Jumbosep™ Centrifugal Device - Working with Samples from 15-60 mL

Centrifugation up to 3,000 x g provides the driving force for filtration, moving sample towards the highly selective, low protein-binding Omega™ UF membrane. Macromolecules larger than the membrane's nominal MWCO are retained in the sample reservoir. Solutes and macromolecules smaller than the MWCO of the membrane pass through the membrane surface into the membrane insert and through the filtrate port into the filtrate receiver. See Figure 2.41 for a diagram showing the components of the Jumbosep centrifugal UF device.

Figure 2.41
Components of Jumbosep Centrifugal UF Device



A. Instructions for Use

1. Separate the filtrate receiver from the sample reservoir.
2. Remove the protective plastic from the membrane insert.

Tip: *The color of the button on the top of the membrane insert indicates the MWCO of the membrane.*

3. Hold the membrane insert by the edge with the filtrate port facing down and drop the insert into the sample reservoir.
4. Place the sample reservoir on a hard surface and, with both thumbs placed on the colored button in the middle of the membrane, press down firmly on the membrane insert. The membrane insert rests on the knobs at the bottom of the sample reservoir.
5. Attach the empty filtrate receiver to the bottom of the sample reservoir.
6. Add 15-60 mL of sample to the sample reservoir. Place the cap on the reservoir to prevent evaporation during centrifugation.
7. Place the device in a swinging bucket rotor that accepts standard 250 mL bottles. Remove any bottle adapters to ensure that the bottom of the bucket is flat. Presence of the adapters might cause deformation of the bottom of the Jumbosep™ device's filtrate receiver.

Tip: *Always counterbalance the rotor with another Jumbosep device containing an equivalent sample volume.*

8. Spin at 1,000-3,000 x g, typically for 15-40 minutes, to achieve desired concentrate volume. It is recommended that spin time and g-force be determined for each application.

CAUTION: *Maximum g-force is 3,000 x g. Higher g-forces may cause retentate leakage into the filtrate.*

9. At the end of spin time, stop the centrifuge and remove the devices.
10. Separate the sample reservoir from the filtrate receiver in the following manner: hold the device so that both palms are placed on the filtrate receiver; with both thumbs placed side by side on the sample reservoir, press upward.
11. To recover retentate, pour off into a storage vessel. Some retentate will remain under the membrane insert. To remove the remaining retentate, twist the insert release onto the sample reservoir. Turn the sample reservoir sideways (taking care that the retentate remains in the sample reservoir). Slide a pipette tip under the dislodged membrane insert and remove the remaining retentate.

B. Diafiltration or Buffer Exchange of Samples

1. Select the Jumbosep device with an MWCO three times smaller than the MWt. of the protein to be retained (see Table 2.40).
2. If the devices have been pre-treated as described in Appendix 6.2, proceed directly to Step 4
3. Add 15-60 mL of high purity water into the sample reservoir. Place cap on reservoir. Centrifuge as described in Steps A7-A8 above. Discard the filtrate.

4. Add up to 15-60 mL of the sample and centrifuge as described in Steps A7-A8. Transfer the filtrate into a clean tube and retain in case the protein of interest was not retained by the UF membrane. At this stage, it is important to achieve concentration of the sample to < 4.0 mL to achieve efficient salt removal or buffer exchange in diafiltration. Transfer the filtrate into a clean tube and retain in case the protein of interest was not retained by the UF membrane.

Tip: *A pilot experiment is usually necessary to confirm that > 99% of the protein target is retained before using this MWCO membrane for diafiltration.*

5. Commence diafiltration by adding 15-60 mL of high purity water or buffer to the sample reservoir. Mix using a pipette (cycle up and down) to thoroughly mix the retentate with the diafiltration solution. Place cap on reservoir. Re-centrifuge as described in Steps A7-A8.
6. Usually three cycles of dilution and concentration will remove over 99% of salts and achieve buffer exchange.

Tip: *Multiple diafiltration steps can decrease overall yields.*

7. Recover the retained sample as described in Step A11.

C. Concentration of Samples

1. Select the Jumbosep™ device with an MWCO three times smaller than the MWt. of the protein to be retained (see Table 2.42).
2. If the devices have been pre-treated, proceed directly to Step 4.
3. Add 15-60 mL of high purity water into the sample reservoir. Place cap on reservoir. Centrifuge as described in Steps A7-A8. Discard the filtrate.
4. Add up to 15-60 mL of the sample and centrifuge as described in Steps A7-A8. Transfer the filtrate into a clean tube and retain in case the protein of interest was not retained by the UF membrane.
5. Recover the retained sample as described in Step A11.

D. Optional Pre-Treatment to Improve Recovery of Samples

If poor sample recovery is experienced with the Omega™ UF membranes and devices, it may be due to non-specific adsorption. It is possible to pre-treat these membranes and devices with protein or detergents to reduce the potential of non-specific adsorption of the target analyte. A protocol is outlined below.

1. Add sufficient volume of 1% (w/v) BSA or 1% (w/v) IgG in phosphate buffered saline (PBS); 5% (w/v) SDS; 5% (v/v) Tween-20; 5% (v/v)Triton*-X100; or 5% (w/v) PEG compound in high purity water to partially fill the UF device. Close the cap and mix by inversion to coat the solution over the internal surfaces of the device.
2. Incubate the solution in the device for at least one hour at room temperature.
3. Discard the above solution and rinse the device thoroughly with high purity water.

Tip: *Rinsing to remove detergents such as SDS, Tween, and Triton may require more than the above wash steps.*

4. To ensure pre-treatment solution is removed from the membrane, add high purity water to the device and spin at the recommended speed for each device to pass the fluid through the UF membrane. Discard the filtrate.

5. The device can be used immediately or stored for later use. If the device is to be used later, add sterile high purity water [rendered sterile by autoclaving or filtration with a 0.2 µm sterilizing grade filter (see Acrodisc® Syringe Filters with Supor® PES membrane for sterile filtration, Pall PN 4612)] to the sample reservoir and store at 4 °C to retard bacterial growth.

Tip: *Do not allow the membrane to dry out once the device has been pre-treated.*

Application Data for Concentration and Diafiltration of Samples (< 60 mL) in a Spin Filter

Improving the Recovery of Proteins from Dilute Solutions (< 0.1 mg/mL) by Pre-treatment

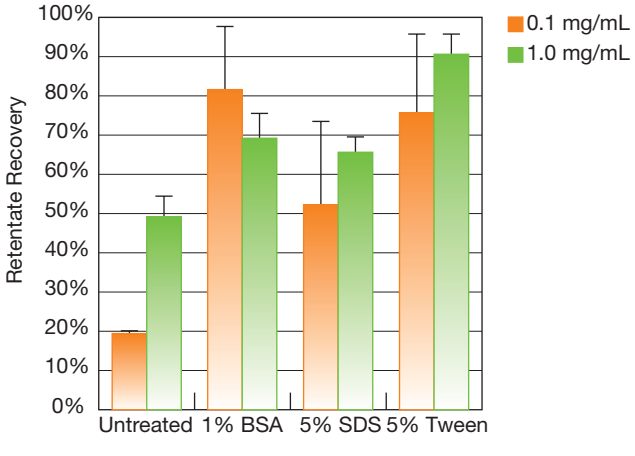
Nanosep® UF centrifugal devices are constructed of materials that minimize non-specific binding (NSB). However, dilute solutions of proteins containing charged or hydrophobic domains can show NSB toward various plastic and membrane surfaces. To reduce this NSB, several approaches have been suggested:

- Pretreatment to occupy NSB sites on the surface.
- Changing the composition of the solution, usually by addition of a protein bulking agent such as albumin, or by inclusion of detergents, or salts to counter the NSB.

In Step D on page 169, a protocol is described for pre-treating Nanosep UF centrifugal devices to reduce NSB, especially with dilute samples. An experiment illustrating the impact of pre-treatment is summarized in Figure 2.42. Pre-treatment with BSA or Tween 20 showed a marked improvement in recovery of the dilute (0.1 µg/mL) protein, achieving 70-82% recovery compared to 20% in the control untreated devices.

Figure 2.42

Use of Pre-Treatment to Increase Protein Recovery in Nanosep UF Centrifugal Devices



Pre-treatment was carried out as described in Protocol Step D on page 169. BSA at 0.1 µg/mL and 1.0 µg/mL in PBS were centrifuged in pre-treated 30K Nanosep centrifugal devices. The retentate recovered and the recovery of BSA was determined in a suitable protein assay.

Salt Removal or Buffer Exchange by Diafiltration

Pall centrifugal UF devices are ideal for the removal or exchange of buffers and salts. Desalting by dialysis is time-consuming and does not concentrate dilute samples and results in further dilution of the original sample. A single round of protein concentration using UF results in a sample with essentially the same buffer or salt composition as the starting material. To remove salts or exchange buffers, the concentrated sample is diluted with the new buffer or water and centrifuged a second time (this process is called discontinuous diafiltration). The dilution/concentration steps can be repeated until the required amount of salt is removed or exchanged. A protocol is described in each device section to remove or exchange buffer of salts from a sample by diafiltration. The results for the removal of 0.5 M NaCl from a 5 mg/mL human serum albumin (HSA) solution are summarized in Table 2.47 for Nanosep® and Microsep™ centrifugal devices. The ionic strength of the sample retentate was monitored by conductivity measurement with a Horiba Model B173 miniature device. This device gave a linear conductivity response from 1.0 M down to 10-100 µm NaCl in high purity water. Protein was monitored by absorbance at 280 nm. The result showed a high recovery of protein and > 99.9% removal of NaCl after three cycles. Total time elapsed was 35 minutes for the Nanosep centrifugal devices and 90 minutes for the Microsep centrifugal devices. After one 10-30 minute spin, > 98% of the NaCl was removed in the filtrate for the two devices. Two more spins achieved complete desalting.

Table 2.47

Diafiltration to Remove NaCl from a BSA Solution in Nanosep and Microsep Centrifugal Devices Using a 10K MWCO Omega™ Membrane

Device	Conductivity of Retentate (mS/cm)				Protein Recovery
	Starting Sample (0.5 M NaCl)	Cycle #1 (% Desalting)	Cycle #2 (% Desalting)	Cycle #3 (% Desalting)	
Nanosep UF Device with a 10K MWCO Membrane	9.25 (100%)	0.150 (99.7%)	0.006 (> 99.9%)	0.004 (> 99.9%)	95.2%
Microsep UF Device with a 10K 9.25 MWCO Membrane	0.69 (100%)	0.048 (98.4%)	0.008 (99.8%)	(99.9%)	94.3%

A BSA sample (5 mg/mL) in 0.5 M NaCl was processed for diafiltration as described in the Protocol sections for Nanosep UF centrifugal devices (page 159) and Microsep UF centrifugal devices (page 160). After each cycle of concentration, 0.45 mL or 3.5 mL of high purity water (conductivity < 0.002 ms/cm) was added to the retentate and mixed with a pipette (5x up and down cycles). A sample (0.1 mL) was removed and measured for conductivity on the Horiba model B173 meter. After measurement, the sample was recovered and returned to the retentate cup of the Nanosep or Microsep UF centrifugal device. The retentate was then re-mixed with a pipette as described above. After the final cycle of diafiltration, 0.2 mL of water was added to the retentate, mixed with a pipette, and transferred to a microcentrifuge tube. An additional 0.2 mL of water was used to rinse the device with a pipette, then removed and added to the original retentate. The pooled volume was measured by aspiration into a 1 mL pipette and the protein content measured by absorbance at 280 nm.

Molecular Weight Fractionation of a Complex Solution

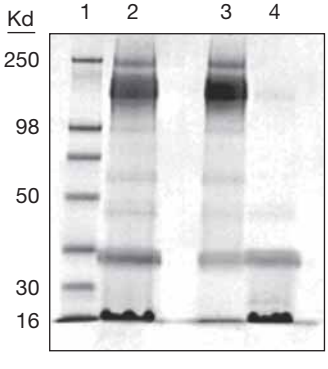
Although UF is primarily used in diafiltration or concentration applications, under some conditions it can be used for the fractionation of proteins that differ significantly in size. In order to fractionate or enrich for a particular protein, the following must be considered:

- The proteins must have at least 10-fold difference in MWt.
- The MWt. of the retained protein should be at least 3x the MWCO of the membrane.
- The protein in the filtrate should be at least 3x smaller than the MWCO of the membrane.
- The sample concentration should be 5 mg/mL or less.
- Centrifugation should be performed at lower than maximum recommended g-force (500-1000 x g) and will take longer than other applications for the Nanosep® UF centrifugal devices.

The separation will rarely be absolute due to the diversity of protein shape and the presence of protein:protein complexes in these native samples. The size-based separation achieved is better viewed as pre-enrichment. In some cases, very high molecular weight proteins such as IgM (970 kD) can be isolated successfully by UF processing.[1, 2] An example of the resolution of intact IgG (156 kD) from Cytochrome C (12.4kD) using a 100K MWCO Omega™ UF membrane is summarized in Figure 2.43. After two spins (not shown), over 95% of Cytochrome C was found in filtrate while more than 85% of the IgG was retained by the membrane.

Figure 2.43

Size Fractionation of a Mixture of IgG and Cytochrome C in Their Native State



A 0.5 mL sample of a 5.0 mg/mL protein solution containing IgG (156 kD) and Cytochrome C (12.4 kD) in PBS was centrifuged at 1,000 x g for 30 minutes in a Nanosep® 100K MWCO UF centrifugal device. The retentate was recovered in 0.5 mL; 0.015 mL samples of the retentate and filtrate were then analyzed on 10% NuPAGE Bis-Tris polyacrylamide gel.
Lane 1 = NOVEX SeeBlue Pre-Stained Protein Standards
Lane 2 = Mixture of IgG and Cytochrome C
Lane 3 = Retentate
Lane 4 = Filtrate

Nanosep UF Concentration as an Alternative to Acetone Precipitation in Sample Preparation for 2DE Gel Electrophoresis

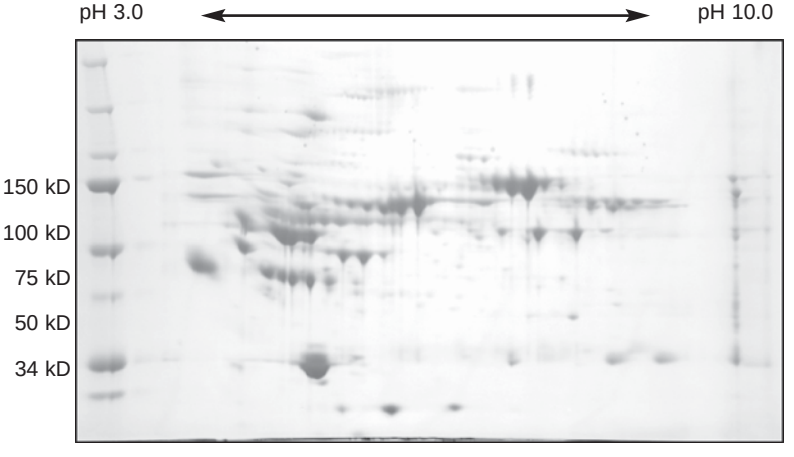
During sample preparation for depletion of abundant proteins (see Section 2.1, page 7), some dilution of the sample can occur. These dilute samples need to be re-concentrated prior to 2DE gel analysis. A common practice is to use acetone precipitation. A comparison of the reconcentration of flowthrough samples analyzed by 2DE that had been depleted of HSA and IgG using the Enchant™ Multi-Protein Affinity Separation Kit (see Section 2.1.4, page 33) is summarized in Figure 2.44 for Nanosep UF reconcentration (Panel A) and acetone precipitation (Panel B). The resulting 2DE stained gel patterns show little or no difference between the two reconcentration methods. The Nanosep UF method was achieved with a

single 30 minute spin. Acetone precipitation can take up to 60 minutes and involves a -20 ºC freezer, a microcentrifuge, and a Speed Vac or equivalent centrifugal evaporator to dry the final pellets to remove acetone.

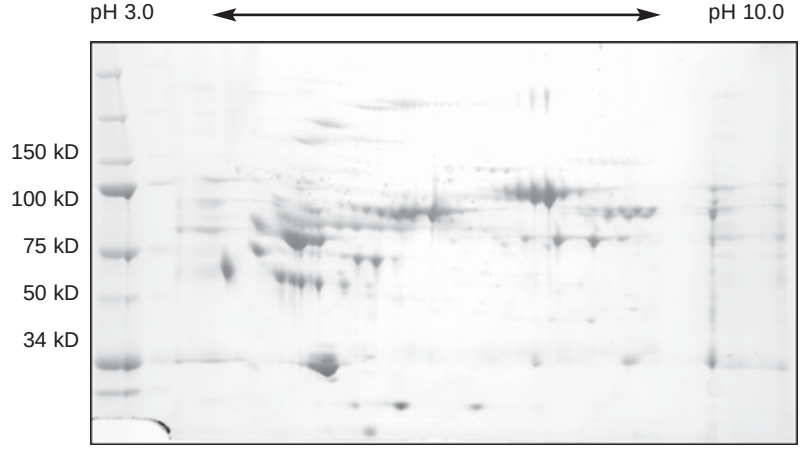
Figure 2.44

Sample Reconcentration for 2DE Gel Analysis – Nanosep® UF Centrifugal Device with a 10K MWCO Membrane vs. Acetone Precipitation

Panel A, Nanosep UF Centrifugal Device Reconcentration of HSA and IgG Depleted Human Plasma



Panel B, Acetone Precipitation of HSA and IgG Depleted Human Plasma



Filtrate sample containing approximately 210-230 µg total protein was concentrated by Nanosep UF or acetone precipitation. a) Add 3 volumes of cold acetone (-20 ºC) and mix, incubate at –20 ºC for 30 minutes. b) Pellet sample by centrifugation at 14,000 x g for 10 minutes at room temperature. c) Remove supernatant with a pipette and then dry the pellets in a Speed Vac or equivalent centrifugal evaporation system. The pellets are then resuspended in 2DE first dimension rehydration buffer prior to isoelectric focusing. Two dimensional gel electrophoresis (2DGE) analysis of human plasma following single and double depletion of HSA and IgG using the Enchant™ Multi-Protein Affinity Separation Kit. Samples were analyzed by focusing in the first dimension on a pH 3-10 non-linear IPG strip and resolving in the second dimension on a 4-12% Bis-Tris gel under reducing conditions. Proteins were visualized with colloidal Coomassie Blue.*

Troubleshooting for Concentration and Diafiltration of Samples (< 60 mL) in a Spin Filter

1. **Common variables that increase molecule passage**
   - Molecular shape at the same MWt. A molecule can exhibit a different hydrodynamic shape or Stokes radii in the linear or globular states.
   - High trans-membrane pressure created by too high a g-force in centrifugal concentrators. (This is especially important in the case of linear molecules, for example DNA fragments. Decreasing the g-force can increase retention of molecules by a membrane.)
   - Buffer composition that leads to dissociation of multi-sub-unit proteins or protein-protein complexes to yield individual sub-units.
   - pH and ionic conditions that induce conformational changes in a molecule leading to a small apparent hydrodynamic shape.
2. **Common variables that decrease molecule passage**
   - Buffer conditions that induce molecular aggregation.
   - Presence of other molecules that increase sample concentration.
   - Lower trans-membrane pressure (in the case of centrifugal concentrators, too low a g-force).
   - Non-specific adsorption to the membrane or device.
   - Low temperature (4 °C versus 24 °C). which can increase solution viscosity or lead to aggregation due to changes in solubility.

Ordering Information for Concentration and Diafiltration of Samples (< 60 mL) in a Spin Filter

Nanosep® Centrifugal Devices with Omega™ Membrane – Volumes < 1 mL

Part Number	Description	Pkg
OD003C33	3K, gray	24/pkg
OD003C34	3K, gray	100/pkg
OD003C35	3K, gray	500/pkg
OD010C33	10K, blue	24/pkg
OD010C34	10K, blue	100/pkg
OD010C35	10K, blue	500/pkg
OD030C33	30K, red	24/pkg
OD030C34	30K, red	100/pkg
OD030C35	30K, red	500/pkg
OD100C33	100K, clear	24/pkg
OD100C34	100K, clear	100/pkg
OD100C35	100K, clear	500/pkg
OD300C33	300K, orange	24/pkg
OD300C34	300K, orange	100/pkg
OD300C35	300K, orange	500/pkg

Microsep™ Centrifugal Devices with Omega™ Membrane – Volumes 1-3.5 mL

Part Number	Description	Pkg
OD001C41	1K, yellow	24/pkg
OD001C46	1K, yellow	100/pkg
OD003C41	3K, gray	24/pkg
OD003C46	3K, gray	100/pkg
OD010C41	10K, blue	24/pkg
OD010C46	10K, blue	100/pkg
OD030C41	30K, red	24/pkg
OD030C46	30K, red	100/pkg
OD050C41	50K, green	24/pkg
OD050C46	50K, green	100/pkg
OD100C41	100K, clear	24/pkg
OD100C46	100K, clear	100/pkg
OD300C41	300K, orange	24/pkg
OD300C46	300K, orange	100/pkg
OD990C41	1000K, purple	24/pkg
OD990C46	1000K, purple	100/pkg

Macrosep® Centrifugal Devices with Omega™ Membrane – Volumes 3.5-15 mL

Part Number	Description	Pkg
OD001C36	1K, yellow	6/pkg
OD001C37	1K, yellow	24/pkg
OD001C38	1K, yellow	100/pkg
OD003C36	3K, gray	6/pkg
OD003C37	3K, gray	24/pkg
OD003C38	3K, gray	100/pkg
OD010C36	10K, blue	6/pkg
OD010C37	10K, blue	24/pkg
OD010C38	10K, blue	100/pkg
OD030C36	30K, red	6/pkg
OD030C37	30K, red	24/pkg
OD030C38	30K, red	100/pkg
OD050C36	50K, green	6/pkg
OD050C37	50K, green	24/pkg
OD050C38	50K, green	100/pkg
OD100C36	100K, clear	6/pkg
OD100C37	100K, clear	24/pkg
OD100C38	100K, clear	100/pkg
OD300C36	300K, orange	6/pkg
OD300C37	300K, orange	24/pkg
OD300C38	300K, orange	100/pkg
OD990C36	1000K, purple	6/pkg
OD990C37	1000K, purple	24/pkg
OD990C38	1000K, purple	100/pkg

Jumbosep™ Centrifugal Devices–Volume 3.5-15 mL

Part Number	Description	Pkg
FD003K65	3K, starter kit, gray	4/pkg
OD003C65	3K, membrane insert, gray	12/pkg
FD010K65	10K, starter kit, blue	4/pkg
OD010C65	10K, membrane insert, blue	12/pkg
FD030K65	30K, starter kit, red	4/pkg
OD030C65	30K, membrane insert, red	12/pkg
FD100K65	100K, starter kit, clear	4/pkg
OD100C65	100K, membrane insert, clear	12/pkg
FD300K65	300K, starter kit, orange	4/pkg
OD300C65	300K, membrane insert, orange	12/pkg

References for Concentration and Diafiltration of Samples (< 60 mL) in a Spin Filter

1. Vollmers, H.P., Wozniak E., Stepien-Botsch, E., Zimmermann, U., Muller-Hermelink, H.K. (1996). A rapid method for purification of monoclonal human IgM from mass culture. *Hum Antibodies Hybridomas*, 7(1), 37-41.
2. Van Oss, C.J., & Bronson, P.M. (1970, August). Removal of IgM from serum by ultrafiltration. *Anal Biochem.*, 36(2), 464-469.
3. Laemmli, U.K. (1970). *Nature*, (227), 680–685.

2.4.2.2 Desalting and Buffer Exchange of Samples (< 1 mL) in an AcroPrep™ 96 and 384 Multi-Well Filter Plate

Biomolecule pre-analytical sample preparation may involve a complex series of steps where molecules are selectively separated using size or a variety of other biochemical properties. Many steps require desalting and buffer exchange as well as sample concentration to prepare the sample for the next step in analysis or purification. Desalting/buffer exchange can be a critical step in the process where yields and biological activity can be significantly reduced. It can be accomplished using methods such as gel filtration, size exclusion HPLC, dialysis, and ultrafiltration (UF). Pall Life Sciences has incorporated the UF membrane technology used in our spin filters into our AcroPrep filter plates. Although the primary basis for UF separation is molecular size, other factors such as molecular shape and charge also play a role. Molecules larger than the membrane pores are retained on the surface of the membrane while smaller molecules pass through the membrane into the filtrate. Highlights of AcroPrep 96 and 384 UF filter plates include:

- Omega™ membrane that is low in biomolecule binding and provides greater than 90% recoveries
- Polypropylene construction that is chemically resistant and biologically inert providing solvent resistance and low biomolecule binding
- Rigid, single-piece construction
- Conformity to SBS guidelines for automation
- Individually sealed membranes that prevent lateral flow and crosstalk
- Outlet tips and splash guards that ensure clean filtrate collection
- Proprietary plate design that minimizes solution/sample weeping
- Ability to simultaneously process up to 96 or 384 samples

Choosing the Correct MWCO

An important first step in the application of UF in sample processing is the selection of the appropriate MWCO membrane able to retain > 90% of protein of interest. In Table 2.48, the retention characteristics of Omega UF membranes for proteins is summarized for an AcroPrep 96 filter plate. For proteins it is best to select an MWCO that is 3x to 6x times smaller than the MWt. of the solute being retained. If flow rate is a consideration, choose a membrane with an MWCO at the lower end of this range (3x). If the main concern is retention, choose a tighter membrane (6x). The retention of a molecule by a UF membrane can be influenced by a number of factors, such as MWt., molecular shape, electrical charge, and the composition of the sample matrix. In choosing UF, it is important to consider performing pilot experiments to verify membrane performance for a particular pre-analytical sample preparation application in Proteomics. An application guideline is provided in Table 2.49 for selecting the right MWCO membrane for the intended study utilizing UF membranes.

Table 2.48
Typical Protein Recovery with Omega™ UF Membranes in AcroPrep™ 96 Filter Plate

Solute	Solute MW (kDa)	MWCO	10K	30K	100K
Vitamin B12 (200 µg/mL)	1.3	% Recovery (+/- SE)	4 ± 1%	5 ± 1%	7 ± 2%
Ovalbumin, Turkey (1 mg/mL)	45	% Recovery (+/- SE)	97 ± 2%	98 ± < 1%	48 ± 7%
IgG, Goat (1 mg/mL)	160	% Recovery (+/- SE)	98 ± 1%	98 ± < 1%	96 ± 1%
Apoferritin, Equine (1 mg/mL)	443	% Recovery (+/- SE)	96 ± 1%	89 ± 1%	93 ± < 1%

Test protein solution (0.3 mL) was added to wells of AcroPrep 96 filter plates with 10, 30 and 100K UF membranes. Each test plate was matched to a receiver plate and the assembly spun at 2,000 x g for 40 minutes. Following centrifugation, retentate samples were collected by adding 0.3 mL of buffer to each assay well, then allowing plate to stand at room temperature for 5 minutes before pipetting up and down 10 times to remove sample to fresh tubes. Protein concentration was determined using UV spectrophotometric analysis. Two independent experiments were performed, n = 18. Error values represent error of the mean.

Table 2.49
Application Guidelines on MWCO Selection

	MWCO UF Membrane		
Application	10K	30K	100K
Concentrate/Desalt Peptides, Proteins, Oligonucleotides, and Nucleic Acids	✓	✓ (PCR primers)	✓ (PCR primers)
Size Fractionation of Complex Mixtures	✓	✓	✓
Clean Up Labeling Reactions – Proteins and Nucleic Acids	✓		
Separate Proteins from SDS-PAGE Gels	✓	✓	
Buffer Exchange or Salt Removal of Chromatography Eluates, Gradient Fractions	✓		
Concentrating Dilute Samples to Enhance Sensitivity for Electrophoresis	✓		
Remove Protein for HPLC Analysis	✓	✓	
Recovery of Antibodies from Cell Culture			✓ (IgM)
Recovery of Low Molecular Weight Compounds from Fermentation Broth	✓	✓	
Free Drug Monitoring in Serum or Plasma	✓		
Natural Products Screening for Medicinal Chemistry	✓	✓	✓
Virus Concentration or Removal			✓

Protocol for Desalting and Buffer Exchange of Samples (< 1 mL) in an AcroPrep™ 96 and 384 Multi-Well Filter Plate

A. Materials Required

1. AcroPrep 96 or 384 filter plate with Omega™ MWCO UF membranes. For specifications, see Table 2.50 for 96-well (0.35 mL) and 384 (0.1 mL) UF plates.

Table 2.50
Specifications of the AcroPrep™ 96 and 384 UF Filter Plate

	Parameter	
Specification	96-well (0.35 mL)	384-well (0.1 mL)
Materials of Construction		
Membrane	Omega™ membrane (low protein-binding, modified polyethersulfone on polyethylene substrate)	
Device	Polypropylene	
Effective Membrane Area	0.25 cm^2	0.05 cm^2
Dimensions		
Length	12.78 cm (5.03 in.)	12.78 cm (5.03 in.)
Width	8.51 cm (3.35 in.)	8.51 cm (3.35 in.)
Height (with Lid)	1.7 cm (0.67 in.)	1.66 cm (0.655 in.)
Height (without Lid)	1.4 cm (0.6 in.)	1.44 cm (0.565 in.)
Tip Length	0.53 cm (0.21 in.)	0.41 cm (0.16 in.)
Capacities		
Maximum Well Volume	0.35 mL	0.1 mL
Recommended Volume	0.30 mL	0.08 mL
Hold-up Volume (Membrane/Support)	< 0.014 mL	< 0.004 mL
Maximum Centrifugal Force	3,000 x g	
Centrifuge	Swinging bucket rotors	
Operating Vacuum	25.4 cm Hg (10 in. Hg)	50.8 cm Hg (20 in. Hg)

2. Appropriate 96 or 384 polypropylene micro-well collection plate (conical well)
3. High purity water or buffer, such as phosphate buffered saline (PBS)
4. Source of vacuum (25.4-50.8 cm Hg (10-20 in. Hg)
5. Centrifuge fitted with a swinging bucket rotor with multi-well plate adapters and ability to spin up to 3,000 x g

B. Operation on a Vacuum Filtration Manifold

1. Optional conditioning, pre-wet membrane with water or buffer for maximal sample recovery.
2. Place an AcroPrep 96 or 384 filter plate of the desired MWCO onto a vacuum manifold. (If the filtrate is to be saved, place a solid-bottom receiver plate into the vacuum manifold.)
3. Add 0.02-0.12 mL of buffer/liquid (no solutes) into the wells of the AcroPrep 96 or 384 UF filter plate.
4. Apply vacuum until all the liquid has evacuated from the wells (see Table 2.51 for flow times).

Table 2.51
Vacuum Processing – Time to Evacuate Well (min: sec) on an AcroPrep™ 96 Filter Plate with a 10K UF Membrane (n = 16)

	Flow Time (+/- SD)	
Sample Volume (mL)	10 in. (25.4 cm) Hg	25 in. (63.5 cm) Hg
0.02	< 1:52 + 0:23	< 0:51 + 0:08
0.05	< 4:01 + 1:02	< 1:53 + 0:45
0.10	< 8:23 + 2:09	< 4:24 + 1:07
0.20	< 16:07 + 3:28	< 10:35 + 3:09
0.30	< 22:37 + 3:49	< 15:03 + 2:17

Indicated volumes of 500 mM NaCl solution were added to wells of each test plate in duplicate. Plates were evacuated with vacuum at 25.4 cm Hg (10 in. Hg).

5. After all fluid has evacuated from the well(s), discard receiver plate.
6. Place the AcroPrep 96 or 384 UF filter plate into vacuum manifold. (If the filtrate is to be saved, insert a new collection plate into the vacuum manifold.)
7. Add biological sample(s), up to 0.3 or 0.08 mL per well.
8. Apply vacuum [25.4 cm Hg (10 in. Hg) for 96-well or 50.8 cm (20 in. Hg) for 384-well plate].
9. When most or all of the liquid has emptied, turn off and release vacuum. Do not release vacuum by pulling the corner of the plate as this can degrade the manifold gasket. If desired, tap plates gently to remove hanging drops from the bottom of the filter plate.
10. To recover protein samples, add 0.02-0.3 mL of buffer to each well, pipette up and down several times without touching the UF membrane, then collect liquid. At this point, samples are generally > 90% desalted (see Table 2.52).

Table 2.52
Desalting of a Range of Solutes Using an AcroPrep™ 96 Filter Plate with a 10K MWCO Omega™ UF Membrane

	% Solute Removal (± SD)				
Sample	Initial	Wash #1	Wash #2	Wash #3	Total
200 mM NaCl	97.0 ± 0.2	2.0 ± 0.1	0.2 ± 0.1	ND	99.2 ± 0.1
500 mM NaCl	98.1 ± 0.3	1.2 ± 0.1	0.2 ± < 0.01	ND	99.5 ± < 0.4
3 M $(NH_4)_2SO_4$	97.9 ± 0.7	0.6 ± 0.04	0.1 ± 0.02	ND	98.6 ± 0.7
50 mM EDTA	94.0 ± 1.1	3.4 ± 0.5	0.2 ± 0.04	ND	97.6 ± 1.2
100 mM $MgCl_2$	96.1 ± 0.4	2.1 ± 0.1	0.08 ± 0.02	ND	98.3 ± 0.5
100 mM K Acetate	92.2 ± 0.3	1.8 ± 0.04	0.05 ± 0.01	ND	94.0 ± 0.3
500 mM K Acetate	96.9 ± 1.0	1.6 ± 0.04	0.11 ± 0.01	ND	98.6 ± 1.0

Test solution (0.3 mL) was added to wells of 10K plates. Test plates were matched with a receiver plate and spun at 2,000 x g for 40 minutes. Retained samples were washed by adding 0.3 mL dH_2O to each assay well followed by centrifugation for 25 minutes at 2,000 x g. The wash procedure was repeated for a total of three washes. Initial filtrate as well as filtrate from the three washes were collected and measured for salt concentration using a Horiba model B173 conductivity meter. The total salt removal was calculated by summing up the percentage of salt removal at each of the filtration steps. n = 5.

11. To achieve simple buffer exchange without a change in sample concentration, use the same or slightly less than the starting volume to recover samples.

12. To achieve sample concentration, add a smaller volume than the starting volume when recovering the sample from the membrane.

13. Optional diafiltration: to further decrease the salt concentration and increase the purity of the sample, repeat Steps B3-B9. (Usually two cycles of buffer exchange will remove > 95% of salts. However, with each added diafiltration step, there is an increased risk of sample loss. Therefore, quality/purity versus yield considerations must be made.)

C. Operation with a Centrifuge

1. Place an AcroPrep 96 or 384 filter plate of the desired MWCO over a solid-bottom receiver plate.

2. Add 0.02-0.12 mL of buffer/liquid (no solutes) into well of the UF filter plate.

3. Place filter and receiver plates together into a standard swinging bucket multi-well plate rotor assembly. Balance with an empty filter and receiver plate if running > 2 plates or balance the sample plates in pairs.

4. Centrifuge at 2,000 x g for 5 minutes. (For approximate filtration times at other centrifugal speeds, see Table 2.53.)

Table 2.53
Centrifugation Processing – Time to Evacuate Well (min: sec) on an AcroPrep™ 96 Filter Plate with a 10K UF Membrane (n = 16)

	Flow Time (+/- SD)			
Sample Volume (mL)	500 x g	1,000 x g	2,000 x g	3,000 x g
0.02	< 30:28 ± 7:52	< 7:40 ± 2:32	< 5:00	< 5:00
0.05	< 31:34 ± 5:36	< 9:32 ± 1:29	< 5:00	< 5:00
0.10	< 29:50 ± 6:01	< 10:00	< 5:00	< 5:00
0.20	< 30:10 ± 5:00	< 12:11 ± 2:31	< 5:00	< 5:00
0.30	< 36:05 ± 5:02	< 15:00	< 10:00	< 5:00

Indicated volumes of 500 mM NaCl solution were added to wells of each test plate in duplicate. Plates were matched with receiver plates and spun at 500 x g, 1,000 x g, 2,000 x g, or 3,000 x g for increments of 5 minutes until complete evacuation was achieved.

5. After all fluid has evacuated from the well(s), discard receiver plate.
6. Place the AcroPrep 96 or 384 filter plate of the desired MWCO over a solid-bottom receiver plate.
7. Add biological sample(s), up to 0.30 mL per well (96-well) or 0.08 mL (384-well).
8. Place filter and receiver plates together into a standard swinging bucket multi-well plate rotor assembly. Balance with an empty filter and receiver plate if running > 2 plates or balance the sample plates in pairs.
9. Centrifuge as in Step C4.
10. Follow Steps B10-B13 above for sample recovery.

Application Data for Desalting and Buffer Exchange of Samples (< 1 mL) in an AcroPrep 96 and 384 Multi-Well Filter Plate

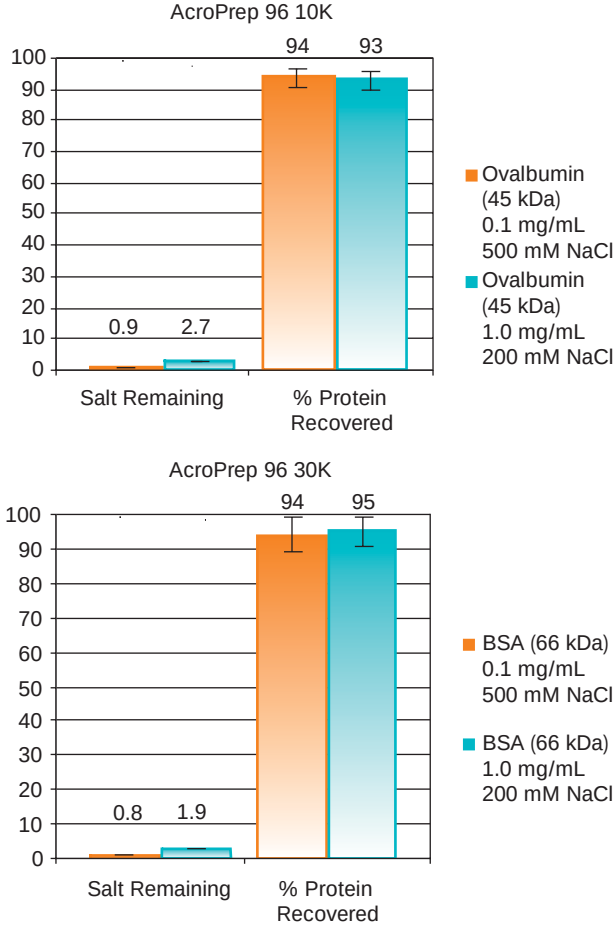
Desalting in an AcroPrep Well Format
UF is a reliable, gentle, and trusted technique for the removal of salts or exchange of buffer components. The AcroPrep 96 or 384 filter plate containing UF membranes provides an excellent platform for high-throughput purification applications. With just a single filtration and re-suspension cycle (no diafiltration), > 90% of the salt is removed (see Table 2.52). With two diafiltration steps added, the purity increases to > 95%.

Concentration in an AcroPrep Multi-Well Plate Format
In addition to desalting, ultrafiltration is also useful for the concentration of biomolecules. Throughout the process of using a UF membrane, the yields and recoveries remain high (see Figure 2.45) allowing the researcher to have confidence that the sample will not only be effectively concentrated and desalted, but not lost in the process. The efficiency of the AcroPrep 96 or 384 UF filter plates to remove salts and other small molecules is not at the expense of sample recovery. Figure 2.45 demonstrates that by using an AcroPrep 96 10K filter plate, recovery of Ovalbumin proteins (45 kDa) at two different concentrations, 0.1 and 1.0 mg/mL,

were greater than 90% with salt removal efficiencies of greater than 95%. Using BSA (66 kDa) and a 30K MWCO membrane, we observed similar protein recoveries and desalting efficiencies.

Figure 2.45

Desalting Ovalbumin and BSA Using an AcroPrep™ 96 Filter Plate with 10K and 30K MWCO UF Membranes



Indicated protein solutions (0.3 mL) were added to wells of 10K or 30K plates. Each test plate was matched to a receiver plate and the assembly spun at 2,000 x g for 40 minutes. Following centrifugation, retained proteins were collected by adding 300 µL of buffer to each assay well, then allowing plate to stand at room temperature for 5 minutes before pipetting up and down 10 times to remove sample to fresh tubes. Protein concentration was determined using UV spectrophotometer analysis (n = 3). Salt removal was determined using a conductivity meter.

Troubleshooting for Desalting and Buffer Exchange of Samples (< 1 mL) in an AcroPrep™ 96 and 384 Multi-Well Filter Plate

Common variables that increase molecule passage:

- Molecular shape, when at the same MWt. A molecule can exhibit a different hydrodynamic shape or Stokes radii in the linear or globular states.
- High trans-membrane pressure created by too high a centrifugal g-force in spin concentrators.
- Buffer composition that leads to dissociation of multi-sub-unit proteins or protein-protein complexes to yield individual sub-units.
- pH and ionic conditions that induce conformational changes in a molecule leading to a small apparent hydrodynamic shape.

Common variables that decrease molecule passage:

- Buffer conditions that induce molecular aggregation.
- Presence of other molecules that increase sample concentration.
- Lower trans-membrane pressure. (In the case of spin concentrators, too low a centrifugal g-force.)
- Non-specific adsorption to the membrane or device.
- Low temperature (4 °C versus 24 °C) which can increase solution viscosity or lead to aggregation due changes in solubility.

Ordering Information for Desalting and Buffer Exchange of Samples (< 1 mL) in an AcroPrep 96 and 384 Multi-Well Filter Plate

AcroPrep 96 Filter Plates, 350 µL Well

Part Number	Description	Pkg
5034	10K Omega™ membrane, natural	10/pkg
5035	30K Omega membrane, natural	10/pkg
5036	100K Omega membrane, natural	10/pkg

AcroPrep™ 384 Filter Plates, 100 µL Well

Part Number	Description	Pkg
5076	10K Omega™ membrane, long tips, natural	10/pkg
5077	10K Omega membrane, short tips, natural	10/pkg
5078	30K Omega membrane, long tips, natural	10/pkg
5079	30K Omega membrane, short tips, natural	10/pkg
5080	100K Omega membrane, long tips, natural	10/pkg
5081	100K Omega membrane, short tips, natural	10/pkg

2.4.3 Desalting with Size Exclusion Media

2.4.3.1 Ultrogel® AcA 202 and Trisacryl® GF-05M Resins

Desalting is an important step in processing samples that have been isolated in the presence of salts or detergents. Additionally, when a protein or nucleic acid is labeled for detection, desalting techniques are used to remove excess unincorporated label. Removal of small molecules from a sample can be achieved by dialysis, ultrafiltration, or gel filtration by using materials (membranes or resins) with appropriate molecular weight separation capabilities. Membrane-based methods (dialysis, ultrafiltration) use materials with specific pore sizes to limit the movement of larger molecules. In the later case, resins are chosen which have low molecular weight "cut-off" (MWCO) properties to exclude larger molecules from entering the resin particles.

Resin-based desalting is achieved by allowing the small molecules to freely enter the resin particles and be retarded in their flow through the packed bed column. The high molecular weight species in the sample are excluded and rapidly exit the column in the flowthrough and first fractions. This process can be carried out in several formats:

- Packed column run under gravity flow ("drip columns") or with a pump.
- A single spin column format where the resin bed is "dewatered" (removal of interstitial and some fluid around the resin particles) and partially rewetted with the sample.
- In a multi-well format, using a similar protocol to the above process.

The latter two processes are very quick and work well with small volumes. They also tend to have little or no sample dilution—one of the major disadvantages with packed column size exclusion chromatography. In addition, spin columns and multi-well plates are typically single use devices that save time and eliminate the possibility of cross contamination. When using these devices, ensure that the materials are chosen for low protein binding (e.g., GHP membrane in Pall devices) to reduce the possibility of target protein loss due to non-specific binding to the device materials. Properties of the BioSepra® desalting resins are highlighted in Table 2.54.

Table 2.54
Properties of Ultrogel® AcA 202 and Trisacryl® GF-05M Resins

	Parameter	
Specification	Ultrogel AcA 202 Resin	Trisacryl GF-05M Resin
Particle Size	60–140 µm	40–80 µm
Monomer	20% (w/v) acrylamide	N-acryloyl-2-amino-2-hydroxymethyl-1,3-propanediol
Cross-linker	2% (w/v) agarose	Hydroxylated acrylic bifunctional monomer
Exclusion Limit	22,000	3,000
Linear Fractionation Range	1,000–15,000	200–2,500
Resolving Power (plates/m)	3,000	2,500
Working pH Range	3–10	1–11

Tip: *Often spin devices containing ultrafiltration (UF) membranes are used for desalting applications. For more information on the use of spin devices with UF membranes for desalting, see Section 2.4.1, page 152.*

Protocol for Ultrogel AcA 202 and Trisacryl GF-05M Resins

A. Materials Required

1. Container/column for size exclusion resin, one of the following:
   - **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29924, for up to 10 mL volume)
   - **b.** Nanosep® MF centrifugal device with 0.45 µm GHP membrane (PN ODGHPC34)
   - **c.** AcroPrep™ filter plate, 1 mL well, 0.45 µm GHP membrane (PN 5054)
2. Collection plates [e.g., 96 well polypropylene V bottom, 0.5 mL (Axygen PN P96450V) or 1.64 mL round bottom, (Axygen PN PDW20)]
3. Separation apparatus (if filtration is used)
   - **a.** Source of vacuum [25.4 cm Hg (10 in. Hg), (Pall vacuum manifold, PN 5017)]
   - **b.** Centrifuge fitted with a swinging bucket rotor with multi-well plate adapters and able to spin up to 3,000 x g
4. Degassed 50% (v/v) slurry of the AcA 202 or GF-05M resins
5. Degassed water or a suitable buffer, such as phosphate buffered saline (PBS).

Tips on Handling Ultrogel® AcA 202 and Trisacryl® GF-05M Resins:
For packed columns, use only degassed liquids. For batch mode processing, degassing is not necessary.

Some BioSepra® media are supplied as concentrated slurries and may be difficult to resuspend. DO NOT use magnetic stir bars with BioSepra media as they can damage the beads. When adding slurry to any device, mix well between additions.

If it is necessary to prepare a 50% (v/v) slurry, use a clean spatula to remove some of the packed media and transfer to a graduated glass cylinder containing buffer. DO NOT add any media back to the storage bottle to avoid contamination of the bulk media. Thoroughly mix and allow settling. Note the volume of settled resin. Decant the supernatant and add back an equal volume of water or buffer to make 50% (v/v) slurry.

For packed columns, removal of fines may be necessary. Prepare the slurry in desired buffer, mix, and allow settling for approximately 5 minutes or enough time that the beads have settled but that small particles are still in solution. Decant off the suspension of fine particles and add fresh buffer and re-mix. Repeat the process until particles settle within approximately 5 minutes and leave a clear supernatant.

B. Packing Ultrogel AcA 202 and Trisacryl GF-05M Resins

1. Gravity flow column format

- **a.** To equilibrate column, bring the degassed 50% gel slurry, and degassed buffer solution (or high purity water) to room temperature.
- **b.** Secure a bottom cap on the column tip and clamp the column (10 mL bed volume column, e.g., Pierce PN 29924) upright in a laboratory stand.
- **c.** Add a sufficient volume of degassed buffer/water to the column to fill it up to the reservoir (wide-mouth) portion, and then gently tap the end and side of the column to dislodge any air bubbles.
- **d.** Float a porous disc on top of the liquid within the column.
- **e.** Using the reverse end of a Pasteur pipette or reverse end of a serum separator (e.g., Pierce PN 69710), push the disc evenly to the bottom of the column.
- **f.** Decant most of the liquid from the empty column, being sure to avoid getting air bubbles in the tip region of the column below the inserted disc. Place the column back in its stand with bottom cap still in place.
- **g.** Add sufficient volume of degassed gel slurry to obtain the desired settled gel volume of 10 mL (depending on the sample quantity).
- **h.** Allow gel to settle in the column for at least 5 minutes.
- **i**. Position a second porous disc on top of the settled gel bed by floating it on the liquid within the column and pushing it down to just above the settled gel. Leave 1-2 mm of space between the top of the gel bed and the top disc. Do not compress the gel bed.
- **j.** Wash the inside top part of the column with buffer/water to remove residual gel that may have remained along the sides during packing.
- **k.** Packed column is now ready for storage at 4 ºC or immediate use.
- **l.** Refer to Section C on page 192 for use instructions.

Tip: *Store the packed column upright at 4 °C with the gel bed submerged under 1-2 mL of buffer and a top column cap securely in place. Sodium azide added to the storage buffer to a concentration of 0.02% will help to prevent microbial growth. Always remove the top cap before the bottom cap to avoid drawing air bubbles down into the gel bed. Prevent air bubbles from forming in the gel bed by using only degassed buffer and sample solutions. Degassing involves subjecting a solution to vacuum to remove off excess dissolved air. Use of too high a vacuum can lead to evaporation of solvent from the solution. Check the final volume after degassing and, if necessary, add more solvent to return to original volume.*

2. Nanosep® spin filter column format

a. Remove the spin filter from the Nanosep collection tube (PN ODGHPC34) and place in a rubber stopper (with a suitable hole to form a tight fit to the spin filter) on the vacuum flask. Apply a low vacuum of 5-10 mm Hg.

b. Mix the 50% Ultrogel® AcA 202 or Trisacryl® GF-05M slurry (washed in bulk to remove the 20% ethanol preservative with high purity water) and quickly pipette 3x 0.4 mL of the slurry into the Nanosep device. Allow the packing to settle in between additions and use vacuum to drain liquid from the resin bed before adding more slurry.

c. After third addition, apply vacuum to remove the liquid from the resin bed which should fill the Nanosep device.

d. Replace the spin filter in the collection tube for storage.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 ºC for no more than one week.*

e. Refer to Section C on page 192 for use instructions.

3. AcroPrep™ multi-well filter plate format

a. Wash the Ultrogel AcA 202 or Trisacryl GF-05M media with 5 column volumes (CV) of water or buffer to remove the 20% ethanol storage buffer.

b. Place an 0.35 mL AcroPrep 96 filter plate (1 mL well volume) on a suitable vacuum manifold (PN 5017) with a collection plate underneath.

Tip: *A centrifuge can be substituted for vacuum as described in Section 2.4.2.2, page 179.*

c. Mix the 50% slurry and quickly pipette 0.4 mL to the multi-well filter plate. Rapidly follow with a second addition of slurry.

Tip: *In between each addition of the slurry, allow the resin bed to settle.*

d. After final addition, allow the vacuum to remove the liquid from the resin bed which should partially fill the well of the AcroPrep 96 filter plate.

e. Cover the multi-well plate for storage.

f. Refer to Section C on page 192 for use instructions.

Tip: *Because all the preservative has been removed from the resin in these devices, they should be used immediately or stored at 4 ºC for no more than one week.*

C. Desalting Applications

1. Gravity flow column format

- **a.** Prepare a 10 mL column.
- **b.** Wash the Ultrogel® AcA 202 or Trisacryl® GF-05M resin with 5 CV of water or buffer to remove the 20% ethanol storage buffer. Check the conductivity of the effluent with a suitable meter to ensure sufficient washing.
- **c.** Allow the liquid to drain from the column and load the sample up to a 1 mL volume onto the column.
- **d.** Collect the column effluent in 1 mL fractions. Measure absorbance at 280 nm to locate the protein peak.

Tip: *Protein rapidly elutes from the column and should be found in the first three fractions. Slight dilution of the sample will occur during elution. If necessary, the sample can be concentrated in a centrifugal UF spin filter, such as a Nanosep® or Microsep™ centrifugal device, with a 10K MWCO UF membrane (see Section 2.4.1, page 152).*

- **e.** After unretained protein has been eluted, discard the resin from the column.

2. Centrifugal spin filter column format

- **a.** Prepare the spin column.
- **b.** Centrifuge the spin column in a swinging bucket rotor at 1,000 x g for 2 minutes to remove excess fluid from the packed bed.
- **c.** Remove the filtrate from the collection tube.
- **d.** Very carefully pipette the sample (0.05-0.1 mL) onto the top of the dry packed bed in the Nanosep centrifugal device. Replace in a fresh collection tube.
- **e.** Centrifuge the spin column in a swinging bucket rotor at 1000 x g for 4 minutes to pass the sample through the resin bed.
- **f.** Filtrate in the collection tube will be desalted sample.

3. AcroPrep™ multi-well filter plate format

- **a.** Prepare the packed multi-well filter plate.
- **b.** Centrifuge the multi-well plate and collection plate in a suitable swinging bucket rotor at 1,000 x g for 2 minutes to remove excess fluid from the packed bed.
- **c.** Remove the filtrate from the collection tube.
- **d.** Very carefully pipette the sample (0.05-0.1 mL) onto the top of the dry packed bed in the Nanosep device.
- **e.** Centrifuge the multi-well plate and collection plate in a swinging bucket rotor at 1,000 x g for 4 minutes to pass the sample through the resin bed.
- **f.** Desalted samples are recovered from the collection plate.

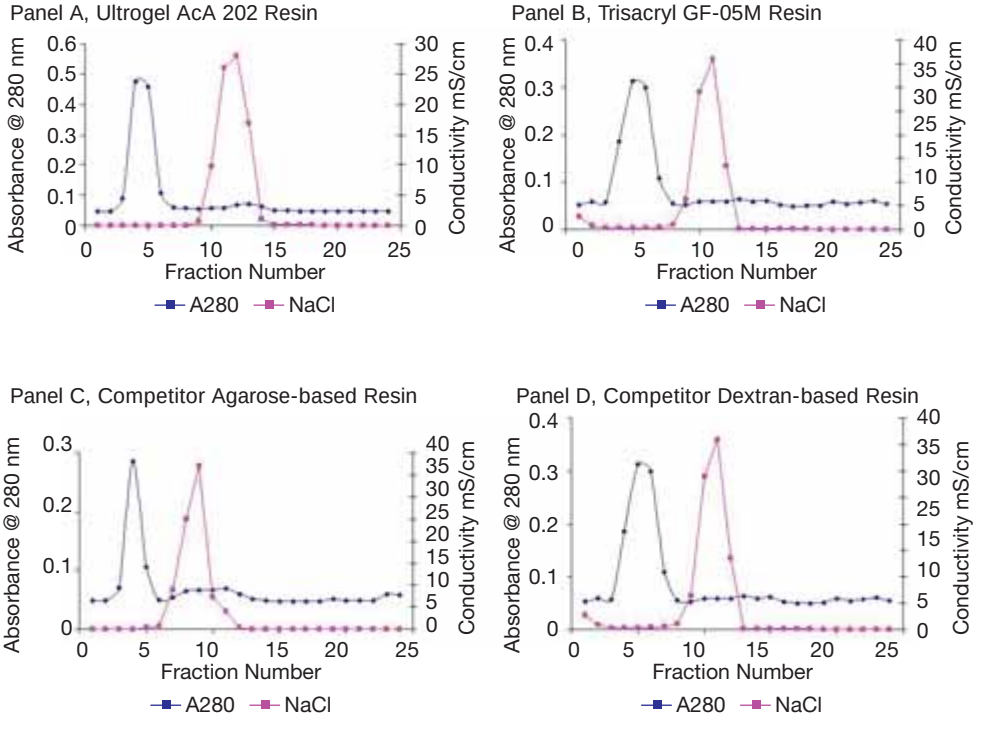
Application Data for Ultrogel® AcA 202 and Trisacryl® GF-05M Resins

Several examples of desalting experiments are represented in this section. The data highlights desalting using both Ultrogel and Trisacryl resin. In addition, results of various column formats are illustrated, i.e., gravity flow columns and spin filters.

Gravity Flow Column Desalting with Ultrogel AcA 202 and Trisacryl GF-05M Resin

Desalting in a gravity drip column format was evaluated with BioSepra® Trisacryl GF-05M and Ultrogel AcA 202 resins against competitive agarose and dextran-based pre-packed columns following directions in the Protocol section. The resulting desalting curves plotting protein (absorbance at 280 nm) and salt elution (m or µS/cm, measured using a Horiba micro-volume conductivity meter) from the above panel are summarized in Figure 2.46. The results all showed excellent separation between the excluded protein peak and the retained salt. Ultrogel AcA 202 gave the best separation between the void fraction containing the proteins of interest and the salt or included peak elution, suggesting that a higher volume of sample could have been loaded on this 10 mL column compared to other resins.

Figure 2.46



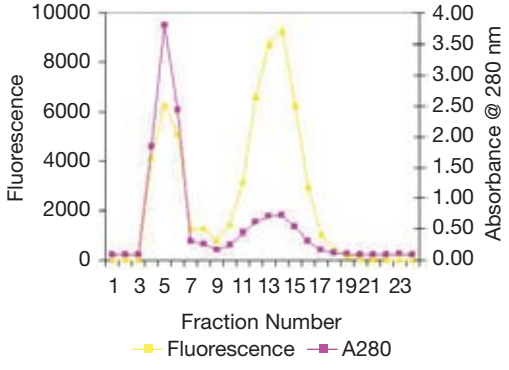
Gravity flow (10 mL) columns were poured with Ultrogel AcA 20 and Trisacryl GF-05M resin and two competitive agarose and Dextran-based media following the process outlined in the Protocol section. A 1 mL protein sample [5 mg/mL human serum albumin (HSA) in 1 M NaCl] was then applied and fractions of 1 mL collected after elution with high purity water. Absorbance was measured at 280 nm and conductivity measured using a Horiba micro-volume meter.

Removal of Excess Unreacted Fluorescein Isothiocyanate (FITC) from HSA by Trisacryl® GF-05M Resin in a Gravity Flow Column Format

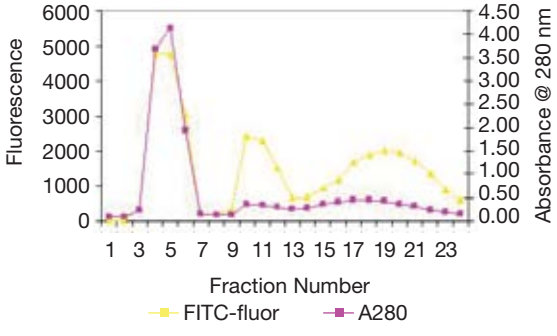
Desalting of albumin to remove free fluorescent label in a gravity flow format was investigated using Trisacryl GF-05M resin against competitive agarose and Dextran pre-packed columns. The sample in this case was HSA that had been reacted with an excess of FITC and contained excess unreacted reagent. Protein was measured by absorbance at 280 nm and FITC fluorescence at an excitation wavelength of 495 nm. The results are summarized in Figure 2.47. Trisacryl GF-05M resin showed the sharpest elution of both the FITC-HSA and the free FITC compared to the competitive products.

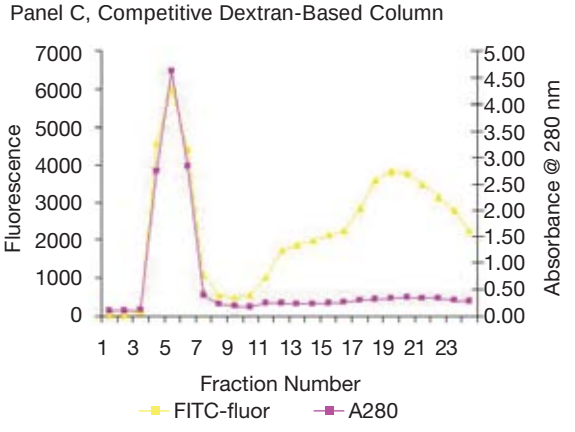
Figure 2.47

Panel A, Trisacryl GF-05M Packed Column



Panel B, Competitive Agarose Column





Gravity flow (10 mL) columns were poured with Ultrogel® AcA 202 and Trisacryl® GF-05M resin and two competitive agarose and Dextran-based media following the process outlined in the Protocol section. A 1 mL protein sample (5 mg/mL HSA in 25 mM Na Bicarbonate buffer pH 9.0 + 1 mg/mL FITC incubated at room temperature for 2 hours) was then applied and fractions of 1 mL collected after elution with high purity water. Absorbance was measured at 280 nm and fluorescence at an excitation of 405 nm.

Nanosep® GHP Spin Column Desalting

Desalting in a Nanosep spin filter column format was evaluated for an Ultrogel AcA 202 and Trisacryl GF-05M resin and a competitive agarose-based resin using the method described in the Protocol section. The results are summarized in Table 2.55. The results showed that Ultrogel AcA 202 resin could remove > 99.9% of the salt in the sample with 100% sample protein volume recovery and no sample dilution. The Trisacryl GF-05M data was not as good, but still better than the competitive agarose media regarded as a standard for this application.

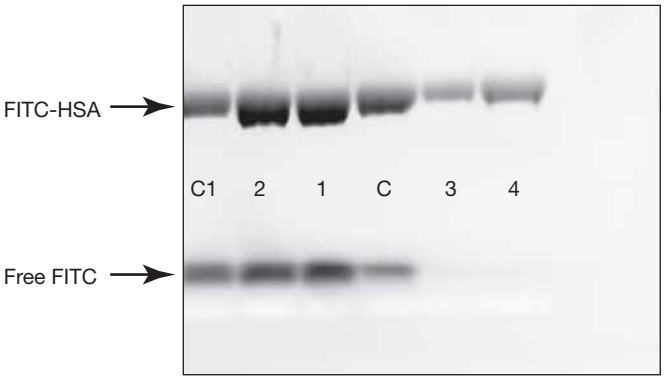
Table 2.55
Desalting Comparison

Resin	Volume Recovered (mL)*	Conductivity (mS/cm)**
Trisacryl GF-05M Resin	0.12	0.45
Ultrogel AcA 202 Resin	0.10	0.021
Competitive Agarose Resin	0.095	1.05

**Initial volume applied to the column is 0.1 mL.*
***< 0.05 mS/cm represents > 99.9% removal of NaCl from the sample.*

Nanosep® GHP Spin Filter Column Removal of Excess Unreacted FITC from a Protein Label Reaction
Removal of free unreacted FITC from an HSA label reaction using a Nanosep device was evaluated for Trisacryl® GF-05M resin and competitive agarose-based media following the Protocol section outline. The results are summarized in Figure 2.48 and show the location of free-FITC and HSA-FITC on an SDS-PAGE gel by illumination with long wavelength UV light. Free FITC ran very close to the dye front on these 12% SDS-PAGE gels. In the case of the Trisacryl GF-05M resin, lanes 3 and 4 had half the amount of material loaded than lanes 1 and 2. The result clearly shows that the Trisacryl GF-05M medium in the Nanosep GHP format device was able to retain all the free unreacted FITC, yielding a very clean FITC-HSA protein sample with no unreacted FITC in the gel image. The agarose competitive media showed a much higher background of free-FITC in the same format.

Figure 2.48
Fast, Efficient Removal of Unincorporated Label from Sample



Nanosep GHP spin columns were prepared with Trisacryl GF-05M resin and a competitive agarose-based media following the process outlined in the Protocol section. A 0.1 mL mL protein sample (5 mg/mL HSA in 25 mM Na Bicarbonate buffer pH 9.0 + 1 mg/mL FITC incubated at room temperature for 2 hours) was then applied and spun at 1,000 x g for 4 minutes. Samples of the filtrate were mixed with SDS sample buffer and run on 12% SDS-PAGE. As the dye front reached the bottom 2 cm of the gel, the run was stopped and the unstained gels imaged under UV illumination. Two sample volumes were tested, 0.02 and 0.1 mL, to get a sense of FITC binding capacity under spin column conditions. Lane C has unprocessed sample, Lanes 1 and 2 are filtrates from the 0.02 mL and 0.1 mL sample load applied to the competitive agarose spin columns. Lanes 3 and 4 are filtrates from the 0.02 and 0.1 mL sample load on the Trisacryl GF-05M spin columns. ***Note:*** *50% less filtrate was loaded on the SDS-PAGE gels in Lanes 3 and 4 as compared to Lanes 1 and 2.*

Ordering Information for Ultrogel® AcA 202 and Trisacryl® GF-05M Resins

Part Number	Description	Pkg
24892-022	Ultrogel AcA 202	100 mL
24892-010	Ultrogel AcA 202	1000 mL
25914-060	Trisacryl GF05 M	100 mL
25914-037	Trisacryl GF05 M	1000 mL

2.5.1 Introduction

Although a basic filtration concept, the clarification and prefiltration of samples remains an important function within protein sample preparation applications. When filtration is used as a prefilter, matching the proper filter media and device to the application is critical. Large pore size filter materials are used to filter solutions prior to more detailed analysis. When selecting the best product for your application, several factors need to be considered. Sample viscosity, sample volume, and sample recovery are just some of the aspects that will drive the selection of the optimal device.

Pall offers a number of media and device options for fast, effective filtration with minimal sample hold-up for both single and high throughput sample processing. From sample volumes of a few microliters to multiple liters, Pall can supply the best product solution for your application.

Figure 2.49
Choose the Best Clarification Device for Your Needs

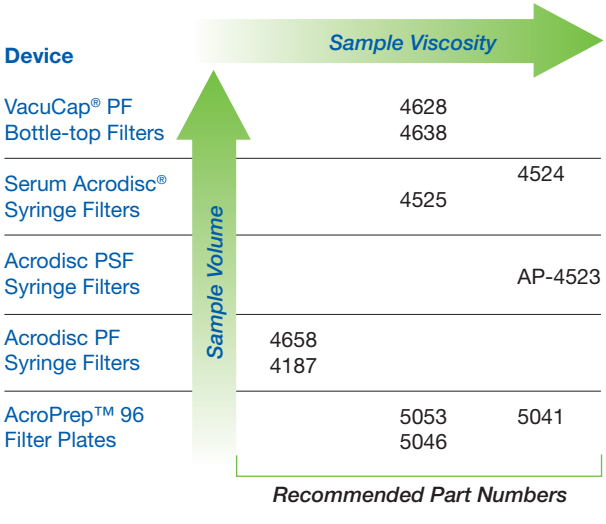
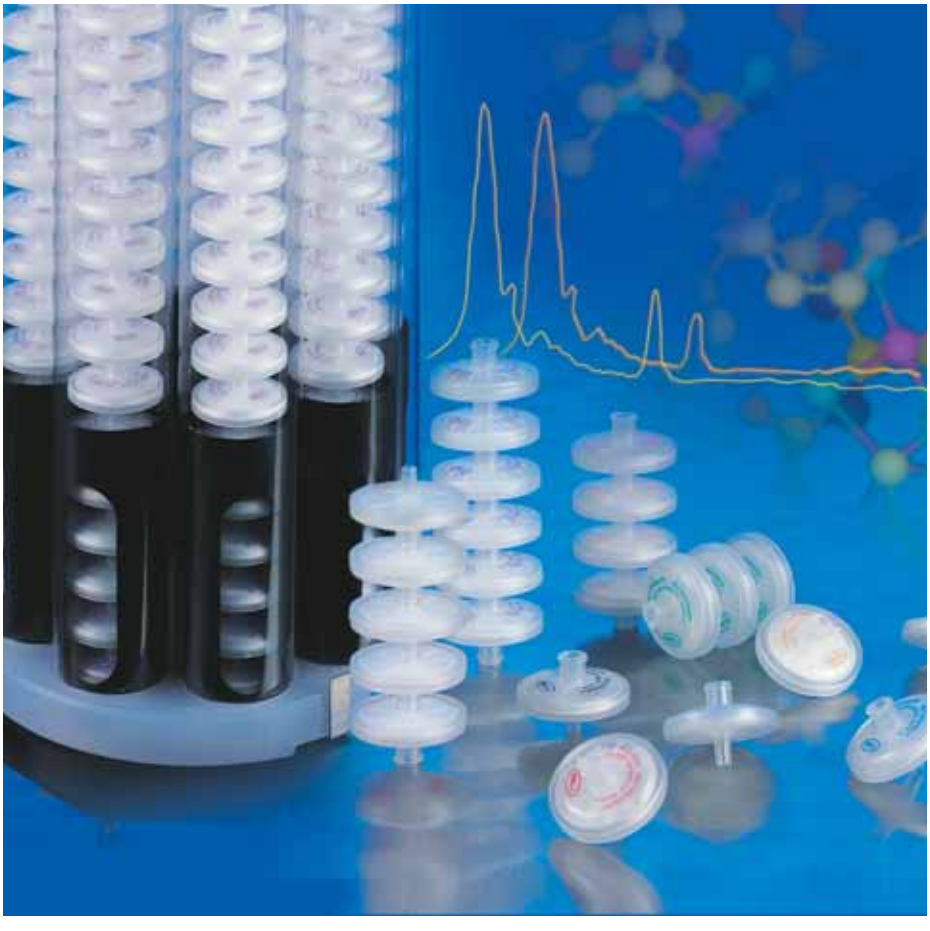


Figure 2.50
Acrodisc® Syringe Filters for Analytical Sample Preparation



2.5.2 AcroPrep™ 96 and 384 Multi-Well Filter Plate for 0.1-1.0 mL Samples

Uncharged microporous membrane filters remove particles from a fluid stream by the sieving or screening mechanism. Particles larger than the pore diameter do not pass through the filter. Such membranes initially show at least 2 to 3 log removal of particles equal to or larger than the rated pore size. There are two classic types of microfiltration (MF) processes:

- Depth filtration with matted fibers or materials compressed to form a matrix that retains particles by random adsorption or entrapment.
- Screen filters and microporous membranes with inherently uniform structures which, like a sieve, retain all particles larger than the precisely controlled pore size within their structure.

When fluid passes through the filter, particles larger than the spaces within the filter matrix are retained, accumulating primarily on the surface of the filter. The distinction between filters is important because the two classes serve very different functions. Depth filters are usually used as prefilters because they are an economical way to remove 98% of suspended solids and protect elements downstream from fouling or clogging. Screen or microporous filters remove 99.99% of suspended solids and may be used as either pre-filters or clarifying filters.

Small-volume multiple samples (0.1–1.0 mL) can be clarified in parallel by microfiltration in the AcroPrep 96 and 384 multi-well filter plates. These filter plates, made to SBS standards, are available in 0.2, 0.45, and 1.2 µm pore sizes and in depth filter/microporous membrane combinations employing 1.0 and 3.0 µm pore size glass fiber media or a 54 µm screen material. The membranes available are:

- GHP, a modified hydrophilic polypropylene membrane
- Hydrophilic, low protein binding Supor® PES, polyethersulfone membrane
- Bio-Inert®, a modified low protein binding nylon membrane
- PTFE hydrophobic membrane
- Glass fiber and glass fiber prefilter combinations

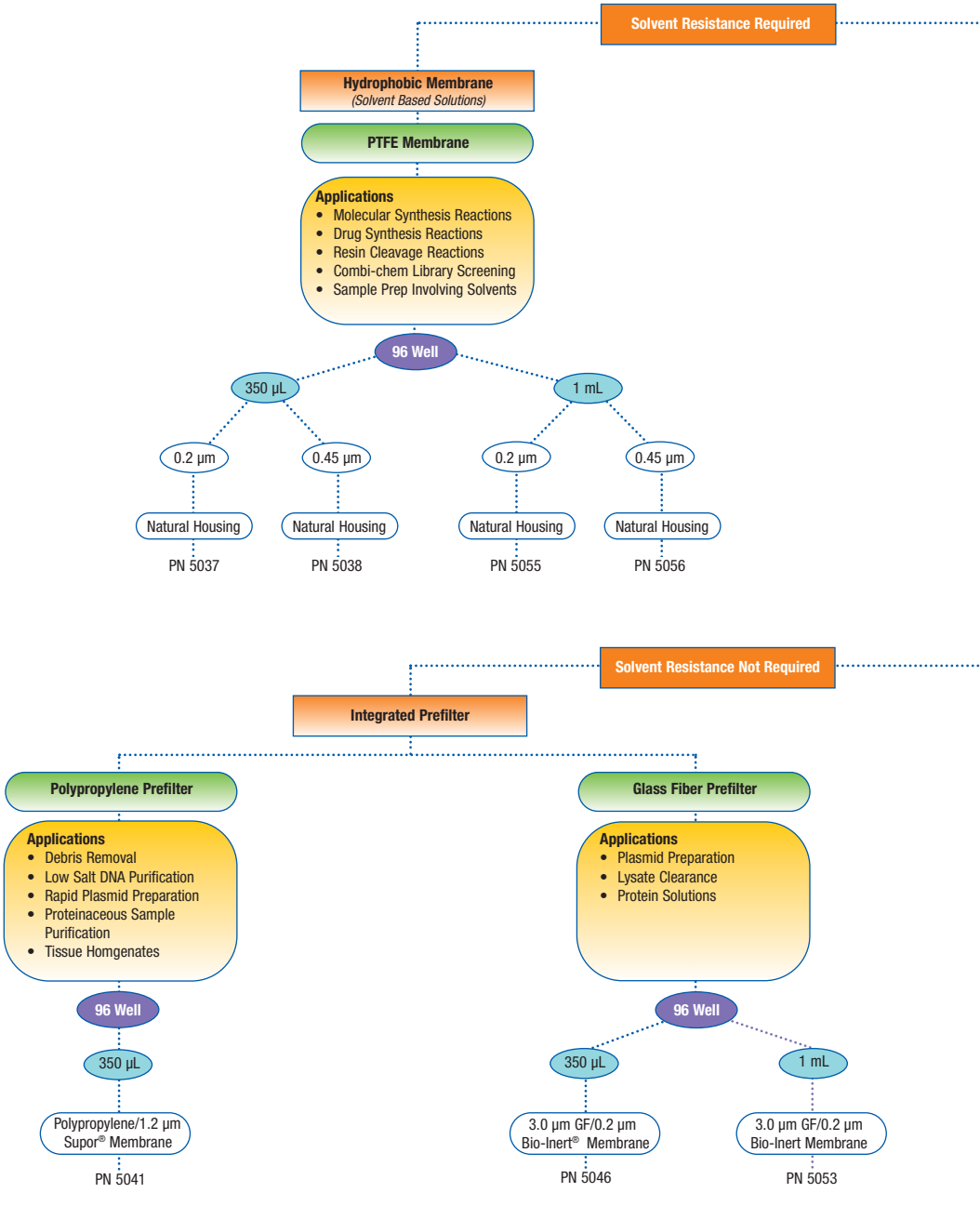
For viscous or heavy particle-containing samples, combination filters are available with integrated prefiltration membranes such as glass fiber prefilter over Bio-Inert membrane and polypropylene (PP) over Supor PES. The AcroPrep 96 and 384 multi-well filter plate properties are summarized in Table 2.56.

Table 2.56
Properties of the AcroPrep™ 96 and 384 MF Multi-Well Filter Plates

	Parameter		
Specification	96-well 350 µL Format	96-well 1 mL Format	384-well 100 µL Format
Materials of Construction			
Membrane	Bio-Inert® (modified low protein-binding Nylon) GHP (hydrophilic polypropylene) PTFE (hydrophobic PTFE) Glass fiber (borosilicate glass without binder) Supor	GHP (hydrophilic polypropylene) PTFE (hydrophobic PTFE) Glass fiber (borosilicate glass without binder) Supor®	
Device	Polypropylene	Polypropylene	
Effective Membrane Area	0.25 cm^2	0.05 cm^2	
Dimensions			
Length	12.78 cm (5.03 in.)	12.78 cm (5.03 in.)	12.78 cm (5.03 in.)
Width	8.51 cm (3.35 in.)	8.51 cm (3.35 in.)	8.51 cm (3.35 in.)
Height (with Lid)	1.66 cm (0.655 in.)	3.35 cm (1.32 in.	1.66 cm (0.655 in.)
Height (without Lid)	1.44 cm (0.565 in.)	2.87 cm (1.13 in.)	1.44 cm (0.565 in.)
Tip Length	0.53 cm (0.21 in.)	0.53 cm (0.21 in.)	0.41 cm (0.16 in.)
Capacities			
Maximum Well Volume	0.35 mL	1.0 mL	0.1 mL
Recommended Volume	0.30 mL	0.9 mL	0.08 mL
Hold-up Volume (Membrane/Support)	< 0.014 mL	< 0.018 mL	< 0.004 mL
Maximum Centrifugal Force	3,000 x g		
Centrifuge	Swinging bucket rotors		
Operating Vacuum	25.4 cm Hg (10 in. Hg)	50.8 cm Hg (20 in. Hg)	

Multi-well filter plates have a wide variety of applications and are key to some analytical and screening processes in biosciences. An application guideline for selection of membrane types is provided in Figure 2.51 and a listing of some applications arranged by membrane type is summarized in Table 2.57.

Figure 2.51
Filter Plate Quick Selection Guide



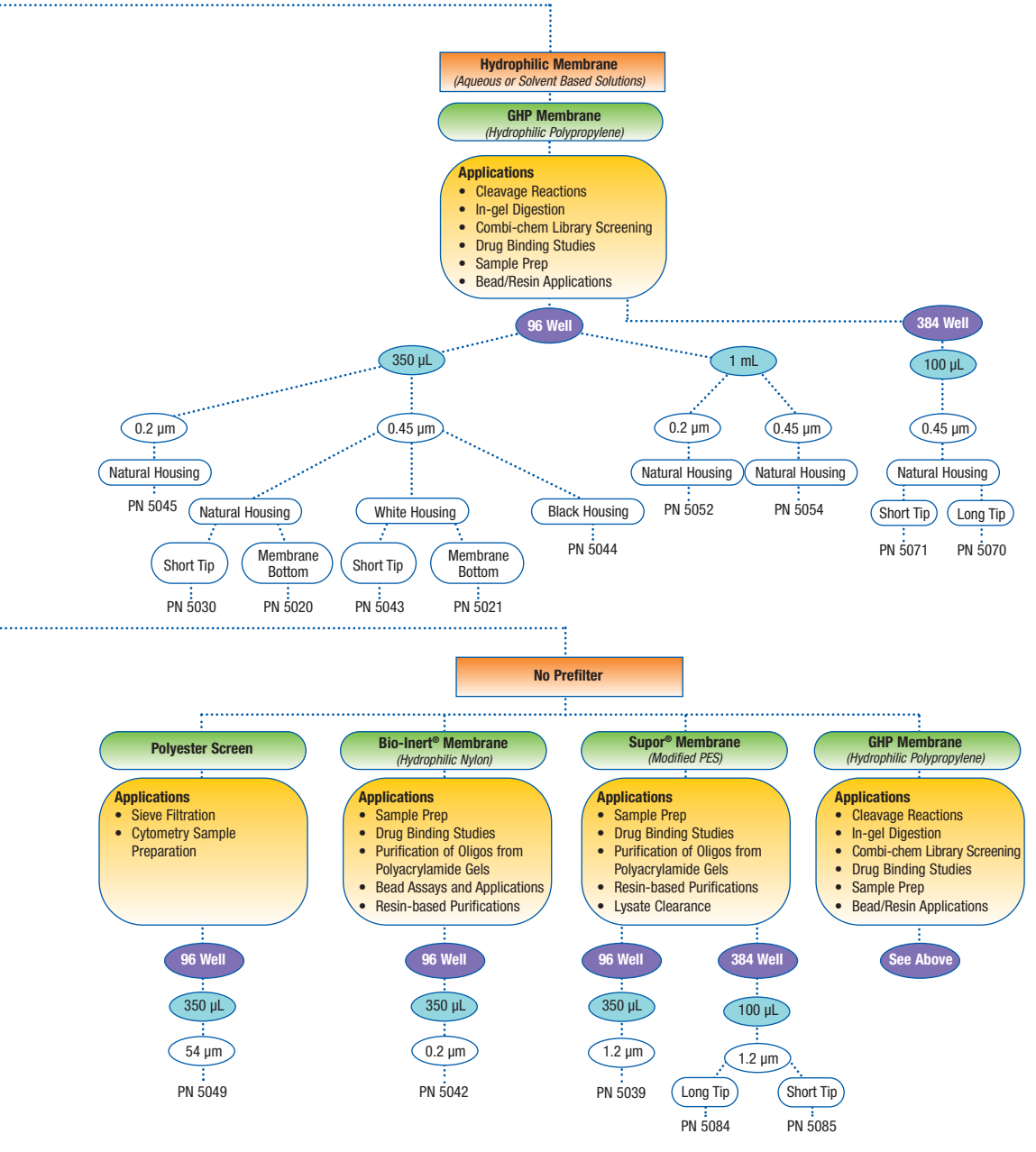

Hydrophilic Membrane
(Aqueous or Solvent Based Solutions)
GHP Membrane
(Hydrophilic Polypropylene)
Applications
• Cleavage Reactions
• In-gel Digestion
• Combi-chem Library Screening
• Drug Binding Studies
• Sample Prep
• Bead/Resin Applications
96 Well
384 Well
350 µL
1 mL
100 µL
0.2 µm
0.45 µm
0.2 µm
0.45 µm
0.45 µm
Natural Housing
Natural Housing
White Housing
Black Housing
Natural Housing
Natural Housing
Natural Housing
Short Tip
Membrane Bottom
Short Tip
Membrane Bottom
Short Tip
Long Tip
PN 5045
PN 5030
PN 5020
PN 5043
PN 5021
PN 5044
PN 5052
PN 5054
PN 5071
PN 5070
No Prefilter
Polyester Screen
Bio-Inert® Membrane
(Hydrophilic Nylon)
Supor® Membrane
(Modified PES)
GHP Membrane
(Hydrophilic Polypropylene)
Applications
• Sieve Filtration
• Cytometry Sample Preparation
Applications
• Sample Prep
• Drug Binding Studies
• Purification of Oligos from Polyacrylamide Gels
• Bead Assays and Applications
• Resin-based Purifications
Applications
• Sample Prep
• Drug Binding Studies
• Purification of Oligos from Polyacrylamide Gels
• Resin-based Purifications
• Lysate Clearance
Applications
• Cleavage Reactions
• In-gel Digestion
• Combi-chem Library Screening
• Drug Binding Studies
• Sample Prep
• Bead/Resin Applications
96 Well
96 Well
96 Well
384 Well
See Above
350 µL
350 µL
350 µL
100 µL
54 µm
0.2 µm
1.2 µm
1.2 µm
Long Tip
Short Tip
PN 5049
PN 5042
PN 5039
PN 5084
PN 5085

Table 2.57
Application Summary for AcroPrep™ Multi-Well MF Filtration Plates – Organized by Membrane Type

GHP Membrane	• Cleavage reactions for combinatorial chemistry library screens • Receptor-ligand screens • Cell- and bead-based receptor-ligand screens • HPLC sample prep and clarification • Metabolic studies • Micro-volume parallel filtration • Molecular synthesis reactions • Enzyme assays • In-gel digestion • Fluorescence assays • Protein scouting – mini columns • Processing of bead bound targets
Supor® Membrane	• Lysate clearance • Protein scouting – mini columns • Processing of bead bound targets • General filtration
PTFE Membrane	• Molecular synthesis reactions using harsh reagents • Drug synthesis reactions • Solubility studies • Cleavage reactions for combinatorial chemistry library screens • Bead-based applications
Glass Fiber Media	• Prefiltration of high solid and viscous samples • Lysate clarification • Radio labeled cell assays • Enzyme assays
Glass Fiber over Bio-Inert® Membrane	• Clarification of cell lysate and tissue homogenates • Preparation of proteinaceous solutions that may have a high solid content • Applications that require prefiltration due to high sample solid or viscosity
Polypropylene over Supor Membrane	• Fast lysate clearance at low salt conditions • Clarification of high particulate-laden solutions • Preparation of proteinaceous solutions that may have a high solid content
54 µm Polyester Screen	• Sample preparation for cell analysis/flow cytometry assays • Separation of cell, organisms and large particulates

Protocol for AcroPrep™ 96 and 384 Multi-Well Filter Plate for 0.1-1.0 mL Samples

A. Materials Required

1. AcroPrep 96 and 384 filter plate with MF membranes. For specifications, see Table 2.56.
2. Collection plates [(Axygen 96-well polypropylene V-bottom, 0.5 mL (PN P96450V); or round bottom, 1.64 mL (PN PDW20); or 384-well polypropylene, 0.24 mL (PN P384240SQ)]
3. High purity water or buffer, such as phosphate buffered saline (PBS)
4. Separation apparatus
   a. Source of vacuum [25.4–50.8 cm Hg (10–20 in. Hg) (Pall vacuum manifold PN 5017)]
   b. Centrifuge fitted with a swinging bucket rotor with multi-well plate adapters and ability to spin up to 3,000 x g

B. Vacuum Manifold Filtration

1. During use, hold the plate so that the outlets on the bottom of the plate are not touched to prevent liquid flow due to wicking.
2. Place a receiver plate into the vacuum manifold sized to collect the volume of the filter plate.
3. Place the filter plate on the vacuum manifold. Add 0.1 mL water or buffer.
4. Apply vacuum to manifold to initiate liquid flow. Recommended vacuum is 25.4 cm Hg (10 in. Hg) for 96-well plates.

Tip: *Do not exceed 38.1 cm Hg (10 in. Hg). Most house vacuum and aspirators do not exceed 38.1 cm Hg (15 in. Hg). For 384-well plates, vacuum up to 50.8 cm Hg (20 in. Hg) can be used.*

5. Release vacuum and discard the first filtrate.

Tip: *Do not release vacuum by pulling the corner of plate. The maifold gasket will ddegrade.*

6. Add the sample to be filtered to the plate. Incubate if required.
7. Gently tap the plate to remove any hanging droplets.
8. Release vacuum from the manifold and recover filtrate from the collection plate.
9. If needed, additional water or buffer solutions can be used. Collect wash filtrate separately and pool with first filtrate as needed.

Tip: *Some sample dilution may occur and the resulting filtrate may require reconcentration using an AcroPrep 96 or 384 filter plate with a 10K MWCO UF membrane.*

C. Filtration Facilitated by Centrifugation in a Swinging Bucket Rotor

1. Place the AcroPrep 96 or 384 filter plate on top of a receiver plate sized to collect the volume of the filter plate.
2. Insert the two plates into a standard multi-well filter plate swinging bucket rotor assembly.
3. Place a duplicate pair of plates matching the weight of the test plate (add water to the receiver plate and match weight of the test plate).

Tip: *An imbalance can result with a single test plate if no counterbalancing plate is used. If different volumes of sample are used in multiple plates, they will need to be balanced in pairs by addition of water to empty wells.*

4. Centrifuge at 500-3,000 x g for 1-2 minutes.

Tip: *The centrifugal force and time parameters can be varied to optimize the filtration rate of fluids in contact in the well of the plate.*

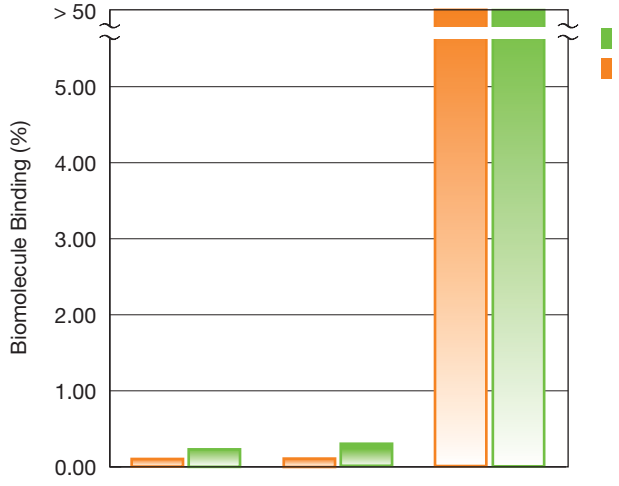
5. If needed, additional water or buffer solutions can be used. Collect wash filtrate separately and pool with first filtrate as needed.

Tip: *Some sample dilution may occur and the resulting filtrate may require reconcentration using an AcroPrep™ 96 or 384 filter plate with a 10K MWCO UF membrane.*

Application Data for AcroPrep 96 and 384 Multi-Well Filter Plate for 0.1-1.0 mL Samples

The modified polypropylene GHP membrane exhibits very low DNA and protein non-specific binding. An example of a comparison of macromolecule binding to GHP and a high binding nylon Biodyne® B membrane are summarized in Figure 2.52. The data clearly shows very low DNA or protein binding to the hydrophilic modified polypropylene membrane.

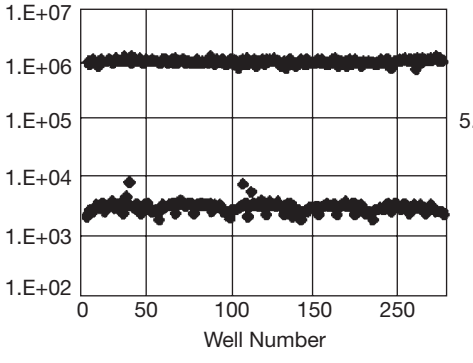
Figure 2.52
Hydrophilic Modified Polypropylene (GHP) Membrane Is Extremely Low in DNA and Protein Non-Specific Binding



^{125}I-labeled BSA (1.6 µg) or ^{32}P-labeled DNA (500 ng) were diluted to 5 mL in PBS (BSA) or Tris-EDTA buffer, pH 8.0 (DNA), and filtered through a 13 mm disc of the indicated membrane. Filtration was carried out using a 10 mL syringe at a flow rate of 1.0 mL/min. Binding was determined by comparing the amount of radioactivity remaining in the membrane (triplicate) to the activity of the starting material by placing the disc or solution directly into a scintillation cocktail and counting in a scintillation counter. Biodyne B membrane is designed for biomolecules binding and was used as a positive control.

The construction of the multi-well filter plate leads to reduced “crosstalk” between adjacent wells during processing of parallel samples. An example of an experiment designed to measure “crosstalk” is summarized in Figure 2.53. The results clearly show no observable “crosstalk” between wells on the plate.

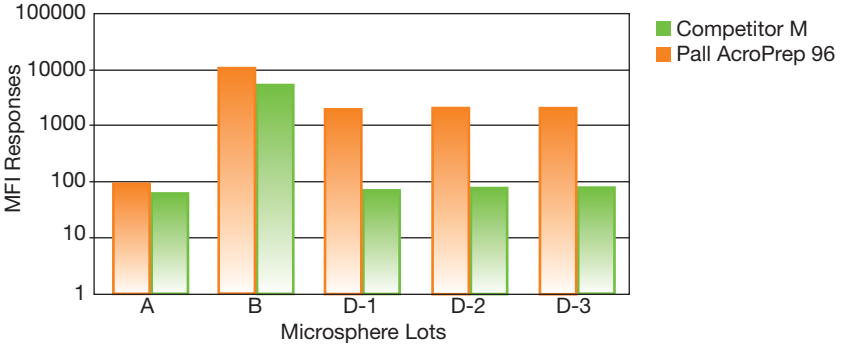
Figure 2.53
Observed Crosstalk in AcroPrep™ 96 Filter Plate



Fluorescein dye (0.200 mL of a 2 µg/mL in water stock) was added to wells of AcroPrep 96 filter plates with GHP membrane (5 plates total) in a checkerboard pattern. Alternate wells were filled with 0.200 mL of water. The fluid was evacuated from each filter plate using vacuum filtration at 30 cm Hg (12 in. Hg) for 15 seconds and the filter plate was read in a PerkinElmer, Wallac VICTOR 1420 Multilabel Counter. Wells filled with water that show a CPS reading above the dashed line (5.2 x 10^4 CPS, which is = "Average" Background + 5% Signal) in the graph constitute a crosstalk event. CPS = Counts Per Second.*

An example of the impact of an AcroPrep filter plate on a Thyroid Stimulating Hormone (TSH) immunoassay application was presented at the Planet xMAP Symposium in April 2005 in conjunction with Luminex Corp. This study showed that the Pall Life Sciences AcroPrep 96 filter plates with 1.2 µm Supor® membrane show a significant reduction in the presence of false positives compared to competitive filter plates when used with the Luminex xMAP technoloy for serological TSH immunoassays. An example of this reduced occurrence of false positives is reproduced in Figure 2.54. In all lots of microspheres tested, the AcroPrep filter plates exhibited a marked reduction in non-specific reactivity than competitive plates.

Figure 2.54
Reduced Occurrence of False Positives in Serological TSH Immunoassays with AcroPrep 96 Filter Plates



Utilizing multiple lots of xMAP microspheres (Luminex Corp) in both AcroPrep 96 filter plates (green bars) and Competitor M filter plates (orange bars), serological immunoassay results were read using a Luminex LX100 instrument. The responses represent the reactivity towards the microspheres without proteins coupled to them which in turn gives an indication of false positives or 'non-specific' reactivity by the microspheres. For all lots of microspheres tested, the AcroPrep 96 filter plates exhibited a reduction in non-specific reactivity when compared to the competitive filter plate.

METHOD: Pipette 10 µL of microspheres to each well of the filter plate. Add 20 µL of 1:20 diluted serum sample (in PBS-4% BSA). Incubate for 1 hour at room temperature. Apply vacuum to the filter plate and wash each well twice with 100 µL PBS-1% BSA. After washing, apply 50 µL of target conjugated antibody to IgG to each well and incubate for 1 hour. Using vacuum, wash each well twice with 100 µL of buffer per wash. After washing, add 100 µL of buffer to each well, mix gently, and insert the plate into the LX100 instrument for reading.

Ordering Information for AcroPrep™ 96 and 384 Multi-Well Filter Plate for 0.1-1.0 mL Samples

AcroPrep 96 Filter Plates, 350 µL Well – MF Filter < 1.0 µm Pore Size

Part Number	Description	Pkg
5045	0.2 µm GHP membrane, natural	10/pkg
5030	0.45 µm GHP membrane, natural	10/pkg
5043	0.45 µm GHP membrane, white,	10/pkg
5044	0.45 µm GHP membrane, black	10/pkg
5042	0.2 µm Bio-Inert® membrane, natural	10/pkg
5037	0.2 µm PTFE membrane, natural	10/pkg
5038	0.45 µm PTFE membrane, natural	10/pkg
5029	0.45 µm Supor® membrane, natural	10/pkg

AcroPrep 96 Filter Plates, 350 µL Well – MF and Screen Filters > 1.0 µm Pore Size

Part Number	Description	Pkg
5031	1.0 µm glass fiber, natural	10/pkg
5032	1.0 µm glass fiber, white	10/pkg
5039	1.2 µm Supor membrane, natural	10/pkg
5041	Prefilter material 1.2 µm Supor membrane, natural	10/pkg
5046	3.0 µm glass fiber media, 0.2 µm Bio-Inert membrane, natural	10/pkg
5049	54 µm screen, natural	10/pkg

AcroPrep™ 96 Filter Plates, 1 mL Well – MF Filter < 1.0 µm Pore Size

Part Number	Description	Pkg
5052	0.2 µm GHP membrane, natural	5/pkg
5054	0.45 µm GHP membrane, natural	5/pkg
5055	0.2 µm PTFE membrane, natural	5/pkg
5056	0.45 µm PTFE membrane, natural	5/pkg

AcroPrep 96 Filter Plates, 1 mL Well – MF and Screen Filters > 1.0 µm Pore Size

Part Number	Description	Pkg
5051	1.0 µm glass fiber media, natural	5/pkg
5053	3.0 µm glass fiber media/0.2 µm Bio-Inert® membrane, natural	5/pk

AcroPrep 384 Filter Plates, 100 µL Well – MF Filter < 1.0 µm Pore Size

Part Number	Description	Pkg
5070	0.45 µm GHP membrane, long tips, natural	10/pkg
5071	0.45 µm GHP membrane, short tips, natural	10/pkg
5084	1.2 µm Supor® membrane, long tips, natural	10/pkg
5085	1.2 µm Supor membrane, short tips, natural	10/pkg

AcroPrep 384 Filter Plates, 100 µL Well – MF and Screen Filters > 1.0 µm Pore Size

Part Number	Description	Pkg
5072	1.0 µm glass fiber media, long tips, natural	10/pkg
5072W	1.0 µm glass fiber media, long tips, white	10/pkg
5073	1.0 µm glass fiber media, short tips, natural	10/pkg
5073W	1.0 µm glass fiber media, short tips, white	10/pkg
5084	1.2 µm Supor membrane, long tips, natural	10/pkg
5085	1.2 µm Supor membrane, short tips, natural	10/pkg

2.5.3 Clarification of Samples (< 1 mL) in Spin Filters (Nanosep® Centrifugal Devices)

Uncharged microporous membrane filters remove particles from a fluid stream by the sieving or screening mechanism. Particles larger than the pore diameter do not pass through the filter. Such membranes initially show at least 2 to 3 log removal of particles which are equal to or larger than the rated pore size. There are two classic types of micro-filtration (MF) processes:

- Depth filtration with matted fibers or materials compressed to form a matrix that retains particles by random adsorption or entrapment.
- Screen filters and microporous membranes with inherently uniform structures which, like a sieve, retain all particles larger than the precisely controlled pore size within their structure.

When fluid passes through the filter, particles larger than the spaces within the filter matrix are retained, accumulating primarily on the surface of the filter. The distinction between filters is important because the two classes serve very different functions. Depth filters are usually used as prefilters because they are an economical way to remove 98% of suspended solids and protect elements downstream from fouling or clogging. Screen or microporous filters remove 99.99% of suspended solids and may be used as either pre-filters or clarifying filters.

Application Guidelines for Clarification of Samples (< 1 mL) in Spin Filters (Nanosep Centrifugal Devices)

Small-volume samples (< 1.0 mL) can be clarified by microporous surface filtration in the Nanosep MF centrifugal devices. MF filters are available in two pore sizes, 0.45 and 0.2 µm; and in two different membrane chemistries, Bio-Inert®, a modified low protein binding nylon surface; and GHP, a hydrophilic modified polypropylene surface. The centrifugal MF device family properties are summarized in Table 2.58. Typical applications of micro-volume MF clarification in proteomics include:

- Clarification of thawed plasma or serum that may contain cryoprecipitate material.
- Prefiltration of samples prior to injection into an HPLC system to remove fine colloidal material that could block a small particle diameter column.
- Prefiltration of samples prior to 1D and 2D electrophoresis to remove aggregated or insoluble material prior to analysis.
- Filter sterilization of components or additives for cell culture that are heat labile.

Table 2.58
Properties of the Nanosep® MF Centrifugal Devices

Property	Parameter
MF Filtration Membrane	Bio-Inert® is a modified nylon GHP is a hydrophilic polypropylene
Filtrate Receiver	Polypropylene
Effective Membrane Area	0.28 cm^2
Dimensions Overall Length (with Cap)	 4.5 cm (1.8 in.)
Capacities Maximum Sample Volume Final Retentate Volume Final Receiver Volume Hold-up Volume (Membrane/Support)	 0.5 mL 0.05 mL 0.5 mL < 0.005 mL
Operating Temperature Range	0–40 °C (32–104 °F)
pH Range	3–14
Maximum Centrifugal Force	14,000 x g
Centrifuge	1.5 mL microcentrifuge rotors
Sanitization	70% ethanol

Protocol for Clarification of Samples (< 1 mL) in Spin Filters (Nanosep® Centrifugal Devices)

A. Materials Required

1. Nanosep MF centrifugal devices with Bio-Inert® or GHP membranes with a collection tube. For specifications, see Table 2.58.
2. Extra collection tubes for the Nanosep MF device
3. Degassed high purity water or buffer, such as phosphate biffered saline (PBS)

B. Nanosep MF Protocol for Working with Samples < 1 mL

For MF filtration with 0.45 or 0.2 µm pore sizes, samples should not be too heavily loaded with suspended solids or be too highly viscous, as this will severely impact the MF filtration flux in these limited area filter devices. In many cases, the filter will plug and will only pass a very small volume of filtrate. If necessary, prefiltration with a depth filter or sample dilution may be required. For example, for clarification of thawed frozen plasma or serum prior to abundant protein depletion, samples should be diluted 1:4 in PBS. Depending on the amount of turbidity of the thawed sample, it may be necessary to prefilter with a GF or Serum Acrodisc® syringe filter before using the 0.45 or 0.2 µm centrifugal MF filters.

1. Select the Nanosep MF device with a 0.45 or 0.2 µm pore size.

Tip: *For most applications, 0.45 µm pore size filters are sufficient to remove particulate material prior to chromatography applications. If removal of microorganisms is required, then the 0.2 µm pore size filter is recommended.*

2. Add 0.5 mL of the sample and centrifuge at 14,000 x g for 5–10 minutes depending on the pore size membrane used.
3. If there is sample left in the retentate cup, mix and repeat Step B2.
4. If sample appears to be flowing through the filter very slowly, consider a pre-filtration step.
5. Transfer the filtrate into a clean tube. Confirm that the protein sample of interest has been recovered in the filtrate with acceptable recovery. If not, the sample may have become aggregated and retained on the MF filter.
6. Recover the sample from the Nanosep MF retentate cup by adding PBS and mixing as a first step. If the retained material is still not recovered, it may be necessary to add solubilization agents, such as detergents (CHAPS, Triton* X-100), chaotropic agents (urea or thiourea) or denaturants (guanidine HCl).

Ordering Information for Clarification of Samples (< 1 mL) in Spin Filters (Nanosep® Centrifugal Devices)

Nanosep MF Centrifugal Devices with Bio-Inert® Membrane

Part Number	Description	Pkg
ODM02C33	0.2 µm, aqua	24/pkg
ODM02C34	0.2 µm, aqua	100/pkg
ODM02C35	0.2 µm, aqua	500/pkg
ODM45C33	0.45 µm, wildberry	24/pkg
ODM45C34	0.45 µm, wildberry	100/pkg
ODM45C35	0.45 µm, wildberry	500/pkg

Nanosep MF Centrifugal Devices with GHP Membrane

Part Number	Description	Pkg
ODGHPC34	0.45 µm, clear	100/pkg
ODGHPC35	0.45 µm, clear	500/pkg

2.5.4 Clarification of Samples (1-100 mL) in an Acrodisc® Syringe Filter

Uncharged microporous membrane filters remove particles from a fluid stream by the sieving or screening mechanism. Particles larger than the pore diameter do not pass through the filter. Such membranes initially show at least 2 to 3 log removal of particles equal to or larger than the rated pore size. There are two classic types of microfiltration (MF) processes:

- Depth filtration with matted fibers or materials compressed to form a matrix that retains particles by random adsorption or entrapment.
- Screen filters and microporous membranes with inherently uniform structures which, like a sieve, retain all particles larger than the precisely controlled pore size within their structure.

When fluid passes through the filter, particles larger than the spaces within the filter matrix are retained, accumulating primarily on the surface of the filter. The distinction between filters is important because the two classes serve very different functions. Depth filters are usually used as prefilters because they are an economical way to remove 98% of suspended solids and protect elements downstream from fouling or clogging. Screen or microporous filters remove 99.99% of suspended solids and may be used as either pre-filters or clarifying filters.

Larger-volume samples (10-50 mL) can be clarified by MF in an Acrodisc syringe filter. MF filters are available in 0.1, 0.2, 0.45, 0.8, 1.2, and 5.0 µm pore sizes with:

- GHP hydrophilic modified polypropylene membrane.
- GxF glass fiber prefilter plus GHP combination filter to handle viscous or heavy particle containing samples (GHP plus GHP/GxF only available in 0.2 and 0.45 µm pore sizes).
- Hydrophilic low protein binding Supor® PES (polyethersulfone) membrane.

In the case of the Supor PES membranes, 0.8/0.2 µm and 1.2/0.45 µm membrane combinations are also available for small pore size filtration of higher viscosity samples, such as serum or plasma with cryoprecipitate present. The Acrodisc MF syringe filter properties are summarized in Table 2.59 for 13, 25, and 32 mm diameter devices.

Table 2.59
Properties of the Acrodisc® MF Syringe Filter

Specification	Parameter
Membranes	
GHP	GHP is a hydrophilic polypropylene
Supor® PES	Hydrophilic polyethersulfone (PES)
GxF Prefilter	Binder free borosilicate glass fiber
Device	Polypropylene
Effective Filtration Area	
13 mm	1.0 cm^2
25 mm	3.9 cm^2
32 mm	5.8 cm^2
Pore Size	
GHP	0.2, 0.45 µm
Supor PES	0.1, 0.2, 0.45, 0.8, 1.2, 5.0 µm
Sample Volume	
13 mm	< 10 mL
25 mm	< 50 mL
Hold-up Volumes	
13 mm	0.014-0.028 mL
25 mm	0.100-0.125 mL
25 mm plus GxF	0.100-0.200 mL
Maximum Temperature	55 °C
Maximum Pressure Limit	
13 mm	6.3 bar (630 kPa, 92 psi)
25 mm	4.1 bar (410 kPa, 60 psi)
32 mm	5.2 bar (520 kPa, 75 psi)
Typical Water Flow Rate (mL/min)	
13 mm (0.2 µm) at 10-45 psi	17-35 (PES)
13 mm (0.45 µm)	28-35 (PES)
25 mm (0.1 µm) at 30-45 psi	35 (PES)-175
25 mm (0.2 µm)	175 (PES)-195
25 mm (0.45 µm)	195-300 (PES)
25 mm (0.8 µm)	700
25 mm (1.2 µm)	1700
25 mm (0.8/0.2 µm)	145
32 mm (0.1 µm) at 45 psi	100
32 mm (0.2 µm)	490
32 mm (0.45 µm)	700
32 mm (1.2 µm)	1700
32 mm (5.0 µm)	1750
32 mm (0.8/0.2 µm)	440
Inlet/Outlet Connectors	
13 mm	Female inlet, luer-lok minispike outlet
25 mm	Female luer-lok inlet, male slip luer outlet
32 mm	Female luer-lok inlet, male slip luer outlet

Application Guidelines for Clarification of Samples (1-100 mL) in an Acrodisc® Syringe Filter

Syringe adapted filtration devices, such as Acrodisc syringe filters, have a wide variety of applications and are key to some analytical and screening processes in biosciences. Typical applications of Acrodisc MF syringe filter clarification in Proteomics include:

- Clarification of dilute (1:4) plasma or serum samples prior to abundant protein depletion to remove cryoprecipitates generated when thawing samples.
- Prefiltration of samples prior to injection into an HPLC system to remove fine colloidal material that could block a small particle diameter column.
- Filtration of buffers and solvents used in liquid chromatography and HPLC.
- Filter sterilization of components or additives for cell culture that are heat labile.
- Clarification of reagents and stains used in proteomics.

Protocol for Clarification of Samples (1-100 mL) in an Acrodisc Syringe Filter

A. Materials Required

1. Acrodisc syringe filter with MF membranes. For specifications, see Table 2.59.
2. Empty syringes (10-60 mL volume)
3. High purity water or buffer, such as phosphate buffered saline (PBS)

B. MF Acrodisc Filtration in Syringe Adapted Format

1. Before filling the syringe with sample, draw approximately 1 mL of air into the device. This will allow the air to follow the sample out of the syringe. This “air purge” minimizes fluid retention in the filter device.
2. Fill the syringe with the solution to be filtered.

Tip: *Use of syringes smaller than 10 mL can generate excessive pressure on the filter, which may exceed maximum operating pressure.*

3. Holding the filter device in one hand and the filled syringe in the other, secure (without excessive force) the filled syringe to the filter device with a twisting motion.
4. Apply gentle pressure to begin filtration. (A gentle pressure helps assure maximum throughput.)

Tip: *As the filter removes particulate, filtration will become more difficult (the syringe plunger will be harder to push), and pressure will rapidly increase on the filter. Change filters when resistance becomes excessive. Failure to change filter may result in housing rupture, which results in particulate contaminating the filtrate.*

Tip: *These filters are for SINGLE USE ONLY.*

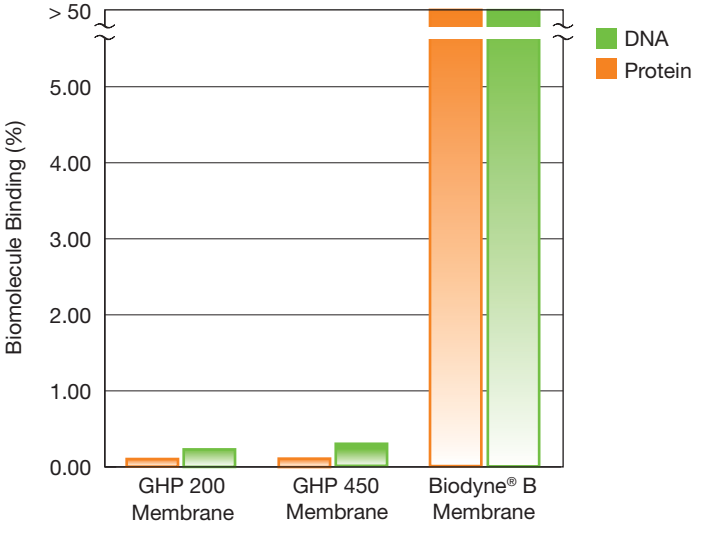
Application Data for Clarification of Samples (1-100 mL) in an Acrodisc Syringe Filter

The modified polypropylene GHP membrane exhibits very low DNA and protein non-specific binding. An example of a comparison of macromolecule binding to GHP and a nylon Biodyne® B membrane are summarized in Figure 2.55. The data clearly shows very low DNA or protein binding to the hydrophilic modified polypropylene membrane. Low protein

binding also leads to high recovery of protein in the dilute filtrates. An example of such a study with a Supor® 0.2 µm PES membrane is summarized in Figure 2.56, with a control of Posidyne® nylon membrane showing poor recovery.

Figure 2.55

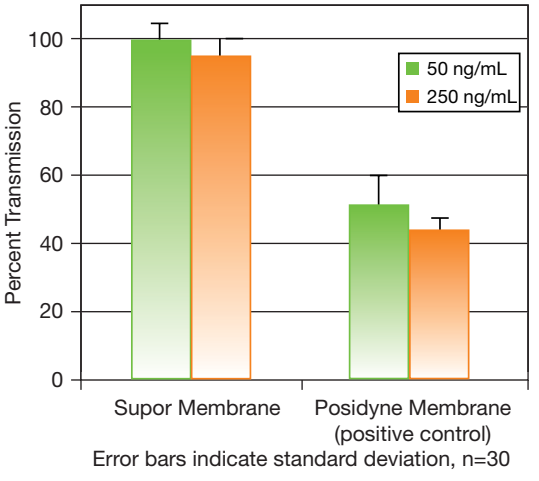
Hydrophilic Modified Polypropylene (GHP) Membrane Is Extremely Low in DNA and Protein Non-Specific Binding



^{125}I-labeled BSA (1.6 µg) or ^{32}P-labeled DNA (500 ng) were diluted to 5 mL in PBS (BSA) or Tris-EDTA buffer pH 8.0 (DNA) and filtered through a 13 mm disc of the indicated membrane. Filtration was carried out using a 10 mL syringe at a flow rate of 1.0 mL/min. Binding was determined by comparing the amount of radioactivity remaining in the membrane (triplicate) to the activity of the starting material by placing the disc or solution directly into a scintillation cocktail and counting in a scintillation counter. Biodyne® B membrane is designed for biomolecules binding and was used as a positive control.

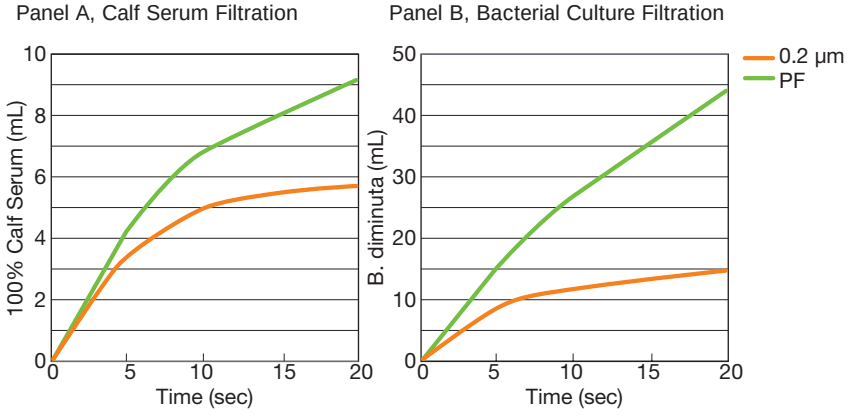
Figure 2.56

Protein Passage Through Supor® PES vs. Posidyne® Nylon Filter Membranes



The addition of a 0.8 µm PES prefilter to a 0.2 µm PES sterilization membrane considerably improves throughput with viscous, particulate-laden or high protein content solutions, such as serum or plasma. The PES 0.2 µm membrane shows high throughput for filtration of high viscosity samples, such as Dextran, Trypticase Soy Broth, and BSA. When a prefilter is also present, further improvement in filtration throughput can be seen. A comparison of filtration of bovine serum or a bacterial culture with a 0.8/0.2 µm Supor® PES Acrodisc® syringe filter is summarized in Figure 2.57. The prefilter considerably extends the filter life of these samples beyond 10-20 seconds when the 0.2 µm PES filter reaches a plateau of throughput.

Figure 2.57
Increased Filtration Throughput with a 0.8/0.2 µm Supor PES Acrodisc Syringe Filter Compared to a Single 0.2 µm Membrane



*Acrodisc 0.2 µm and Acrodisc PF 0.8/0.2 µm Supor syringe filters were challenged with Panel A, calf serum or Panel B, a bacterial culture (*B. diminuta *at 10^7 cfu/mL) at a constant pressure of 1.4 bar (140 kPa, 20 psi).*

Ordering Information for Clarification of Samples (1-100 mL) in an Acrodisc® Syringe Filter

Acrodisc Syringe Filters with GHP Membrane, 13 mm

Part Number	Description	Pkg
4554	0.2 µm, minispike outlet	100/pkg, 300/cs
4567	0.2 µm, minispike outlet	1000/pkg
4556	0.45 µm, minispike outlet	100/pkg, 300/cs
4563	0.45 µm, minispike outlet	1000/pkg

Acrodisc PSF Syringe Filters with GHP Membrane, 25 mm

Part Number	Description	Pkg
AP-4564	0.2 µm	50/pkg, 200/cs
AP-4566	0.2 µm	1000/pkg
AP-4560	0.45 µm	50/pkg, 200/cs
AP-4562	0.45 µm	1000/pkg

Acrodisc PSF GxF Syringe Filters with GHP Membrane, 25 mm

Part Number	Description	Pkg
AP-4307	GxF/0.2 µm	50/pkg, 200/cs
AP-4306	GxF/0.2 µm	1000/pkg
AP-4559	GxF/0.45 µm	50/pkg, 200/cs
AP-4558	GxF/0.45 µm	1000/pkg

Acrodisc® Syringe Filters with Supor® Membrane, Non-sterile

Part Number	Description	Pkg
4506	0.2 µm, 25 mm, modified acrylic housing	1000/pkg
4655	0.2 µm, 32 mm, modified acrylic housing	1000/pkg
4508	0.45 µm, 25 mm, modified acrylic housing	1000/pkg
4653	0.45 µm, 32 mm, modified acrylic housing	1000/pkg
4509	0.8 µm, 25 mm, modified acrylic housing	1000/pkg
4504	0.8/0.2 µm, 25 mm, modified acrylic housing	1000/pkg
4659	0.8/0.2 µm, 32 mm, modified acrylic housing	1000/pkg
4660	1.2 µm, 32 mm, modified acrylic housing	1000/pkg
4661	1.2/0.45 µm, 32 mm, modified acrylic housing	1000/pkg
4662	5 µm, 32 mm, modified acrylic housing	1000/pkg

2.5.5 Filtration of Reagents (> 50 mL) with a VacuCap® Vacuum Filtration Device

Uncharged microporous membrane filters remove particles from a fluid stream by the sieving or screening mechanism. Particles larger than the pore diameter do not pass through the filter. Such membranes initially show at least 2 to 3 log removal of particles which are equal to or larger than the rated pore size. There are two classic types of microfiltration (MF) processes:

- Depth filtration with matted fibers or materials compressed to form a matrix that retains particles by random adsorption or entrapment.
- Screen filters and microporous membranes with inherently uniform structures which, like a sieve, retain all particles larger than the precisely controlled pore size within their structure.

When fluid passes through the filter, particles larger than the spaces within the filter matrix are retained, accumulating primarily on the surface of the filter. The distinction between filters is important because the two classes serve very different functions. Depth filters are usually used as prefilters because they are an economical way to remove 98% of suspended solids and protect elements downstream from fouling or clogging. Screen or microporous filters remove 99.99% of suspended solids and may be used as either prefilters or clarifying filters.

Larger volume (> 50 mL) reagents used in analysis can be clarified by microfiltration in the VacuCap and VacuCap PF vacuum filtration devices. These devices employ Supor® hydrophilic polyethersulfone 0.1, 0.2, and 0.45 µm pore size membranes which offer high flow rates and throughputs, and are ideal for solutions where low protein binding is required. In addition, PF devices with 0.8/0.2 µm pore size combination membranes offer high filtration throughput in sample streams with high numbers of particles. The VacuCap and VacuCap PF device family properties are summarized in Table 2.60 for 60 and 90 mm diameter devices.

Figure 2.58
VacuCap Filtration Devices

Table 2.60
Properties of the VacuCap® and VacuCap PF Vacuum Filtration Devices

Specification	Parameter
Membranes	
Supor® PES	Hydrophilic polyethersulfone
Device	
Membrane Support	Polyester
Housing	Modified acrylic
Inlet Tubing	Medical grade PVC
Sinker on Inlet Tubing	Glass-filled polyurethane elastomer
Sealing Gasket	Silicone rubber
Effective Filtration Area	
60 mm	30 cm^2
90 mm	60 cm^2
Pore Size	0.1, 0.2, 0.45, 0.8/0.2 µm
Sample Volume	
60 mm	0.5-1.0 L
90 mm	1.0-5.0 L
Hold-up Volumes	3.4 mL
Maximum Temperature	55 °C
Maximum Vacuum	63.5 cm Hg (25 in. Hg)
Typical Water Flow Rate (mL/min) at 25.4 cm Hg (10 in. Hg)	
60 mm (0.1 µm)	50
60 mm (0.2 µm)	200
60 mm (0.45 µm)	280
60 mm (0.8/0.2 µm)	200
90 mm (0.1 µm)	100
90 mm (0.2 µm)	400
90 mm (0.45 µm)	560
90 mm (0.8/0.2 µm)	400
Collection Vessel	
60 mm	Can be used on receptacles with openings ranging from 60 mm, 2-5 cm (0.8-1.9 in.) and 90 mm, 2-6.5 cm
90 mm	(0.8-2.5 in.)

Application Guidelines for Filtration of Reagents (> 50 mL) with a VacuCap® Vacuum Filtration Device

Vacuum filtration devices have a wide variety of applications and are key to some analytical and screening processes in biosciences. Typical applications of VacuCap MF clarification in proteomics includes:

- Filter sterilization of components or additives for cell culture that are heat labile.
- Clarification of reagents and stains used in proteomics.

Tip: *All bulk solid reagents contain a variable percent of their content as in-soluble solids.*

- Stains and detection reagents used in electrophoresis and Western blotting to remove in-soluble solids.

Tip: *Many of these reagents are colored or somewhat opaque and it is not possible to see in-soluble material in suspension or as precipitates in their containers. This can lead to non-specific background staining or detection.*

Protocol for Filtration of Reagents (> 50 mL) with a VacuCap Vacuum Filtration Device

A. Materials Required

1. VacuCap filtration devices with MF membranes. For specifications, see Table 2.60.
2. Vacuum source [max 63.5 cm Hg (25 in. Hg)].

B. VacuCap MF Filtration of > 50 mL Samples

1. Remove VacuCap device from the sterile packing and place with gasket seated on the rim of the receiving bottle.
2. Connect the feed tubing to the port marked "INLET" on the top of the device. Place the opposite end of the feed tubing in the vessel from which the unfiltered sample will be drawn.

Tip: *Very viscous solutions, such as serum-containing culture media, may prematurely clog the VacuCap device. If the device does clog before the entire batch can be filtered, try the VacuCap PF (with 0.8 µm PES prefilter) device.*

3. Connect the vacuum tubing to the port marked "VACUUM" on the top of the device. Refer to product insert for safety precautions.
4. While holding the device securely on the receiving bottle, start the vacuum. The device will seal securely to the receiving bottle and fluid will be drawn.
5. When filtration is complete, switch off the vacuum pump and allow the vacuum inside the receiving bottle to dissipate.

Tip: *These filtration devices are for SINGLE USE ONLY.*

Application Data for Filtration of Reagents (> 50 mL) with a VacuCap® Vacuum Filtration Device

Media preparation for cell culture applications can be a tedious process, depending on the volume and viscosity of the material to be filtered. The sterile VacuCap vacuum filtration device was engineered for this purpose. PF devices, designed with a built-in prefilter, use the ingenuity of the standard VacuCap device and increase its throughput performance with serum-containing media or other viscous solutions. Other applications requiring 0.2 µm filtration will also see the throughput benefits of VacuCap PF devices when encountering high particle load in a solution. A summary of vacuum filtration of tissue culture media and calf serum is presented in Table 2.61 for the VacuCap 90 mm device with the 0.2 µm and 0.8/0.2 µm PES membranes. The data clearly shows rapid filtration of high viscosity fluids, such as tissue culture media with the PES membranes, in this bottle-top filtration format. This is a useful configuration for a wide range of large volume liquid samples used in analysis.

Table 2.61
VacuCap 90 PF Device Filtration Efficiency[1]

	Filtrate Volume (mL)			
Sample	1.5 min.	3.0 min.	5.0 min.	6.0 min.
Tissue Culture Fluid (RPMI + 10% calf serum)				
0.2 µm Supor® PES	950	2200	ND	3600
0.8/0.2 µm Supor PES	950	2200	ND	3600
Whole Calf Serum				
0.2 µm Supor PES	ND	ND	160	ND
0.8/0.2 µm Supor PES	ND	ND	320	ND

Filtration conducted at 21 °C (70 °F) with 50 cm Hg (20 in. Hg) vacuum for a 5 minute filtration time. Actual results may vary depending upon type and concentration of serum, liquid temperature, and applied vacuum.

Ordering Information for Filtration of Reagents (> 50 mL) with a VacuCap® Vacuum Filtration Device

VacuCap 60 Devices, Supor® Membrane

Part Number	Description	Pkg
4631	0.1 µm, 60 mm, sterile	10/pkg
4632	0.2 µm, 60 mm, sterile	10/pkg
4634	0.45 µm, 60 mm, sterile	10/pkg
TA4632*	0.2 µm, 60 mm, sterile	10/pkg
4638	PF 0.8/0.2 µm, 60 mm, sterile	10/pkg

VacuCap 90 Devices, Supor® Membrane

Part Number	Description	Pkg
4621	0.1 µm, 90 mm, sterile	10/pkg
4622	0.2 µm, 90 mm, sterile	10/pkg
4624	0.45 µm, 90 mm, sterile	10/pkg
TA4622*	0.2 µm, 90 mm, sterile	10/pkg
4628	PF 0.8/0.2 µm, 90 mm, sterile	10/pkg

**"TA" products are supplied with individually attached tubing for each filter device. Standard products are supplied with one piece of tubing per 10 filter devices.*

2.5.6 Clarification of Plasma or Serum Samples to Remove Cryoprecipitate Using Acrodisc® Syringe Filters with a Prefilter

Uncharged microporous membrane filters remove particles from a fluid stream by the sieving or screening mechanism. Particles larger than the pore diameter do not pass through the filter. Such membranes initially show at least 2 to 3 log removal of particles that are equal to or larger than the rated pore size. When filtering viscous or heavy particle laden fluids, the small pore size filters rapidly clog and their throughput is very low. Depth filters (matted fibers or materials compressed to form a matrix that retains particles by random adsorption or entrapment) can be used as prefilters to remove 98% of suspended solids and protect small pore size surface filters downstream from fouling or clogging.

The Serum Acrodisc syringe filter was developed for syringe filtration of serum or plasma. The filter contains a glass fiber prefilter upstream of a 0.2 µm Supor® PES filter in a 37 mm diameter Acrodisc syringe filter. The properties of this device are summarized in Table 2.62.

Table 2.62
Properties of the Serum Acrodisc MF Syringe Filter, 32 mm Device

Specification	Parameter
Materials of Construction Membrane Supor PES Prefilter	 Hydrophilic polyethersulfone (PES) Binder free borosilicate glass fiber
Device	ABS plastic
Effective Filtration Area	5.8 cm^2
PES Membrane Pore Size Glass Fiber Prefilter Pore Size	0.2 µm 1 µm (nominal) binder-free borosilicate glass
Hold-up Volume	< 0.1 mL
Maximum Temperature	55 °C
Maximum Pressure Limit	5.2 bar (520 kPa, 75 psi)
Typical Water Flow Rate [mL/min at 3.1 bar (310 kPa, 45 psi)]	425
Inlet/Outlet Connectors	Female luer-lok inlet, male slip luer outlet
Endotoxin Test	< 0.25 EU/mL using Limulus Amoebocyte Lysate (LAL) test
Sterilization	Sterilized by gamma irradiation and individually packaged

Protocol for Clarification of Plasma or Serum Samples to Remove Cryoprecipitate Using Acrodisc® Syringe Filters with a Prefilter

A. Materials Required

1. Serum Acrodisc syringe filter (PN 4525) with GF plus MF membranes. For specifications, see Table 2.62.
2. Empty syringes (10-30 mL volume)

B. Serum Acrodisc Filtration in Syringe Adapted Format

1. Before filling the syringe with sample, draw approximately 1 mL of air into the syringe. This will allow the air to follow the sample out of the syringe. This "air purge" minimizes fluid retention within the filter device.
2. Fill the syringe with the solution to be filtered.

Tip: *Use of syringes smaller than 10 mL can generate excessive pressure on the filter, which may exceed maximum operating pressure.*

3. Holding the filter device in one hand and the filled syringe in the other, secure (without excessive force) the filled syringe to the filter device with a twisting motion.
4. Apply gentle pressure to begin filtration. A gentle pressure helps assure maximum throughput.

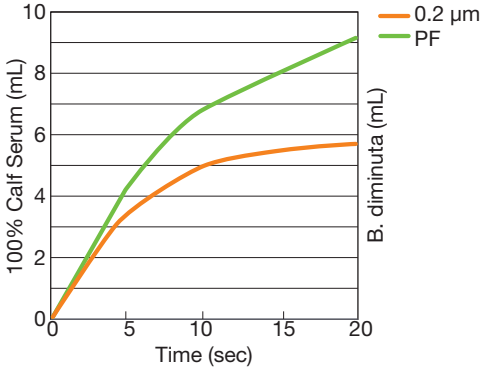
Tip: *As the filter removes particulate, filtration will become more difficult. The syringe plunger will be harder to push. Pressure will rapidly increase on the filter. Change filters when resistance becomes excessive. Failure to change filter may result in housing rupture, which results in particulate contaminating the filtrate.*

These filters are for SINGLE USE ONLY.

Application Data for Clarification of Plasma or Serum Samples to Remove Cryoprecipitate Using Acrodisc Syringe Filters with a Prefilter

The PES 0.2 µm membrane shows high throughput for filtration of high viscosity samples, such as Dextran, Trypticase Soy Broth, and BSA. The addition of a 0.8 µm PES prefilter to a 0.2 µm PES sterilization membrane considerably improves throughput with viscous, particulate-laden or high protein content solutions, such as serum or plasma. A comparison of filtration of bovine serum or a bacterial culture with a 0.8/0.2 µm Supor® PES membrane Acrodisc filter is summarized in Figure 2.59. The prefilter considerably extends the filtration of these samples beyond 10-20 seconds when the 0.2 µm PES filter reached a plateau of throughput. When a glass fiber prefilter is used, improvement in filtration throughput occurs.

Figure 2.59
Increased Serum Filtration Throughput with a 0.8/0.2 µm Supor® PES Filter Compared to a Single 0.2 µm Membrane



Acrodisc® 0.2 µm (PN 4612) and Acrodisc PF 0.8/0.2 µm (PN 4187) syringe filters with Supor membrane were challenged with calf serum at a constant pressure of 1.4 bar (140 kPa, 20 psi).

Ordering Information for Clarification of Plasma or Serum Samples to Remove Cryoprecipitate Using Acrodisc Syringe Filters with a Prefilter

Acrodisc Syringe Filters, Supor Membrane

Part Number	Description	Pkg
4525	Serum Acrodisc syringe filter, GF/0.2 µm, 37 mm, sterile	20/pkg
4187	Acrodisc PF syringe filter, 0.8/0.2 µm, 25 mm, sterile	50/pkg
4658	Acrodisc PF syringe filter, 0.8/0.2 µm, 32 mm, sterile	50/pkg

2.5.7 Prefiltration of Plasma or Serum Samples Using Acrodisc® Syringe Filters with Glass Fiber

Plasma or serum when stored frozen can yield variable amounts of aggregated material on thawing. This aggregated material, if not removed, will rapidly block a microfiltration (MF) filter. As an alternative to centrifugation, this material can be removed by prefiltration using glass fiber. This process is usually used in conjunction with Serum Acrodisc MF final filtration or Acrodisc PF series 0.8/0.2 and 1.2/0.45 µm combination Supor® PES membrane filtration. It is important to employ a final MF filtration step to trap any glass microfibers that may be shed from the Acrodisc prefilter media.

The Glass Acrodisc syringe prefilter is available with single layer glass fiber or with a multi-layered glass fiber prefilter over glass fiber media. The GxF multi-layered prefilter provides two to four times the throughput of standard glass fiber prefilter devices, allowing for quick and easy filtration of your most difficult-to-filter samples, reducing clogging in subsequent MF filtration steps. It is used alone or in series with a final membrane filter to increase flow rate and throughput. For extremely heavy particulate loads, a larger 37 mm size is available.

Protocol for Prefiltration of Plasma or Serum Samples Using Acrodisc Syringe Filters with Glass Fiber

A. Materials Required

1. Acrodisc PSF GxF syringe filter (PN AP-4523). For specifications, see Table 2.63.
2. Empty syringes (10-30 mL volume)

Table 2.63
Properties of the Acrodisc PSF GxF Syringe Filter, 25 mm

Specification	Parameter
Membrane Prefilter	Binder free borosilicate glass fiber
Device	Polypropylene
Effective Filtration Are	3.9 cm^2
GxF Prefilter Pore Size	1-40 µm (depth filter)
Hold-up Volumes (with Air Purge)	< 0.125 mL
Maximum Temperature	82 °C
Maximum Pressure Limit	4.1 bar (410 kPa, 60 psi) at 21-24 ºC
Typical Water Flow Rate (mL/min) at 1.0 bar (100 kPa, 15 psi)	650
Inlet/Outlet Connectors	Female luer-lok inlet, male slip luer outlet

B. Prefiltration with an Acrodisc® GxF Membrane

1. Before filling the syringe with sample, draw approximately 1 mL of air into the syringe. This will allow the air to follow the sample out of the syringe. This "air purge" minimizes fluid retention within the filter device.
2. Fill the syringe with the solution to be filtered.

CAUTION: *Use of syringes smaller than 10 mL can generate excessive pressure on the filter, which may exceed maximum operating pressure.*

3. Holding the filter device in one hand and the filled syringe in the other, secure (without excessive force) the filled syringe to the filter device with a twisting motion.
4. Apply gentle pressure to begin filtration. A gentle pressure helps assure maximum throughput.

Tip: *As the filter removes particulate, filtration will become more difficult. The syringe plunger will be harder to use. Pressure will rapidly increase on the filter. Change filters when resistance becomes excessive. Failure to change filter may result in housing rupture, which results in particulate contaminating the filtrate. These are for SINGLE USE ONLY.*

Ordering Information for Prefiltration of Plasma or Serum Samples Using Acrodisc Syringe Filters with Glass Fiber

Acrodisc PSF GxF Syringe Filters, Glass Fiber

Part Number	Description	Pkg
AP-4523	GxF/Glass, 25 mm	50/pkg, 200/cs
AP-4529	GxF/Glass, 25 mm	1000/pkg

2.6.1 Endotoxin Removal from Water Buffer, Neutral Sugars, and Certain Biological Solutions

Endotoxins are complex aggregates of acidic lipopolysaccharides (LPS) and consist of an innermost core of hydrophobic fatty acid groups and a central and outermost region composed of hydrophilic polysaccharides. In aqueous solutions, endotoxins can exist in various states of aggregation up to 1 MDa. Divalent cations, such as Ca^{2+} and Mg^{2+}, are found to stabilize the aggregated structure of LPS, whereas detergents help to break down the structure into smaller sub-units. When producing recombinant proteins in *E. coli* and other gram-negative bacteria, it is often necessary to remove LPS from the final product. This is especially important when carrying out immunological readouts and when developing manufacturing processes. Endotoxin can cause false readings in cell-based assays. There are limits to the amounts of endotoxin allowed in human products.

Cause of Endotoxin Contamination

Endotoxins are continuously shed from the outer membrane of viable gram-negative bacteria and released when the bacterial cell dies. Although bacteria are often removed by using a 0.2 µm sterilizing grade filter, LPS itself is difficult to remove or inactivate because it is extremely heat and pH stable. The pyrogenic threshold of an endotoxin reaction is on the order of 1 EU (endotoxin unit ~0.1 ng) per kg of body weight. This amount of endotoxin can come from 10^5 bacterial cells.

Removal of endotoxin is one of the most difficult downstream processes during protein purification. Many commercially available products are unable to remove endotoxin satisfactorily, or require time-consuming incubation steps. In many cases, complete endotoxin removal is only achieved with massive substrate loss. Because endotoxins are negatively charged at pH above 2, a positively-charged membrane surface can remove endotoxin. The Acrodisc® unit with positively-charged, hydrophilic Mustang® E membrane is ideal for the removal of endotoxins from solution due to its highly crosslinked quaternized amine charged surface. This gives very high dynamic capacities under selected conditions for the removal of endotoxin from process feedstreams, buffers, and water. The Acrodisc syringe filter has a high performance endotoxin removal capability of approximately 500,000 EU/unit in saline. The device properties are summarized in Table 2.64.

Table 2.64
Properties of the Acrodisc® Unit with Mustang® E Membrane, 25 mm

Specification	Parameter
Materials of Construction	
Membrane	Mustang E (positively-charged quarternary amine surface)
Housing	Polypropylene
Effective Filtration Area	3.9 cm^2
Membrane Pore Size	0.2 µm
Membrane Bed Volume	0.12 mL
Mean Endotoxin Removal Capacity (EU)*	~500,000 EU/Acrodisc unit from saline**
Maximum Pressure Limit	5.5 bar (550 kPa, 80 psi)
Mean Flow Rate	1-4 mL/min
Inlet/Outlet Connectors	Female luer-lok inlet, male slip luer outlet
Sterilization	Sterilized by gamma irradiation and individually packaged

**The endotoxin removal capacity may vary by flow rate, protein surface charge, type and concentration of protein, pH, salt concentration, or other components of the sample solution such as surfactants and glycols.*

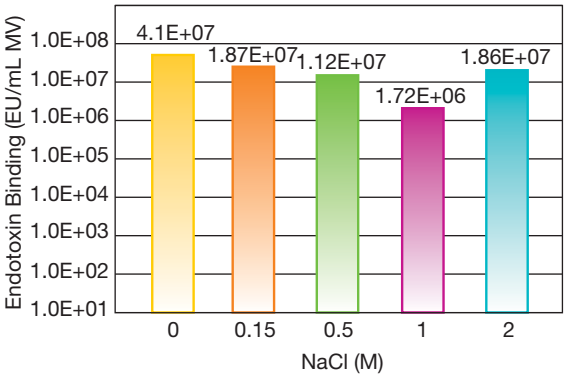
***Endotoxin source:* Escherichia coli *strain 055:B5 (no dispersing agents) at a minimum challenge level of 10,000 EU/mL in a volume of 60 mL of 0.9% saline.*

Application Data for Endotoxin Removal from Water Buffer, Neutral Sugars, and Certain Biological Solutions

The impact of composition of the sample medium was assessed for ionic strength and pH on the dynamic binding capacity of the Acrodisc unit with Mustang E membrane. Data is summarized in Figure 2.60 for NaCl and Figure 2.61 for pH. The results show that NaCl at all concentrations reduced the dynamic binding capacity suggesting that an ion exchange mechanism is responsible for adsorption of endotoxin onto the Mustang E surface. Interestingly the dynamic binding capacity appeared to be higher at 2.0 M than at 1.0 M NaCl, suggesting that a secondary hydrophobic interaction may be present under high salt conditions.

Figure 2.60

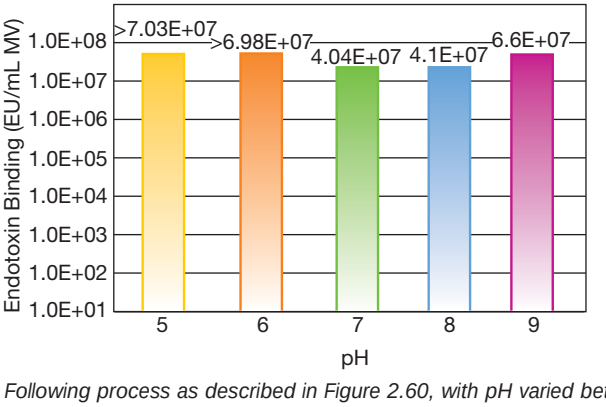
Effect of NaCl Concentration in 25 mm Tris (pH 8) on Endotoxin Dynamic Binding Capacity to Acrodisc® Unit with Mustang® E Membrane



Endotoxin source: Escherichia coli *strain 055:B5 (no dispersing agents) at a minimum challenge level of 10,000 EU/mL. A range of NaCl concentrations were tested in a volume of 60 mL. The dynamic binding capacity was calculated at 10% of the breakthrough curve.*

Figure 2.61

Effect of pH on Endotoxin Dynamic Binding Capacity to Acrodisc Unit with Mustang E Membrane



Following process as described in Figure 2.60, with pH varied between 5.0 and 9.0.

Ordering Information for Endotoxin Removal from Water Buffer, Neutral Sugars, and Certain Biological Solutions

Acrodisc® Unit with Mustang® E Membrane

Part Number	Description	Pkg
MSTG25E3	0.2 µm, 25 mm, sterile, blister packs	10/pkg

References for Endotoxin Removal from Water Buffer, Neutral Sugars, and Certain Biological Solutions

1. Nelsen, L. (1977). Filtration removal of endotoxin in solution in different states of aggregation. *Applied and Environmental Microbiology*, (34), 382–385.
2. Olson, W.P. (1995). Separations Technology, *Pharmaceutical and Biotech Appl.*, 57–194.
3. Zimmerman, G., et al. (1976). Drugs made in Germany-pyrogen elimination from paren. med. by means of UF. (19), 123–128.
4. Karbachsch, M., et al. (1984). Drugs made in Germany-depyrogen of soln of low Mol. Wt. Subs. by UF. 27(72), 74–76.