4.1.1 Introduction

In many areas, chromatography resins are the media of choice for chromatography applications. In some instances, where resin-based methods have limitations (e.g., purification of viruses or large molecules), membranes have proven to be a robust, scalable, and economical alternative to resins. Chromatography membranes offer faster flow rates as compared to their resin counterparts.

For ion exchange applications (see Table 4.1), Pall offers Mustang ion exchange membrane. This technology is available in several formats including a syringe filter column and a 96-well filter plate.

Table 4.1
Ion Exchange Applications

Type of Ion Exchanger	Common Abbreviation	Functional Group	Product Name	Description
Strong Anion	Q	Quarternary Ammonium	Acrodisc® Unit with Mustang Q AcroPrep™ Filter Plate with Mustang Q	Syringe filter column 96-well filter plate (350 µL or 1 mL)
Strong Cation	S	Sulfonic Acid	Acrodisc Unit with Mustang S AcroPrep Filter Plate with Mustang S	Syringe filter column 96-well filter plate (350 µL or 1 mL)

4.1.2 Purification on an Acrodisc® Unit with Mustang Q Membrane

Pall Life Sciences' disposable 25 mm Acrodisc unit is a chromatography device containing high capacity Mustang Q membrane, an anion exchanger with a polyethersulfone (PES) base modified with quaternary amines. Mustang Q membrane delivers efficient and rapid flow rates with a convective pore structure combined with high dynamic binding capacity for plasmid DNA (3.6 mg/Acrodisc unit), negatively charged proteins (10 mg), and viruses (10^{12} viral particles). Processing time is much shorter and more efficient than the conventional bead- or resin-based technology. Mustang devices have throughputs of up to 100 times that of traditional columns, with no associated loss of capacity. This cartridge format can directly scale up to large capsules with Mustang Q membrane for larger-volume applications. See Table 4.2 for specifications. Typical applications:

- Provides contaminant removal such as DNA viral particle, host cell proteins, or endotoxin.
- Ideal for isolation via capture and release of plasmid DNA, virus, or target protein from a complex mixture.
- Offers protein polishing for negatively-charged proteins.
- Purifies virus and oligo nucleotides.

Table 4.2
Specifications of the Acrodisc Unit with Mustang Q Membrane

Specification	Parameter
Materials of Construction	
Membrane	Mustang Q modified Supor® PES
Device	Polypropylene
Membrane Bed Volume	0.18 mL
Pore Size	0.8 µm
Hold-up Volumes - 25 mm	< 0.1 mL
Maximum Temperature	70-75 °C
Maximum Pressure Limi -25 mm	5.5 bar (550 kPa, 80 psi)
Typical Water Flow Rate	1-4 mL/min
Inlet/Outlet Connectors	Female luer-lok inlet, male slip luer outlet
Typical Mean Dynamic Binding Capacity*	
DNA	3.6 mg DNA /Acrodisc unit or 20 mg/mL membrane volume
Protein	10 mg BSA/Acrodisc unit or 56 mg/mL membrane volume

**The yield is contingent on type of DNA, size, and copy number of plasmid, concentration of protein, ionic strength, and pH of buffer.*

Protocol for Purification on an Acrodisc® Unit with Mustang Q Membrane

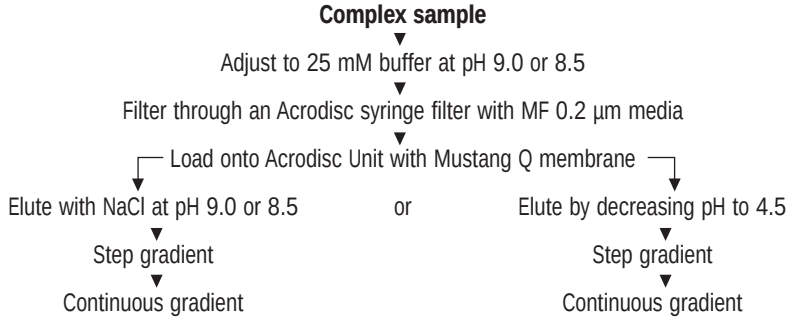
A. Materials Required

1. Syringes (5-25 mL) with luer lock fittings.
2. Chromatography fittings to transition 1/8 inch tubing connections to male luer-lock inlet and female slip luer fitting on the outlet (UpChurch or equivalent).
3. Degassed and filtered suitable buffers; Pump A, 25 mM Tris HCl pH 8.0; and Pump B, 1 M NaCl in buffer A or 25 mM sodium acetate pH 4.5.

B. Ion Exchange Purification Can Be Carried Out by Two Approaches

1. Changing the pH of the buffer.
2. Introducing a counter ion into the loading buffer in the form of a salt gradient.
3. In both cases, proteins elute from the ion exchange surface.
   - **a.** They become neutral or acquire the same charge as the ion exchange support.
   - **b.** They are displaced by the presence of a small counter ion in the form of salt.
4. The two approaches are useful in developing an optimal purification strategy and are summarized in Table 4.3. Either strategy or a combination of both can be applied to the purification of components from a complex sample in the Acrodisc device format. These devices can be operated by syringe (step gradient elution) or pumped flow (stepped and gradient elution) using a chromatography workstation.

Table 4.3
Summary of Purification Options for Acrodisc Unit with Mustang Q Membrane



C. Syringe Protocol

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 cartridge.
2. Fill the syringe with equilibration buffer A.

Tip: *Use of syringes smaller than 10 mL can generate excessive pressure on the cartridge, 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 passing fluid through the device. (A gentle pressure

helps to assure maximum throughput.)

5. Collect the column effluent in 0.5 mL fractions. Measure the A_{280} to locate the protein peak.

Tip: *Protein rapidly elutes from the cartridge 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® centrifugal device, with a 10K MWCO UF membrane.*

6. Retained fractions can then be eluted by step gradient of buffer pH, up to 1.0 M salt, or a combination of both.
7. Each step of the gradient should be at least 2-5 column volumes (CV). Fractions of 0.5 mL should be collected. After protein has eluted, the device can be regenerated by 5 CV of 1.0 M NaCl followed by equilibration back to initial buffer conditions.

D. Syringe Protocol on Chromatography Workstation

1. Place luer fitting adaptors onto the Acrodisc® device. Connect to a chromatography workstation.
2. Set flow to 100% buffer A at 1 mL/min and fill the device with fluid in the reverse flow direction to displace air from the device.
3. Reverse the flow and equilibrate the device for 5-10 CV of buffer A.
4. Load the sample up to a 2 mL volume onto the column at 1 mL/min flow rate. Monitor the A_{280} of the effluent.
5. Collect the column effluent in 1 mL fractions. Measure the A_{280} to locate the protein peak.

Tip: *Protein rapidly elutes from the cartridge 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 centrifugal device, with a 10K MWCO UF membrane.*

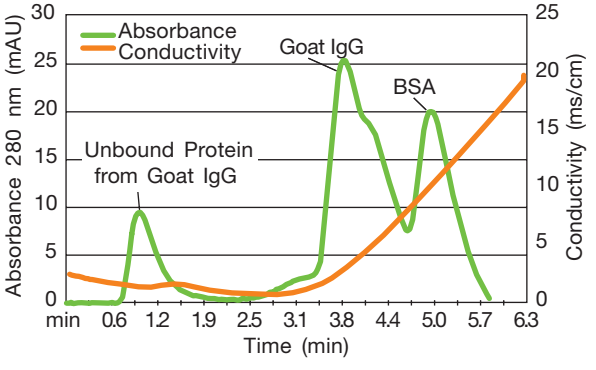
6. Retained fractions can then be eluted by linear gradient of buffer pH, linear gradient up to 1.0 M salt, or a combination of both.
7. The volume of the gradient should be at least 5-10 CV. Fractions of 1 mL should be collected. After protein has eluted, the cartridge can be regenerated by 5 CV of 1.0 M NaCl followed by equilibration back to initial buffer conditions.

Application Data for Purification on an Acrodisc® Unit with Mustang Q Membrane

Resolution of a mixture of BSA and IgG at pH 8.0 is summarized in Figure 4.1. The data clearly shows a rapid (< 10 minutes) separation of the two plasma proteins as well as resolved symmetrical peaks at a very high flow rate of 13 CV/min. The dynamic binding capacity for this Mustang Q device is summarized in Figure 4.2. The cartridge capacity was calculated to be 10 mg BSA with a membrane media capacity of 56 mg/mL, which is very comparable to conventional particle-based media. The Mustang Q Acrodisc device offers a very high flow rate. It is an equivalent capacity device which can yield high resolution separations.

Figure 4.1

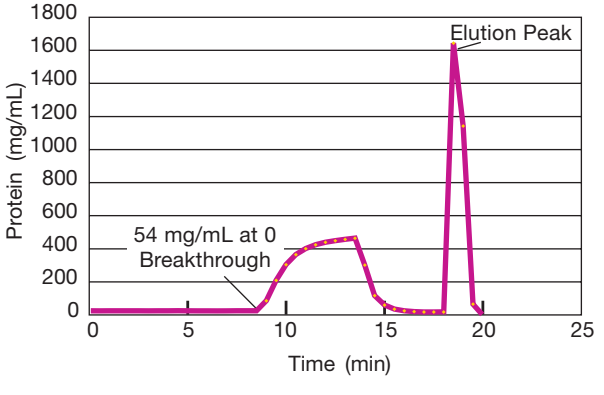
Acrodisc Unit with Mustang Q Membrane: Resolution of BSA and Goat IgG



The conditions used to generate data for the resolution graph above include buffer: 25 mM Tris pH 8.0; salt: 1 M NaCl in 25 mM Tris pH 8.0; gradient: 0 to 0.5 M NaCl in 50 CV; flow rate: 2.3 mL/min (13 cv/min); sample loading: 4% of total binding capacity.

Figure 4.2

Acrodisc Unit with Mustang Q Membrane: Dynamic Binding with BSA



A solution of 0.524 mg/mL BSA was pumped through the Acrodisc unit at 2.3 mL/min. Breakthrough occurred at 8.1 minutes and was calculated as 54 mg/mL using: flow rate (2.3 mL/min) X initial protein BSA concentration (0.524 mg/mL) X time (8.1 minutes) membrane bed volume of Mustang Q membrane in 25 mm Acrodisc unit (0.18 mL).

Ordering Information for Purification on an Acrodisc® Unit with Mustang Q Membrane

Acrodisc Unit with Mustang Q Membrane

Part Number	Description	Pkg
MSTG25Q6	0.8 µm, 25 mm, non-sterile, blister packs	10/pkg

4.1.3 Purification on an Acrodisc® Unit with Mustang S Membrane

Pall Life Sciences' disposable 25 mm Acrodisc unit is a chromatography device containing high capacity Mustang S membrane, a cation exchanger with a polyethersulfone (PES) base modified with sulfonic acid groups. Mustang S membrane delivers efficient and rapid flow rates with a convective pore structure combined with high dynamic binding capacity for positively-charged proteins (10 mg), and viruses. Processing time is much shorter and more efficient than the conventional bead or resin-based technology. Mustang devices have throughputs of up to 100 times that of traditional columns, with no associated loss of capacity. This cartridge format can directly scale up to large capsules with Mustang S membrane for larger-volume applications. See Table 4.4 for specifications.

Table 4.4
Specifications of the Acrodisc Unit with Mustang S Membrane

Specification	Parameter
Materials of Construction	
Membrane	Mustang S modified Supor® PES
Device	Polypropylene
Membrane Bed Volume	0.18 mL
Pore Size	0.8 µm
Hold-up Volumes - 25 mm	< 0.1 mL
Maximum Temperature	70-75 °C
Maximum Pressure Limit - 25 mm	5.5 bar (550 kPa, 80 psi)
Typical Water Flow Rate	1-4 mL/min
Inlet/Outlet Connectors	Female luer-lok inlet, male slip luer outlet
Typical Mean Dynamic Binding Capacity*	
Lysozyme	8 mg Lysozyme/Acrodisc unit or 47 mg/mL membrane volume
IgG	11 mg per Acrodisc unit or 60 mg/mL membrane volume

**The yield is contingent on type of DNA, size, and copy number of plasmid, concentration of protein, ionic strength, and pH of buffer.*

Protocol for Purification on an Acrodisc® Unit with Mustang S Membrane

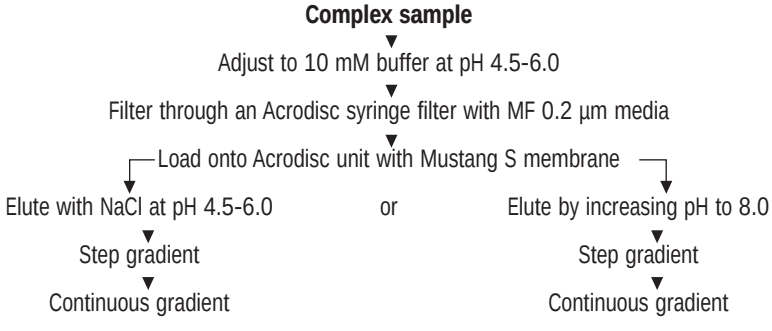
A. Materials Required

1. Syringes (5-25 mL) with luer lock fittings.
2. Chromatography fittings to transition 1/8 inch tubing connections to male luer-lock inlet, and female slip luer fitting on the outlet (UpChurch or equivalent).
3. Degassed and filtered suitable buffers; Pump A, 10 mM MES-NaOH pH 5.5; and Pump B, 1 M NaCl in buffer A or 25 mM Tris HCl pH 8.0.

B. Ion Exchange Purification Can Be Carried Out by Two Approaches

1. Changing the pH of the buffer.
2. Introducing a counter ion into the loading buffer in the form of a salt gradient.
3. In both cases, proteins elute from the ion exchange surface because:
   a. They become neutral or acquire the same charge as the ion exchange support.
   b. They are displaced by the presence of a small counter ion in the form of salt.
4. The two approaches are useful in developing an optimal purification strategy and are summarized in Table 4.5. Either strategy or a combination of both can be applied to the purification of components from a complex sample in the Acrodisc device format. These devices can be operated by syringe (step gradient elution) or pumped flow (stepped and gradient elution) using a chromatography workstation.

Table 4.5
Summary of Purification Options for Acrodisc Unit with Mustang S Membrane



C. Syringe Protocol

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 cartridge.
2. Fill the syringe with equilibration buffer A.

Tip: *Use of syringes smaller than 10 mL can generate excessive pressure on the cartridge, 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 passing fluid through the device. (A gentle pressure helps assure maximum throughput.)
5. Collect the column effluent in 0.5 mL fractions. Measure the A_{280} to locate the protein peak.

Tip: *Protein rapidly elutes from the device 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® centrifugal device, with a 10K MWCO UF membrane.*

6. Retained fractions can then be eluted by step gradient of buffer pH, up to 1.0 M salt, or a combination of both.
7. Each step of the gradient should be at least 2-5 column volumes (CV). Fractions of 0.5 mL should be collected. After protein has eluted, the device can be regenerated by 5 CV of 1.0 M NaCl followed by equilibration back to initial buffer conditions.

D. Syringe Protocol on Chromatography Workstation

1. Place luer fitting adaptors onto the Acrodisc® device. Connect to a chromatography workstation.
2. Set flow to 100% buffer A at 1 mL/min and fill the device with fluid in the reverse flow direction to displace air from the device.
3. Reverse the flow and equilibrate the device for 5-10 CV of buffer A.
4. Load the sample up to a 2 mL volume onto the column at 1 mL/min flow rate. Monitor effluent at 280 nm.
5. Collect the column effluent in 1 mL fractions. Measure the A_{280} to locate the protein peak.

Tip: *Protein rapidly elutes from the device 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 centrifugal device, with a 10K MWCO UF membrane.*

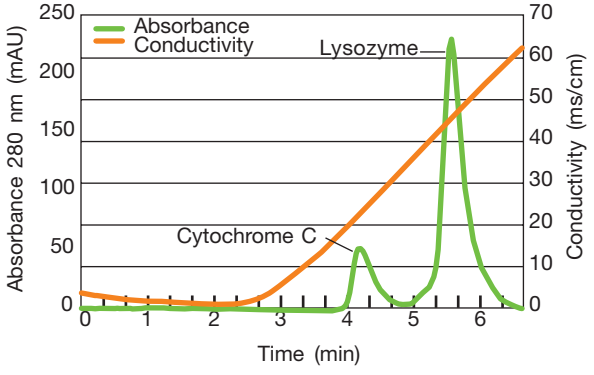
6. Retained fractions can then be eluted by linear gradient of buffer pH, linear gradient up to 1.0 M salt, or a combination of both.
7. The volume of the gradient should be at least 5-10 CV. Fractions of 1 mL should be collected. After protein has eluted, the cartridge can be regenerated by 5 CV of 1.0 M NaCl followed by equilibration back to initial buffer conditions.

Application Data for Purification on an Acrodisc Unit with Mustang S Membrane

Resolution of a mixture of Cytochrome C and lysozyme at pH 5.5 is summarized in Figure 4.3. The data clearly shows a rapid (< 10 minutes) separation of the two proteins as well as resolved symmetrical peaks at a very high flow rate of 13 CV/min. The dynamic binding capacity for this Mustang S device is summarized in Figure 4.4. The cartridge capacity was calculated to be 8 mg lysozyme with a membrane media capacity of 52 mg/mL, which is very comparable to conventional particle-based media. The Mustang S Acrodisc device offers a very high flow rate. It is an equivalent capacity device which can yield high resolution separations.

Figure 4.3

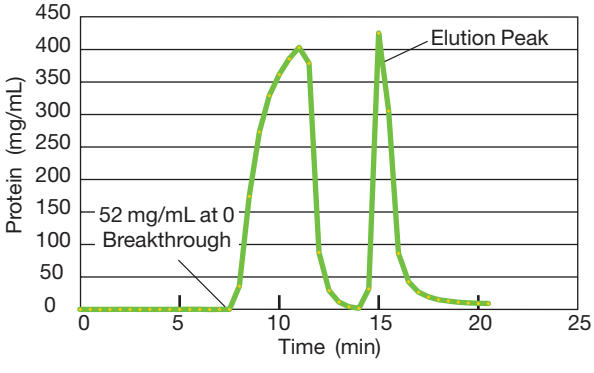
Acrodisc® Unit with Mustang S Membrane: Resolution of Cytochrome C and Lysozyme



The conditions used to generate data for the resolution graph above include buffer: 10 mM MES-NaOH pH 5.5; salt: 1 M NaCl in 10 mM MES-NaOH pH 5.5; gradient: 0 to 0.5 M NaCl in 50 CV; flow rate: 2.3 mL/min (13 cv/min); sample loading: 4% of total binding capacity.

Figure 4.4

Acrodisc Unit with Mustang S Membrane: Dynamic Binding with Lysozyme



A solution of 0.512 mg/mL lysozyme was pumped through the Acrodisc unit at 2.3 mL/min. Breakthrough occurred at 8.0 minutes and was calculated as 52 mg/mL using: flow rate (2.3 mL/min) X initial protein concentration (0.524 mg/mL) X time (8.1 minutes) membrane bed volume of Mustang S membrane in 25 mm Acrodisc unit (0.18 mL).

Ordering Information for Purification on an Acrodisc® Unit with Mustang S Membrane

Acrodisc Unit with Mustang Q Membrane

Part Number	Description	Pkg
MSTG25S6	0.8 µm, 25 mm, non-sterile, blister packs	10/pkg

4.2.1 Introduction

Chromatography continues to be an essential technology for the purification of biomolecules. Pall offers a line of chromatography resin ideal for protein purification applications (see Table 4.6). This broad line of chromatography products exhibits superior performance and is useful for affinity, ion exchange, size exclusion, and hydrophobic interaction chromatography (HIC). Unique mixed-mode BioSepra products also exist to provide solutions to current sample preparation challenges.

The resins Pall offers for small-scale applications are the same ones offered to our customers currently manufacturing biopharmaceuticals. The ability to scale up is essential for those working in drug discovery, development, and manufacturing. These resins can be used in varying size chromatography columns, as well as in batch mode for single or high throughput mode. This is ideal for quick preps or in situations where optimizing purification conditions is required.

Table 4.6
Chromatography Resins

Chromatography Type	Product Name	Description
Ion Exchange	Q Ceramic HyperD®	Strong anionic exchanger, binds negatively-charged target
	S Ceramic HyperD	Strong cationic exchanger, binds positively-charged target
	CM Ceramic HyperD	Weak cationic exchanger
	DEAE Ceramic HyperD	Weak anionic exchanger
Affinity	Protein A Ceramic HyperD	Binds IgG
	Blue Trisacryl®	Binds albumin
	Heparin HyperD	Direct binding to targets that have an affinity for heparin
	Lysine HyperD	Direct binding to targets that have an affinity for lysine
	IMAC HyperCel™	Binds tagged proteins using a immobilized metal compound
	SDR HyperD	Detergent removal
Mixed Mode	MEP HyperCel	Uses several binding mechanisms including hydrophobic interactions
	HA Ultrogel®	Hydroxyapatite
HCIC	MEP HyperCel	Uses several binding mechanism including hydrophobic interactions
Size Exclusion	Ultrogel AcA	Separates targets by size

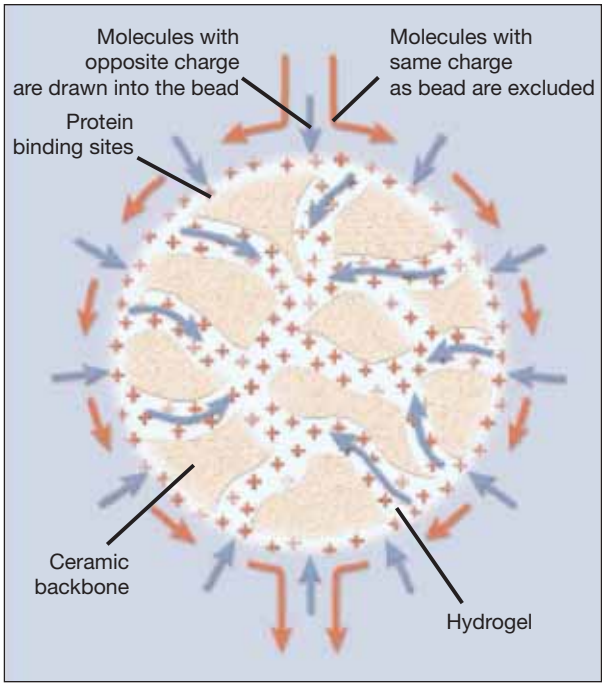
Table 4.7
Available Separation Columns

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

4.2.2 Ceramic HyperD® Ion Exchange Resin

Ceramic HyperD ion exchange resins employ a high-capacity hydrogel polymerized within the gigapores of a rigid ceramic bead. As shown in Figure 4.5, this design combines the desirable characteristics of a soft, high-capacity hydrogel with the absolute dimensional stability of a rigid ceramic bead. Ceramic HyperD resins do not shrink or swell with changes in pH or conductivity. Abundant ion exchange sites in the hydrogel are highly accessible to protein molecules. Proteins diffuse rapidly within the hydrogel, facilitating rapid uptake of product. This mechanism of mass-transfer – known as enhanced diffusion – allows the resins to operate free of operational constraints typically encountered with conventional macroporous ion exchange resins. Specifications of the range of Ceramic HyperD ion exchange resins available are summarized in Table 4.8.

Figure 4.5
Ceramic HyperD Resin – 'Gel in a Shell' Design



Ceramic HyperD resins deliver outstanding dynamic capacity and exceptional dimensional stability. This translates into unsurpassed productivity.

Table 4.8
Ceramic HyperD® Ion Exchange Resin Specifications

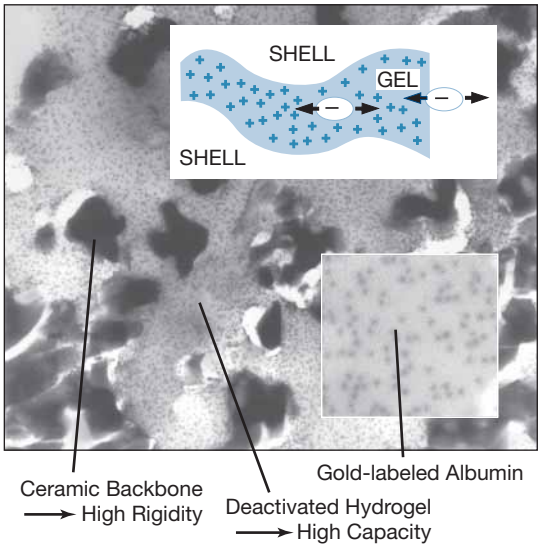
Type of Ceramic HyperD	Q	S	Q	S	DEAE	CM
Grade	20	20	F	F	F	F
Average Particle Size (µm)	~20	~20	~50	~50	~50	~50
Dynamic Binding Capacity (mg/mL)	BSA	Lysozyme	BSA	Lysozyme	BSA	IgG
10% Breakthrough at 200 cm/h	≥ 85*	≥ 85*	≥ 85*	≥ 75**	≥ 85*	≥ 60***
Amount of Ionic Groups (µeq/mL)	≥ 250	≥ 150	≥ 250	≥ 150	≥ 200	250-400
Working pH	2-12					
Cleaning pH	1-14					
Volumes 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.*

The Enhanced Diffusion Concept

Traditional macroporous ion exchangers operate on the basis of classical pore diffusion. Pore diffusion is characterized by rapidly decreasing binding capacity with increased flow rate. In contrast, the unique structure of the Ceramic HyperD resin supports a more rapid mechanism of mass transfer, known as enhanced diffusion. Rapid mass transfer overcomes classical flow rate dependence. Since product is 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 4.6. 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.

Figure 4.6
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 homogeneously throughout the hydrogel.

Protocol for Ceramic HyperD Ion Exchange Resin

In this section, the focus will be on small-scale purification in gravity flow and packed glass columns for use on a liquid chromatography instrument. Methods development and scouting protocols employing small-scale single and multi-well devices are described in small-scale protein pre-fractionation in Section 2.2.2, page 49.

A. Materials Required

1. Choose one of the following:
   - **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene, column, e.g., Pierce PN 29922); or
   - **b.** Glass column 6.6 mm ID x 10 cm length, 1-2 mL volume (e.g., Omnifit PN 006CC-06-10-AF)
2. Degassed 50% (v/v) slurry of the HyperD ion exchange resin
3. 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 4-5 mS/cm as measured with a conductivity meter. **Note:** 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:
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 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 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 HyperD® Ion Exchange Resin

1. Gravity flow column format

- **a.** Equilibrate column, degassed 50% (w/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 storage at 4 ºC for no more than one week or for immediate use.
- **l.** Refer to Section C on page 314 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. Glass chromatography column format

a. Equilibrate column, degassed 50% (v/v) 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 ºC for no more than one week or for immediate use.

i. Refer to Section C below for use instructions.

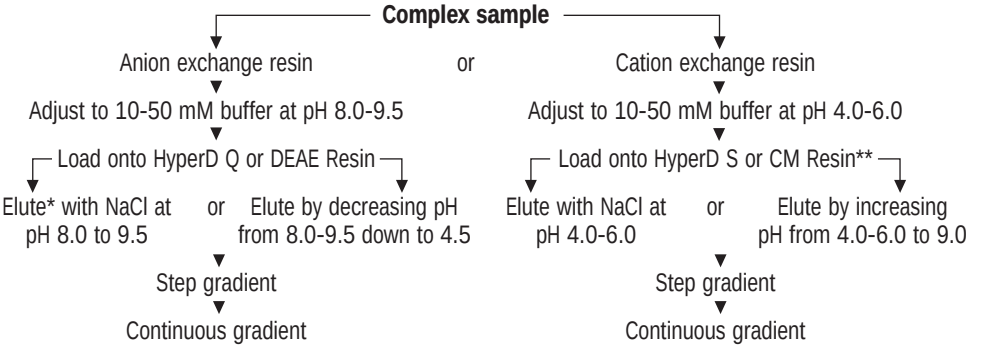
C. Ion Exchange Pre-fractionation

The following two approaches can be used:

- Changing the pH of the buffer; or
- Introducing a counter ion into the loading buffer in the form of a salt gradient.

In both cases, proteins elute from the ion exchange surface because they become neutral or acquire the same charge as the ion exchange support, or are displaced by the presence of a small counter ion in the form of salt. The two approaches are useful in developing an optimal purification strategy and are summarized in Table 4.9. Either strategy or a combination of both can be applied to the purification of components from a complex sample in one of the following formats.

Table 4.9
Summary of Purification 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 HyperD ion exchange resin with 5 column volumes (CV) of buffer to remove the 1 M NaCl, 20% (v/v) ethanol storage buffer.
- **c.** Allow the liquid to drain from the column and load the sample up to a 2 mL volume onto the column.
- **d.** Collect the column effluent in 1 mL fractions. Measure the 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™ centrifigual 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 is attempted.
- **f.** Retained fractions can then be eluted by a series of buffer pH steps, serial steps up to 1.0 3 M salt, 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. 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).*

- **g.** After the last pH or salt 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.

2. Chromatography glass column format

a. Prepare a 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 A_{280} of the effluent.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 4.9.*

c. Collect the column effluent in 1 mL fractions. Measure the A_{280} 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 Macrosep® 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 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 2.4.2, page 154).*

Application Data for Ceramic HyperD® Ion Exchange Resin

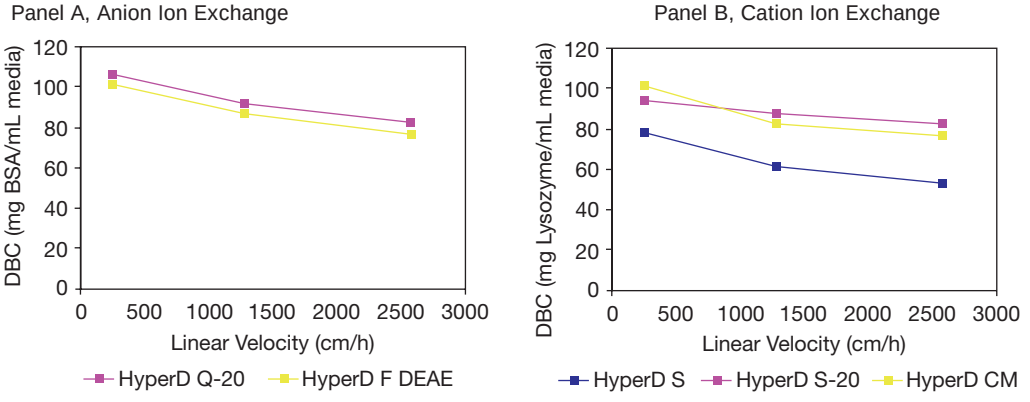
Ceramic HyperD ion exchange resin packed into chromatography columns can be used to carry out small-scale pilot purification optimization when used in conjunction with a fluidic workstation. Under these conditions, flow rates are more controlled and more sophisticated elution conditions are employed, such as binary gradients between pumps for pH or salt-based optimization. Ceramic HyperD ion exchange resins lend themselves very well to pumped flow conditions. Application data is presented below to illustrate the use of Ceramic HyperD ion exchange resin in small-scale purification projects.

Dynamic Binding Capacity in Small Volume Chromatography Columns

Ceramic Hyper D ion exchange resins deliver high dynamic binding capacity at high linear velocity. As shown in Figure 4.7 and Table 4.10, there is only a modest decline in dynamic binding capacity for BSA and lysozyme as linear velocity is increased from 258 cm/h to 280 cm/h.

Figure 4.7

Impact of Linear Velocity on Dynamic Binding with HyperD® Ion Exchange Resin



Ceramic HyperD ion exchange resins (1 mL) were packed into OmniFit glass columns (6.6 mm diameter x 2.8 cm bed height) and equilibrated with 25 mM Tris HCL pH 8.5 (Anion); and 10 mM MES-NaOH pH 5.8 (Cation) at 1 mL/min until a stable pH and conductivity were obtained. Solutions of 5 mg/mL BSA and lysozyme were then pumped onto their respective anion or cation columns at 1 mL/min until "break through" was seen on the absorbance trace at 280 nm. The protein solution pumping continued until a plateau of absorbance was seen, usually after 15 CV. The dynamic binding capacity was then calculated at 10% of the plateau value, allowing for system dead volume and expressed as mg/mL of media. This study was repeated at high flow rates of 5 and 10 mL/min.

Table 4.10

Dynamic Binding Capacity of Ceramic HyperD Ion Exchange Resin Packed in 1 mL Chromatography Glass Columns at a Range of Flow Rates (Linear Velocities)

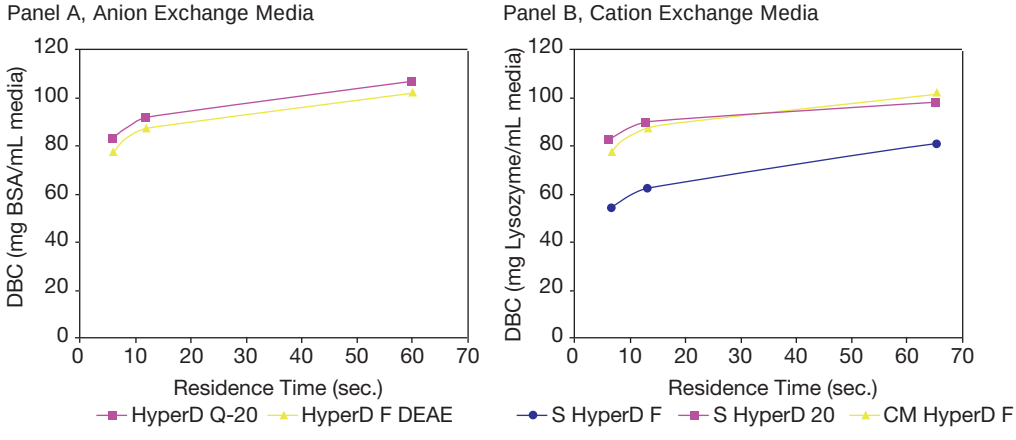
	Dynamic Binding Capacity (mg/mL)*		
Media	1 mL/min (258 cm/h)	5 mL/min (1290 cm/h)	10 mL/min (2580 cm/h)
HyperD Q-20 µm	106.0	91.5	82.5
HyperD F DEAE	101.5	87.5	77.5
HyperD F S	80.5	61.5	53.5
HyperD S-20	97.0	89.5	83.5
HyperD F CM	108.0	87.5	73.5

**Dynamic binding capacity measured by breakthrough curve analysis at 10% of media saturation; a 1 mL volume column of ion exchange resin was packed as described in Protocol Section B on page 313 and equilibrated with 25 mM Tris HCl pH 8.5 (Anion ion exchange) or 10 mM MES-NaOH pH 5.5 (Cation ion exchange) at the flow rates of 1, 5, or 10 mL/min. For anion ion exchange; 5 mg/mL BSA in the above buffer was then pumped onto the column until a break through in absorbance at 280 nm was seen. The flow was continued until a plateau in absorbance was achieved corresponding to 100% protein feed. Dynamic binding capacity was then calculated at 10% of the plateau value, correcting for any "dead volume" in the system and expressed as mg BSA/mL media volume. For cation ion exchange; 5 mg/mL lysozyme was used to test these resins in a similar manner to the anion ion exchange media.*

In moving from small-scale purification to a pilot scale, many process developers prefer to examine the influence of residence time on dynamic binding capacity. This approach allows assessment of resin characteristics without reference to details of column geometry. At a residence time of only 6 seconds, dynamic binding capacity for BSA is 82.5 mg/mL at 10% breakthrough for Ceramic HyperD® F DEAE resin. As shown in Figure 4.8, there is only modest reduction in dynamic binding capacity as residence time is reduced from 60 to 6 seconds. Dynamic binding capacity values ranging from approximately 80 to 106 mg BSA/mL were achieved over the range of conditions studied. The inherently high binding capacity of Ceramic HyperD resins permits operation using columns of moderate volume. By reducing bed volume requirements, buffer volume requirements may also be reduced. High flow velocity, short residence time, reduced bed volume, and reduced buffer volume support high productivity in small-scale applications and enhanced process economics when moved upscale into production.

Figure 4.8

Binding Capacity vs. Residence Time of Q Ceramic HyperD F Resin



Dynamic binding capacity was determined as described in the legend to Figure 4.7 or Table 4.10. Residence time was assumed to be the time (in seconds) for the passage of 1 CV (1 mL) of protein though the column at the flow rates of 1, 5 and 10 mL/min and corresponds to 60, 12, and 6 seconds. Panel A shows the data for anion ion exchange media and Panel B shows cation ion exchange media.

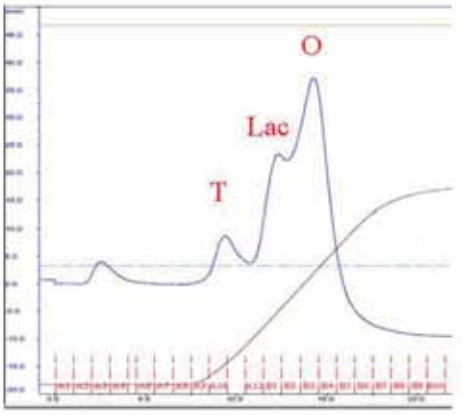
Resolution of Protein Standards

Ceramic HyperD ion exchange resins are capable of high flow and offer protein resolution in gradient applications. A summary of resolution of standard proteins is shown in Figure 4.9 for Anion (Panels A-B) and Cation (Panels C-D) ion exchange chemistries to illustrate the protein resolution performance of the Ceramic HyperD media. Further resolution can be obtained with smaller particle diameter, such as the Ceramic HyperD 20 µm media. A summary of the high resolution performance of this media is shown in Figure 4.10, compared to a competitive 15 µm polymeric media evaluated under the same conditions. The resulting chromatograms show that the smaller particle size Ceramic HyperD ion exchange resins are capable of high protein resolution, equivalent to competitive Anion ion exchange sorbents based on a 15 µm polymeric bead.

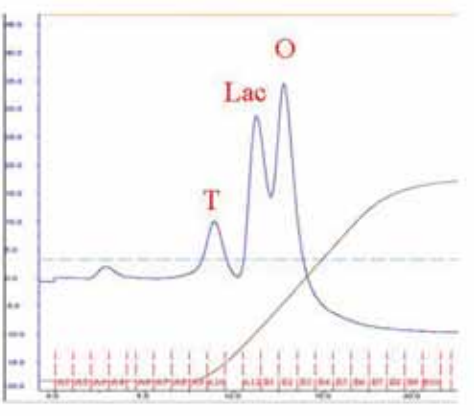
Figure 4.9

Protein Standards Resolved by Ceramic HyperD® Ion Exchange Resin Under Linear Gradient Elution Conditions

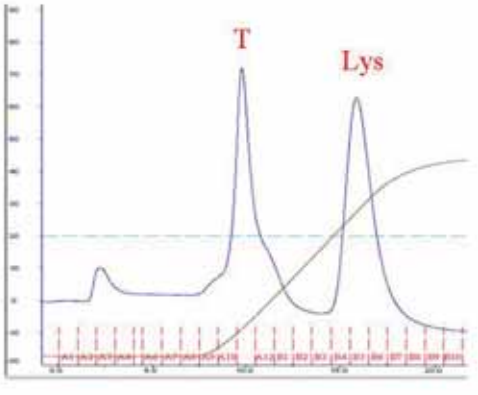
Panel A, HyperD F Q



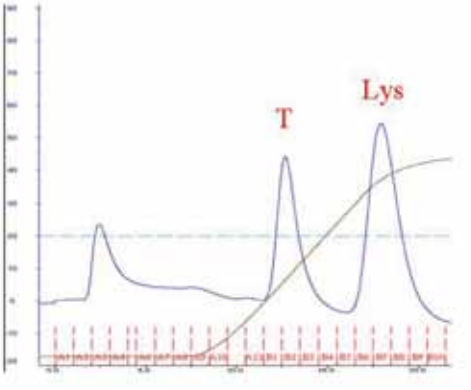
Panel B, HyperD F DEAE



Panel C, HyperD F S



Panel D, HyperD F CM

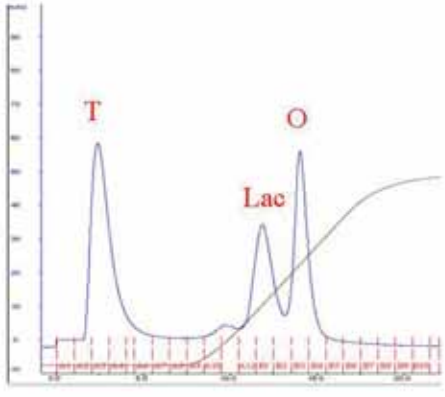


Panels A and B, protein mix (0.5 m/mL Trypsinogen [T, pI 9.3], Ovalbumin [O, pI 5.1/5.3] and Beta Lactoglobulin [Lac, pI 4.6] in 25 mM Tris HCl pH 8.5) loaded (0.5 mL) onto 1 mL volume Ceramic HyperD anion ion exchange resins. Elution with a linear gradient up to 50% B (1 M NaCl in loading buffer) at 1 mL/min. Panels C and D, protein mix (0.5 m/mL Trypsinogen [T, pI 9.8] and Lysozyme [Lys, pI 11.2] in 10 mM MES-NaOH pH 5.8) loaded (0.5 mL) onto 1 mL volume Ceramic HyperD cation ion exchange resins. Elution with a linear gradient up to 50% B (1 M NaCl in loading buffer) at 1 mL/min.

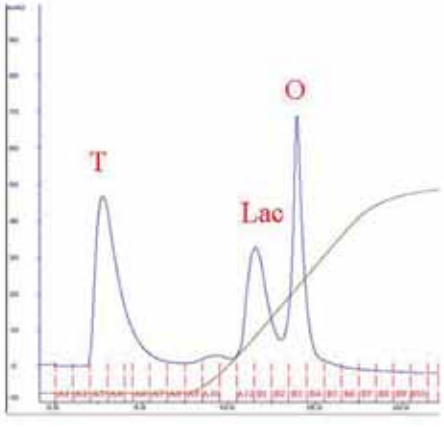
Figure 4.10

Protein Standard Resolution With 15-20 µm Ceramic HyperD® IEX Particles

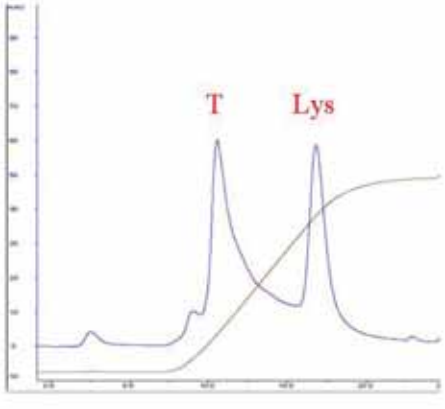
Panel A, Ceramic HyperD Q-20



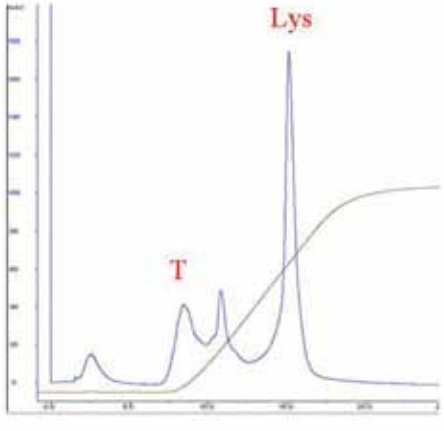
Panel B, Competitive 15-Q Polymeric Particle



Panel C, Ceramic HyperD S-20



Panel C, Competitive 15-S Polymeric Particle



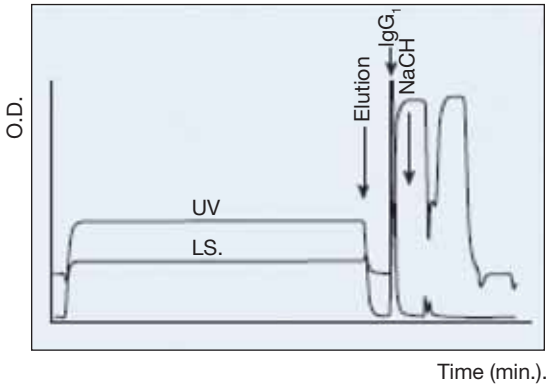
Panels A and B, protein mix (0.5 m/mL Trypsinogen [T, pI 9.3], Ovalbumin [O, pI 5.1/5.3] and Beta Lactoglobulin [Lac, pI 4.6] in 25 mM Tris HCl pH 8.5) loaded (0.5 mL) onto 1 mL volume Ceramic HyperD Q-20 and 15-Q competitive polymeric ion exchange resins. Elution with a linear gradient up to 50% B (1 M NaCl in loading buffer) at 1 mL/min. Panels C and D, protein mix (0.5 m/mL Trypsinogen [T, pI 9.8] and Lysozyme [Lys, pI 11.2] in 10 mM MES-NaOH pH 5.8) loaded (0.5 mL) onto 1 mL volume Ceramic HyperD S-20 and 15-S competitive polymeric ion exchange resins. Elution with a linear gradient up to 50% B (1 M NaCl in loading buffer) at 1 mL/min.

Scale Up Applications

Purification of mouse IgG1 from cell culture supernatant (CCS) on Ceramic HyperD® F CM resin. With its highly substituted hydrogel, the Ceramic HyperD F CM ion exchange resin binds effectively even in the presence of moderate concentrations of salt. As shown in Figure 4.11, IgG1 was harvested from 31 L of clarified CCS using a 330 mL column of Ceramic HyperD F CM resin. Prior to loading, the pH of the CCS was adjusted to pH 4.7. Conductivity of the feedstock was 19 mS/cm, equivalent to about 180 mM sodium chloride. The concentration of IgG in the feedstock was 150 µg/mL. At a linear velocity of 260 cm/h, loading was accomplished in 112 minutes, and chromatography was complete in 164 minutes. Residence time was only 1 minute. Isolated IgG was > 90% pure. Eliminating the need for preliminary diafiltration or dilution will simplify the process and enhance productivity of the scheme.

Figure 4.11

One-step Capture of Mouse IgG1 from CCS on Ceramic HyperD F CM Resin



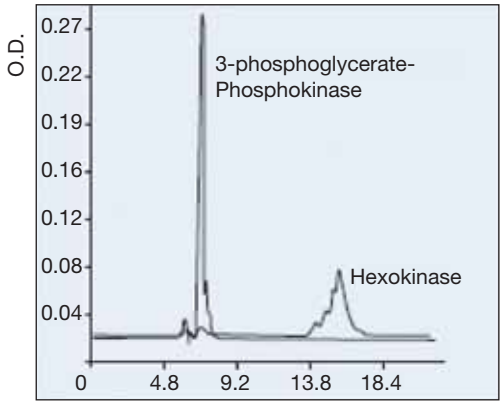
IgG1 purity: 90%; Column: 9 cm ID x 5.2 cm (330 mL); Load: 31 L CCS 100-150 µg/mL adjusted to pH 4.7; Equilibration and post-load wash: 50 mM sodium acetate, 0.1 M NaCl, pH 4.7; Elution: same buffer + 1.5 M NaCl; Duration: 164 minutes; Residence time: 1 minute; Linear velocity: 260 cm/h.

Purification of Hexokinase and 3-phosphoglycerate-Phosphokinase on Ceramic HyperD Q-20 Resin

The 20 µm grade allows rapid method development for enzyme separation using a salt gradient (see Figure 4.12). The rapid high resolution separation at a linear velocity of 1,223 cm/h (4 mL/min) still was able to yield a sharp, well-resolved peak of 3-phosphoglycerate-Phosphokinase. The whole separation was complete in < 20 minutes, illustrating the high throughput efficiency of the small particle ion exchange HyperD resin.

Figure 4.12

Purification of Hexokinase and 3-phosphoglycerate-Phosphokinase on Ceramic HyperD® Q-20 Resin



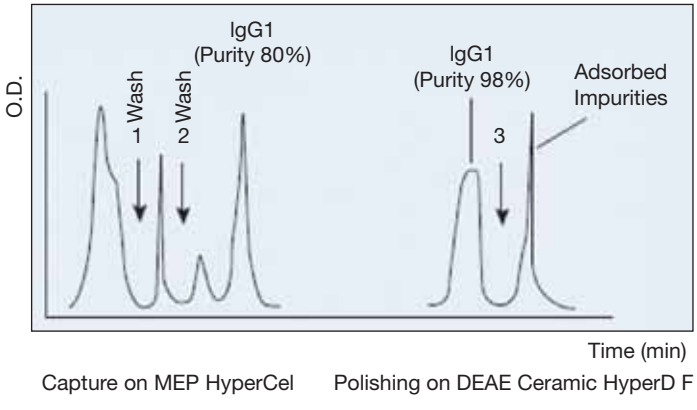
Column: 0.5 cm ID x 10 cm (1.7 mL); Adsorption, washing, equilibration in 50 mM Tris HCl/Tris base, pH 7.2; Elution by 0 to 1 M NaCl gradient; Protein concentration: 1 mg/mL; Linear velocity: 1,223 cm/h (4 mL/min).

Polishing Step on Ceramic HyperD F DEAE Resin After Monoclonal Antibody Capture on MEP HyperCel™ Resin

Ceramic HyperD F DEAE resin has been used in a two-step process for a polishing step to purify a mouse IgG1 from ascites fluid (see Figure 4.13). The first step is a capture of the IgG1 on a MEP HyperCel column (Hydrophobic Charge Induction Chromatography–HCIC–), which results in a good initial capture of the IgG1 (93%). A purity of 98% for the IgG1 is achieved in two steps.

Figure 4.13

Two-step Purification of IgG1 from Ascites Fluid on MEP HyperCel Resin Followed by Ceramic HyperD F DEAE Resin



MEP HyperCel column: First wash with 50 mM Tris HCl buffer, pH 8, second wash with 25 mM sodium caprylate in same buffer (arrow 1), followed by a water wash (arrow 2), to remove albumin. Elution with 50 mM sodium acetate, pH 4.0. The IgG1 enriched fraction is added with Tris base up to pH 8.8 and ionic strength of 7.4 mS/cm, and injected onto the Ceramic HyperD F DEAE column (0.6 cm ID x 10 cm). Wash with same buffer to collect the antibody. Equilibration: 50 mM Tris HCl, pH 8.8; Linear velocity: 160 cm/h. IgG do not bind, adsorbed impurities are eluted by 1 M NaCl (arrow 3).

Ordering Information for Ceramic HyperD® Ion Exchange Resin

Part Number	Description	Pkg
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
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
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
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
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
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

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4.2.3 Blue Trisacryl® M Resin

This resin was developed to extend the use of Cibacron* Blue F3GA to industrial and advanced lab-scale plasma fractionation applications. The molecular structure of Blue Trisacryl M resin is shown in Figure 4.14. This affinity ligand is immobilized through a stable six-carbon spacer arm allowing a better accessibility of the molecular structure even at high flow rates. The properties of this affinity resin are summarized in Table 4.11.

Figure 4.14
Molecular Structure of Blue Trisacryl M or LS Resin

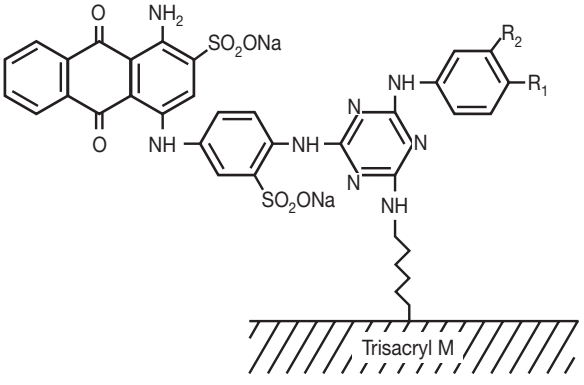


Table 4.11
Properties of Blue Trisacryl M Affinity Resin

Specification	Parameter
Particle Size	40-80 µm
Exclusion Limit	10^7 daltons
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)

Using the properties of Blue Trisacryl M resin, some large scale industrial processes have been developed to purify human albumin to a high degree of purity (> 98% by electrophoresis). The application of this resin to large scale processing and its ability to create sub-fractions of plasma has important application in proteomics, such as bulk albumin depletion of pooled plasma samples. In this section, protocols for packing Blue Trisacryl M resin into chromatography LC and gravity flow columns will be described to provide a purification platform for components from serum or plasma samples.

Protocol for Blue Trisacryl® M Resin

A. Materials Required

1. Choose one of the following:

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

b. Glass column 6.6 mm ID x 10 cm length, 1-2 mL volume (Omnifit PN 006CC-06-10-AF)

2. Degassed 50% (v/v) slurry of the Blue Trisacryl M resin

3. Degassed water or suitable buffer, such as 50 mM Tris HCl pH 8.8

Tips on Handling Blue Trisacryl M 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. Also, these resins are quite dense and settle quickly. When adding slurry to any device, mix well between additions. If it is necessay 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 settled resin. Decant the supernatant and add back an equal volume of water or buffer to make a 50% (v/v) slurry.

B. Packing Blue Trisacryl M Resin

1. Gravity flow column format

a. Equilibrate column, degassed 50% (v/v) 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 ºC for no more than one week or for immediate use.

i. Refer to Section C below 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. Glass chromatography column format

a. Equilibrate column, degassed 50% (v/v) gel slurry, and degassed buffer solution (or high purity water) to room temperature.

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

c. Add a 1 mL of degassed buffer/water 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 ºC for no more than one week or for immediate use.

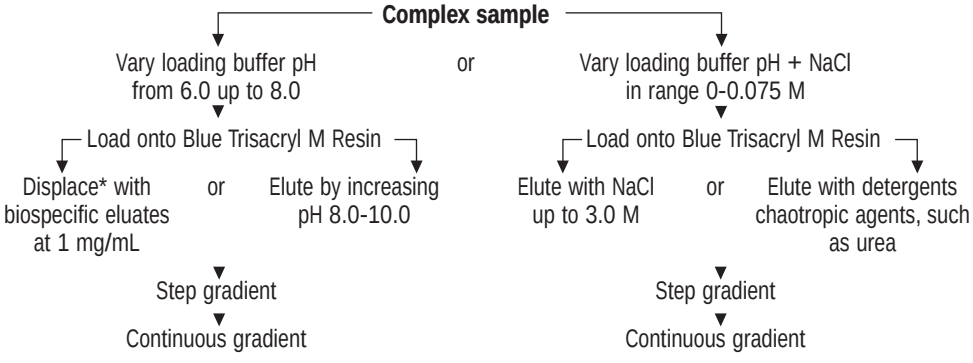
i. Refer to Section C below for use instructions.

C. Application Guidelines for Blue Trisacryl® M Resin in the Above Formats

Blue Trisacryl M resin is a group-specific adsorbent with affinity for a wide variety of enzymes[1,2]. Some proteins interact biospecifically with the dye due to its structural similarity with nucleotide cofactors (ADP, NAD, NADP), while others, such as albumin and interferon, bind in a less specific manner by electrostatic and/or hydrophobic interactions with the aromatic anionic ligand. Biospecifically adsorbed proteins can be eluted by low concentrations of the free cofactor, or increased ionic strength. Less specifically bound proteins require the use of much higher cofactors, or salt concentrations. Desorption with cofactors normally occurs in the range 1-20 mM. Desorption by increasing ionic strength is normally complete at salt concentrations 2 M or less (NaCl or KCl are suitable).

Blue dye mimetic-based chromatographic purification of components of serum or plasma can be carried out by two approaches: changing the pH of the buffer (range pH 6-8) and ionic strength (up to 75 mM NaCl) during adsorption to the resin, and elute bound material by increased salt, pH, or cofactors and the use of detergents or chaotropic agents, such as urea to break up adsorption to the column or protein-protein interactions on the adsorbed abundant protein, such as albumin. The two approaches are useful in developing an optimal purification strategy for components of serum or plasma in one of the following formats and are summarized in Table 4.12.

Table 4.12
Summary of Purification Options for Blue Trisacryl M Resin



**Biospecific elution by cofactors such as ADP, NAD, NADP, NADH.*

1. Gravity flow column format
   - **a.** Prepare a 1-2 mL column as described above.
   - **b.** Wash the Blue Trisacryl M resin with 5 column volumes (CV) of buffer to remove the 1 M NaCl, 20% (v/v) ethanol 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 4.12. The sample should be buffer exchanged (see Section 2.4, page 152 for details on the use of Pall centrifugal UF devices) or dialyzed to reduce the salt concentration in the original sample and adjust the loading conditions.*

   - **d.** After sample has drained into the column, replace with binding buffer and 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).*

   - **e.** After 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 raising the pH up to 10.0, raising the ionic strength up to 3 M NaCl, addition of cofactors up to 1-20 mM, detergent elution with 1% (w/v) sodium deoxycholate, 1% (v/v) Triton* X-100, or elution with urea up to 8 M. Combinations of elution steps should also be evaluated, such as salt gradient elution at different pH values up to 10.0. The elution can be carried out as a series of steps.

g. 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).*

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

2. Packed glass column on a liquid chromatography system

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

b. At this stage, sample loading conditions should be optimized following recommendations in Table 4.12. The sample should be buffer exchanged (see Section 2.4, page 152 for details on the use of Pall centrifugal UF devices) or dialyzed to reduce the salt concentration in the original sample and adjust the loading conditions.

c. Load the sample up to a 2 mL volume onto the column at 0.5-1 mL/min flow rate. Monitor effluent at 280 nm.

d. Collect the column effluent in 1 mL fractions.

Tip: *Flowthrough protein rapidly elutes from the column and should be found in the first three fractions.*

e. The column should then be washed with 5-10 CV of equilibration buffer or until the absorbance trace at 280 nm returns to baseline.

f. Retained fractions can then be eluted by raising the pH up to 10.0, raising the ionic strength up to 3 M NaCl, addition of cofactors up to 1-20 mM, detergent elution with 1% (w/v) sodium deoxycholate,1% (v/v) Triton X-100, or elution with urea up to 8 M. Combinations of elution steps should also be evaluated, such as salt gradient elution at different pH values up to 10.0. The elution can be carried out as a series of steps or a continuous linear gradient.

g. The volume of the gradient should be at least 10 CV. Fractions of 1 mL should be collected. After protein has eluted, the column can be re-used by flushing with 5-10 CV of equilibration buffer to return the system back to initial buffer conditions.

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).*

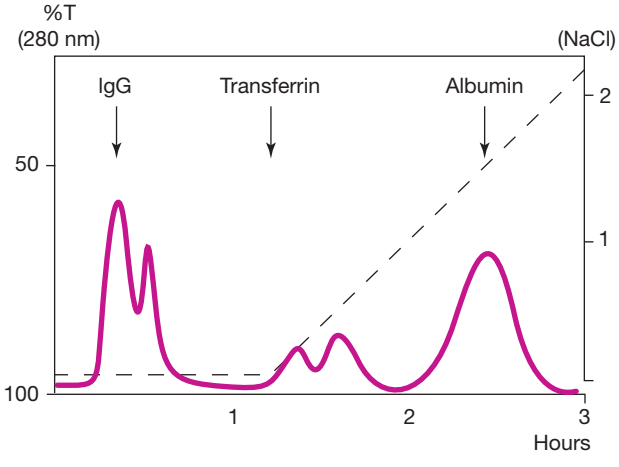
h. If the column will be re-used within 24 hours, store at 4 ºC. If longer storage is required, add 0.02% (w/v) sodium azide to the last 5 CV of equilibration buffer and store at 4 ºC.

Application Data for Blue Trisacryl® M Resin

Analytical Separation of Human Plasma Proteins on Blue Trisacryl M Resin

Fractionation of plasma in a chromatographic separation has been demonstrated for Blue Trisacryl M resin and is summarized in Figure 4.15. The resulting chromatogram shows that this dye mimetic surface can show selectivity towards albumin retention. Using this property, some large-scale industrial processes have been developed to purify human albumin to a high degree of purity (> 98% by electrophoresis). The application of this resin to large scale processing and its ability to create sub-fractions of plasma has important application in proteomics, such as bulk albumin depletion of pooled plasma samples.

Figure 4.15



Column: 1.6 cm I.D. x 10 cm; Buffer: 0 05 M Tris-HCl, pH 8 8. Elution performed by a continuous sodium chloride gradient from 0 to 3 M; Flow Rate: 100 cm/h; Separation time: 180 minutes; Temperature: 20 ºC.

Ordering Information for Blue Trisacryl® M Resin

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

References for Blue Trisacryl M Resin

1. Subramanian, S. (1984). Dye-ligand affinity chromatography: the interaction of Cibacron* Blue F3GA with proteins and enzymes. *CRC Crit Rev Biochem.*, 16(2), 169-205.
2. Ramadoss, C.S., Steczko, J., Uhlig, J.W., & Axelrod, B. (1983, April 15). Effect of albumin on binding and recovery of enzymes in affinity chromatography on Cibacron Blue. *Anal Biochem.*, 130(2), 481–484.

4.2.4 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[1,2,3]. 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 revolution of expression, cell-mapping, and structural proteomics.

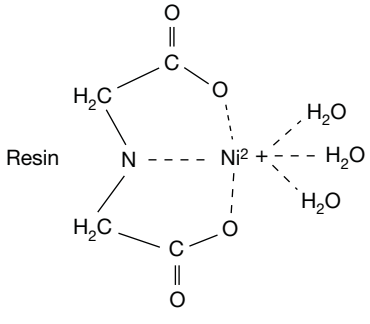
Resins suitable for IMAC are modified to provide strong cation chelation chemistry with a high affinity for transition metals, such as Cu (II), Ni (II), Zn (II), and Fe (III). An example of the tridentate IDA (iminodiacetic acid) chemistry on IMAC HyperCel resin is shown in Figure 4.16, for a Ni (II) 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 Zr (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 4.16

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 4.13. In this section, the focus will be on small-scale purification in gravity flow and packed glass columns for use on a liquid chromatography instrument. Methods development and scouting protocols employing small-scale single and multi-well devices are described in small-scale protein pre-fractionation in Section 2.2.3.3, page 88.

Table 4.13
Properties of IMAC HyperCel™ Resin

Specification	Parameter
Particle Size	80-100 µm (average)
Particle Composition	Crosslinked 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 (44 psi)
Working Pressure	< 1 bar (14 psi)
Thermal Stability	4-121 °C

Protocol for IMAC HyperCel Resin for Immobilized Metal Ion Affinity Chromatography

A. Materials Required

1. Choose one of the following:
   a. Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922); or
   b. Glass column 6.6 mm ID x 10 cm length, 1-2 mL volume (e.g., Omnifit PN 006CC-06-10-AF)
2. Degassed 50% (v/v) slurry of the IMAC HyperCel resin
3. Degassed buffer, such as 20 mM sodium phosphate, pH 7.4, containing 0.14 M NaCl or phosphate buffered saline (PBS)

Tip: *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. 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 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 IMAC HyperCel™ 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 storage at 4 ºC for no more than one week or for immediate use.
- **i.** Refer to Section C on page 335 for use instructions.

Tip: *Store the packed column upright at 4 ºC 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. Glass chromatography column format

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

b. Attach the bottom end fitting onto 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 onto 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 ºC for no more than one week or for immediate use.

i. Refer to Section C below for use instructions.

C. Loading Metal Ion onto 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 1,000 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 4.14 for 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.*

Table 4.14
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 by eye or can be measured at a suitable wavelength for the colored metal ion (measure absorption spectrum of starting metal ion solution).*

f. Final 2x 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% (w/v) sodium azide to the last water wash and place at 4 ºC.

2. Binding in gravity flow format.

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

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

c. Wash the column with 5-10 column volumes (CV) of high purity water to remove any remaining 1 M NaCl, 20% 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 4.14 for 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 by eye 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). Ready for use at this step.

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

E. Immobilized Metal Ion-based (IMAC) Fractionation

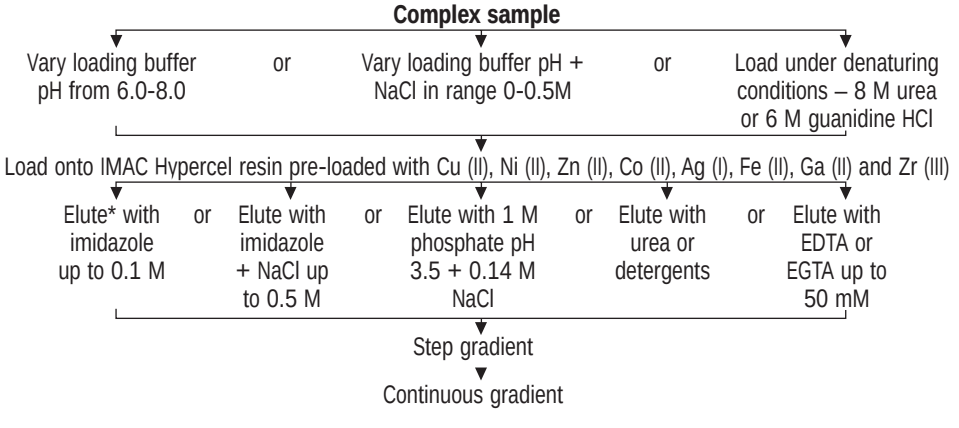
One of the following approaches can be used:

- Investigating the impact of metal ions, such as Fe (III), Cu (II), Zn (II), Co (II), Ni (II), Ag (II), Ga (II), and Zr (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, 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 SDS as a dissociating agent. Combinations of the above approaches are useful in developing an optimal pre-fractionation strategy, and are summarized in Table 4.15. Either strategy or a combination of both can be applied to pre-fractionate a complex sample in one of the following formats.

Table 4.15

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.*

1. Gravity flow column format

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

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 4.15.*

c. Collect the column effluent in 1 mL fractions. Measure the 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. For details, see Section 2.4, page 152.*

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

e. 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 spin filter with a 10K MWCO membrane (see Section 2.4, page 152).*

h. 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. Chromatography glass column format

a. Prepare a 1-2 mL column as described above with resin that has been pre-loaded with metal ion.

b. If necessary, on-column metal ion column loading can be carried out as outlined below.

(1) Prime (with high purity water) the solvent delivery system, lines, and valves with a column bypass loop in place to assure that all air is displaced.

(2) Prime buffer line B with 0.15 M metal ion solution (see Table 4.14 for 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 to remove any insoluble material.*

(3) Prime buffer line C with 1 M NaCl.

(4) Adjust the pump to provide a volumetric flow rate (i.e., mL/min) equivalent to 600-1000 cm/h linear velocity. Check that this volume is being delivered using a measuring cylinder and timer.

(5) Stop liquid flow.

(6) Disconnect the column bypass loop and reinitiate flow with buffer A (water) in the reverse direction. Attach column and pump air up out of the top fittings. Attach to the liquid chromatography system. Continue flow until air in the system is displaced to waste.

(7) Reverse flow top to bottom and equilibrate with buffer A for 5-10 CV, or until a stable low conductivity baseline is achieved.

(8) Switch to buffer line B and pump metal ion solution onto the column until a stable plateau of conductivity is achieved. Continue flow for an additional 10 CV to fully saturate the metal ion binding capacity of the resin.

(9) Switch to buffer line A and wash till a low conductivity baseline is achieved.

Tip: *If an absorbance detector in the visible light range is available, monitor the specific wavelength for the colored metal ion in use.*

(10) Switch to buffer line C and flow until a plateau of conductivity is achieved and continue for 10 CV.

(11) Finally, switch back to buffer line A and wash the column back down to low baseline.

c. Equilibrate the column to IMAC loading conditions.

Tip: *At this stage sample loading conditions should be optimized following recommendations in Table 4.15.*

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

e. Collect the column effluent in 1 mL fractions. Measure the A_{280} 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 Macrosep® centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).*

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

g. After protein has eluted, the column can be regenerated by 5 CV of 3.0 M NaCl followed by equilibration back to initial buffer conditions. Some slight dilution of the sample will occur during elution. If necessary these fractions can be concentrated with a centrifugal UF spin filter, such as a Nanosep or Macrosep centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).

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	1000 mL

References for IMAC HyperCel Resin for Immobilized Metal Ion Affinity Chromatography

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4.2.5 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 complementary 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 crosslinked agarose beads with microcrystals entrapped within 60-180 µm agarose particles. The agarose particles are crosslinked 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 4.16. In this section, the focus will be on small-scale purification in gravity flow and packed glass columns for use on a liquid chromatography instrument. Methods development and scouting protocols employing small-scale single and multi-well devices are described in small-scale protein pre-fractionation in Section 2.2.4.2, page 121.

Table 4.16
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% 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. Choose one of the following:
   - **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922); or
   - **b.** Glass column 6.6 mm ID x 10 cm length, 1-2 mL volume (e.g., Omnifit PN 006CC-06-10-AF)
2. Degassed 50% (v/v) slurry of the HA Ultrogel resin
3. Degassed buffer, such as 1 mM phosphate buffer pH 6.8

Tips on Handling HA Ultrogel 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. 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 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 storage at 4 ºC for no more than one week or for immediate use.

i. Refer to Section C on page 344 for use instructions.

Tips: *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. Glass chromatography column format

a. Equilibrate column, degassed 50% (v/v) 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 ºC for no more than one week or for immediate use.

i. Refer to Section C below for use instructions.

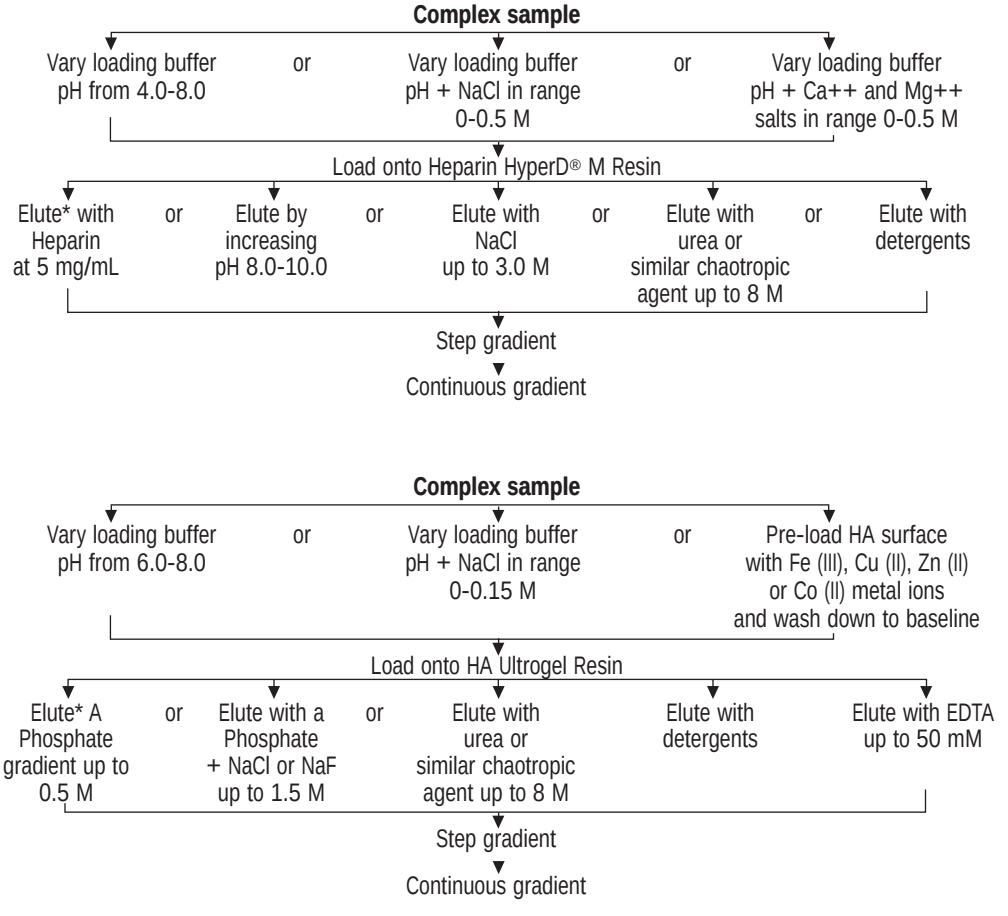
C. Hydroxyapatite Pseudo-affinity-based Pre-fractionation

One of 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; or
- 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: 1) use of a phosphate buffer gradient up to 0.5 M; 2) addition of NaCl to the above phosphate buffer gradient up to 1.5 M; 3) use of chaotropic agents such as urea up to 8 M; and 4) 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 4.17. Either strategy or a combination of both can be applied to pre-fractionate a complex sample in one of the following formats.

Table 4.17
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.*

1. Gravity flow column format
   - **a.** Prepare a 1-2 mL column as described above.
   - **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 4.17.*

   - **c.** Collect the column effluent in 1 mL fractions. Measure the 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. For details, see Section 2.4, page 152.*

d. After unretained protein has been eluted, the column should be washed with 5 column volumes (CV) of loading buffer before elution of retained material is attempted.

e. 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).*

f. 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. Chromatography glass column format

a. Prepare a 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 effluent at 280 nm.

Tip: *At this stage sample loading conditions should be optimized following recommendations in Table 4.17.*

c. Collect the column effluent in 1 mL fractions. Measure the A_{280} 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 Macrosep® centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).*

d. 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 the gradient should be at least 10 CV. Fractions of 1 mL should be collected.

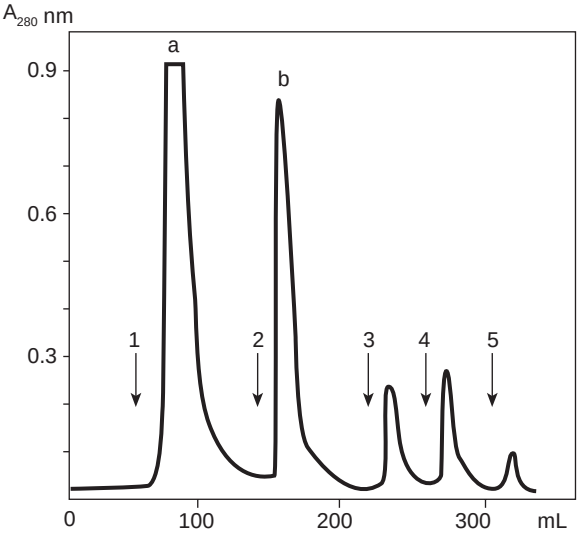
e. After protein has been eluted, the column can be regenerated by 5 CV of 3.0 M NaCl followed by equilibration back to initial buffer conditions. Some slight dilution of the sample will occur during elution. If necessary these fractions can be concentrated with a centrifugal UF spin filter, such as a Nanosep® or Macrosep® centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).

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 4.17 and 4.18). 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.

Figure 4.17

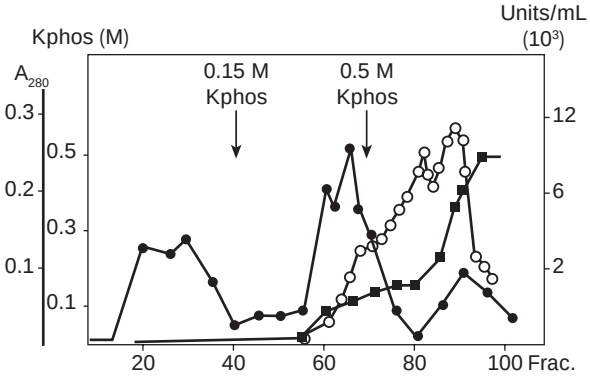
Separation of Glycohydrolases from a Crude Enzyme Extract of Buckwheat



Column: 2 x 6 cm; Sample: 40 mg of lyophilized extract in 1 mL of 1 mM phosphate buffer, pH 6.8; Discontinuous elution gradient of phosphate buffer; Flow rate: 7.1 cm/h; Temperature: 4 °C. Peak a: proteins with no glycohydrolase activity; peak b: glucosidase. Courtesy of R. Rourbouze & F. Percheron, Biochemistry Lab., Faculty of Pharmacy, Paris.

Figure 4.18

Purification of Damage-Specific DNA Binding Protein from Human Placenta



The sample was previously pre-purified from human placenta by ammonium sulfate precipitation, ion exchange chromatography and gel filtration. Column: 2.5 x 6 cm; Initial buffer: 10 mM potassium phosphate, pH 8 containing 5% glycerol and 13 mM 2-mercapto-ethanol; 1st elution (arrow): 0.15 M potassium phosphate buffer; 2nd (arrow): 0.5 M potassium phosphate buffer; Fraction volume: 1.8 mL. ●—●A280 nm ■—■ DNA binding activity o—o conductivity. Courtesy of R.S. Feldberg, et al., J. Biol. Chem. 257 (1982) 6394–401.

Hydroxyapatite has been reported to have several unique applications[1] in the purification of monoclonal antibodies:

- Adsorption of Protein A leaching off affinity columns.
- Removal of endotoxin.
- Adsorption of DNA.
- Resolution of IgG aggregates in samples eluting from Protein A supports at low pH.

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

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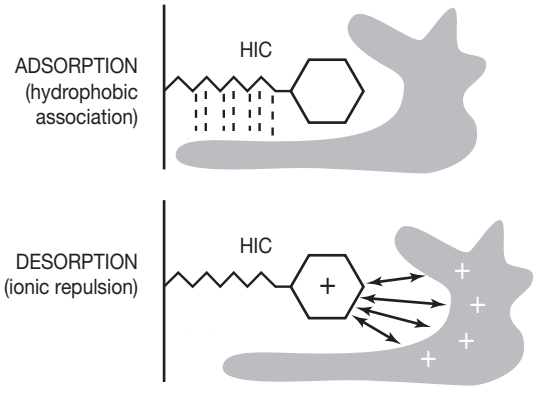
4.2.6 MEP HyperCel™ Resin for Purification of Antibodies by Hydrophobic Charge Induction Chromatography (HCIC)

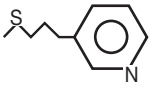
Hydrophobic Charge Induction Chromatography (HCIC) is based on the pH-dependent behavior of ionizable, dual-mode ligands. MEP HyperCel resin carries an antibody-selective ligand, 4-Mercapto-Ethyl-Pyridine (4-MEP). As shown in Figure 4.19, adsorption is based on mild hydrophobic interaction and is achieved without addition of lyotropic or other salts. Desorption is based on charge repulsion. It is performed by reducing the pH.

Figure 4.19
Protein Adsorption and Elution Mechanism on MEP HyperCel Resin



In contrast to traditional hydrophobic interaction chromatography, HCIC is controlled on the basis of pH rather than salt concentration. Antibody elution is conducted at low ionic strength, eliminating the need for extensive diafiltration in applications where ion exchange chromatography will follow capture. Compared to chromatography on Protein A sorbents, elution from MEP HyperCel resin is achieved under relatively mild conditions (pH 4.0). Thus, aggregate formation is reduced and antibody activity is preserved. MEP HyperCel resin is composed of cellulose matrix to which 4-Mercapto-Ethyl- Pyridine (4-MEP) is linked. The cellulose bead confers high porosity, chemical stability, and low non-specific interaction. Bead size is 80-100 µm to allow a good compromise between capacity and flow property. 4-MEP was chosen for its high selectivity and capacity for antibodies, and for its pKa which is 4.8. It contains a hydrophobic tail and an ionizable headgroup. At physiological pH, the aromatic pyridine ring (see Figure 4.20) is uncharged and hydrophobic. Additional contributions to binding are provided by the aliphatic spacer arm. Antibody binding is further enhanced by interaction with the other group. Both ligand structure and ligand density are designed to provide effective binding in the absence of lyotropic or other salts. When pH of the mobile phase is adjusted to values below 4.8 (typically pH 4.0), the ligand takes on a distinct positive charge. Under such conditions, antibody molecules also carry a positive charge. Electrostatic repulsion is induced and antibody is desorbed.

Figure 4.20
MEP HyperCel™ Resin Mimetic Affinity Ligand



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

MEP HyperCel resin provides high binding capacity and high flow rates, ideal for small- and large-scale antibody purification applications. Purification properties are summarized in Table 4.18. In this section, the focus will be on small-scale purification in gravity flow and packed glass columns for use on a liquid chromatography instrument. Methods development and scouting protocols employing small-scale single- and multi-well devices are described in small-scale protein pre-fractionation in Section 2.2.4.1.

Table 4.18
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 (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 Hhuman 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 IgG1	37
Murine IgG2a	34

Protocol for MEP HyperCel™ Resin for Purification of Antibodies by Hydrophobic Charge Induction Chromatography (HCIC)

A. Materials Required

1. Choose one of the following:
   - **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922); or
   - **b.** Glass column 6.6 mm ID x 10 cm length, 1-2 mL volume (e.g., Omnifit PN 006CC-06-10-AF)
2. Degassed 50% (v/v) slurry of the MEP HyperCel resin
3. Degassed water or suitable loading buffer, such as 50 mM Tris HCl pH 8.0

Tips on Handling MEP 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. 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 the bulk media. Thoroughly mix and allow settling, noting 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.

B. Packing MEP HyperCel Resin

1. Gravity flow column format
   - **a.** Equilibrate column, degassed 50% (v/v) 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 the 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 ºC for no more than one week or for immediate use.

i. Refer to Section C below 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. Glass chromatography column format

a. Equilibrate column, degassed 50% (v/v) gel slurry, and degassed buffer solution (or high purity water) 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/water 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.

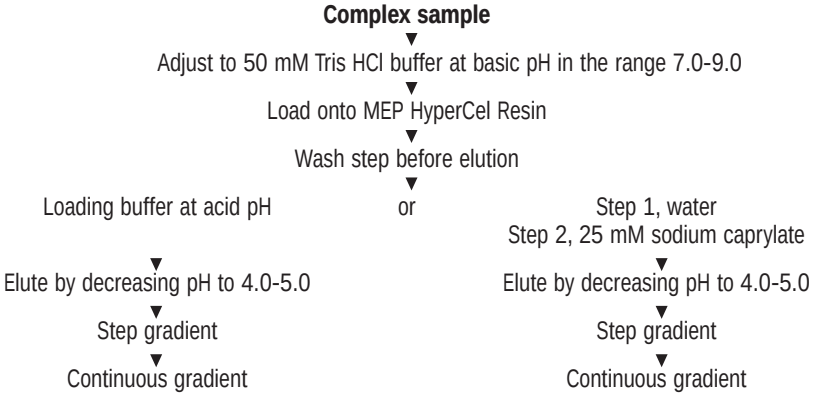
h. Packed column is now ready for storage at 4 ºC for no more than one week or for immediate use.

i. Refer to Section C below for use instructions.

C. Antibody Purification from Ascites or Cell Culture Media

A summary of the binding and elution conditions of MEP HyperCel™ resin are summarized in Table 4.19. There are some variables such as loading buffer pH and ionic strength that can be optimized to bind antibodies to the MEP mixed mode chromatography supports. The optimal elution conditions for MEP HyperCel resin are at acidic pH. Combinations of the above approaches are useful in developing an optimal purification strategy in one of the following formats.

Table 4.19
Summary of Purification Options for MEP HyperCel™ Mixed Mode Resin



1. Gravity flow column format
   a. Prepare a 1-2 mL column as described above.
   b. Wash the MEP HyperCel resin with 5 column volumes (CV) of water or 50 mM Tris HCL pH 8.0 to remove the 0.02% (w/v) sodium azide storage buffer.
   c. Allow the liquid to drain from the column and load the sample onto the column.

Tip: *Due to the unique low salt mediated mechanism of MEP HyperCel resin adsorption of IgG, it is possible to load large volumes of dilute protein-free and 5% (v/v) fetal calf supplemented culture supernatant. The sample should be prefiltered with an Acrodisc® syringe filter or a VacuCap® and VacuCap PF Vacuum Filtration system (see Section 2.5.5, page 221) to ensure that the MEP HyperCel column will not be blocked by particulate material. For ascites, dilute the sample 1:1 with loading buffer to reduce viscosity.*

   d. After loading the sample, wash with loading buffer or phosphate buffered saline (PBS) until the effluent shows no absorbance at 280 nm.

Tip: *In the case of 5% supplemented culture media, two extra wash steps are required; water followed by 25 mM sodium caprylate promotes desorption of albumin.*

   e. Elute the bound IgG with 50 mM sodium acetate buffer pH 4.0.
   f. Collect the column effluent in 1 mL fractions. Measure the A_{280} 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 (see Section 2.4, page 152).*

2. Chromatography glass column format

a. Prepare a 1-2 mL column as described above and equilibrate with 50 mM Tris HCl pH 8.0.

b. Load the sample onto the column at 1 mL/min flow rate. Monitor effluent at 280 nm.

c. After sample loading, wash column to baseline with 10 CV of loading buffer or PBS.

d. If using 5% supplemented culture fluid, add additional 5 CV water and 5 CV Sodium Caprylate wash steps.

e. Elute with a step gradient down to pH 4.0 with 50 mM sodium acetate buffer.

f. 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 (see Section 2.4, page 152.)*

D. Stability and Cleaning

The physical and chemical properties of MEP HyperCel™ resin are well suited to both laboratory and process scale use. 0.5-1.0 M sodium hydroxide is recommended for cleaning. Submitted to a series of 200 clean-in-place cycles with 1 M sodium hydroxide (1 hour contact/cycle), the resin maintained its initial properties. Ligand density was unchanged. Other useful cleaning agents include 4-6 M guanidine, 8 M urea, and 50/50 ethylene glycol/water.

Application Data for MEP HyperCel Resin for Purification of Antibodies by Hydrophobic Charge Induction Chromatography (HCIC)

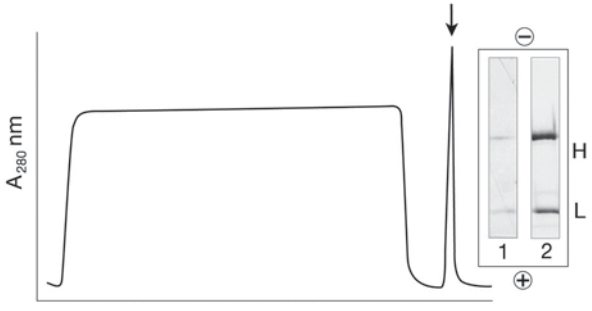
Monoclonal Antibody Purification from Cell Culture Media

Capture of antibody from both protein-free and serum-supplemented (5% 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 4.21 and results are summarized in Table 4.20. 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 4.21

Monoclonal Antibodies Purification on MEP HyperCel™ Resin from Cell Culture Supernatants (CCS)

Panel A, from serum-free cell culture media



Sample A = 300 mL protein-free CCS. Sample B = 300 mL CCS containing 5% fetal bovine serum; Equilibration: 50 mM Tris HCl, pH 8; Elution: 50 mM acetate, pH 4; Flow rate: 70 cm/h. In curve B, (a) and (b) are respectively water and 25 mM sodium caprylate washings; SDS-PAGE (reduced conditions): (1) = crude sample; (2) = purified IgG. H = Heavy chain; L = Light chain.

Panel B, from 5% serum supplemented cell culture media

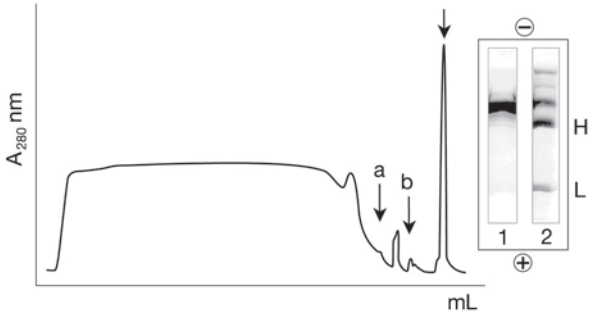


Table 4.20

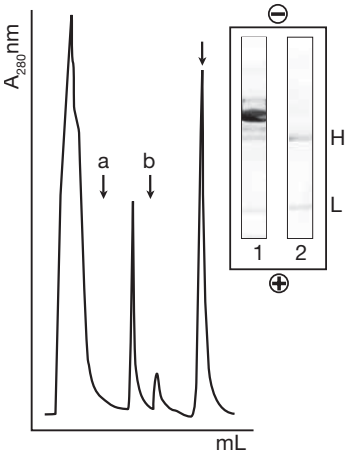
Summary of Monoclonal Antibody Purification on MEP HyperCel Resin

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

Monoclonal Antibody Purification from Ascites Fluid

IgG was isolated from ascites fluid using the same procedure as described above for use with serum-supplemented CCS. The chromatogram appears in Figure 4.22. Results are summarized in Table 4.20. Isolated product was 83% pure. Purity of the IgG fraction could be brought to 98% using the anion exchange procedure summarized below.

Figure 4.22
Immunoglobulin Capture from Ascites Fluid on MEP HyperCel™ Resin



(a), (b) = Contaminant elution peaks after the 2 washing steps; same conditions as those of Figure 4.21. SDS-PAGE (reduced conditions) analysis: (1) = crude sample; (2) = purified IgG.

MEP HyperCel Resin Followed by Anion Exchange Chromatography
Efficient removal of residual albumin present in the IgG fraction can be accomplished using Ceramic HyperD® F DEAE resin. The column is equilibrated with 25 mM Tris buffer, pH 8.8, containing sodium chloride sufficient to provide a conductivity of 10 mS. The IgG fraction obtained following HCIC is diluted with two volumes of the same buffer and applied to the column. After loading, elution is continued with the above buffer. Antibody appears as an unretained peak, while albumin is retained. The latter is desorbed using 25 mM Tris buffer containing 0.5-1.0 M sodium chloride, pH 8.8.

Ordering Information for MEP HyperCel™ Resin for Purification of Antibodies by Hydrophobic Charge Induction Chromatography (HCIC)

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

4.2 – Section 4.2.6

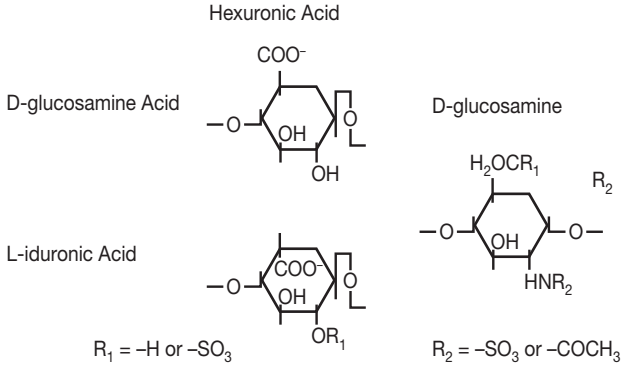
References for MEP HyperCel Resin for Purification of Antibodies by Hydrophobic Charge Induction Chromatography (HCIC)

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.

4.2.7 Heparin HyperD® Resin

Heparin is a mucopolysaccharide consisting of a repeating dimer of Hexuronic acid, and D-glucosamine (see Figure 4.23), 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, e.g. 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 4.23
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.

Immobilized heparin has been used to purify enzymes (mast cell proteases, lipoprotein lipase, coagulation enzymes superoxide dismutase); serine protease inhibitors (antithrombin III, protease nexins); growth factors (fibroblast growth factor, Schwann cell growth factor, endothelial cell growth factor); extracellular matrix proteins (fibronectin, vitronectin, laminin, thrombospondin, collagens); nucleic acid-binding proteins (initiation factors, elongation factors, restriction endonucleases, DNA ligase, DNA, and RNA polymerases); hormone receptors (oestrogen, androgen receptors); and lipoproteins.

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 based in HyperD particles is presented in Table 4.21. In this section, the focus will be on small-scale purification in gravity flow and packed glass columns for use on a liquid chromatography instrument. Methods development and scouting protocols employing small-scale single- and multi-well devices are described in small-scale protein pre-fractionation in Section 2.2.3.1, page 72.

Table 4.21
Properties of Heparin HyperD® 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, 1015 psi)

**Determined using human ATIII at 72.5 UI/mL in 20 mM TrisHCl, 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 Resin

A. Materials Required

1. Choose one of the following:
   - **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922); or
   - **b.** Glass column 6.6 mm ID x 10 cm length, 1-2 mL volume (e.g., Omnifit PN 006CC-06-10-AF)
2. Degassed 50% (v/v) slurry of the Heparin HyperD M resin
3. Degassed buffer, such as 20 mM Tris HCl, 0.3 M NaCl, pH 7.4

Tips on Handling Heparin HyperD 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. 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 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 Heparin HyperD® M 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 storage at 4 ºC for no more than one week or for immediate use.
- **i.** Refer to Section C on page 362 for use instructions.

Notes: *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. Glass chromatography column format

a. Equilibrate column, degassed 50% (v/v) 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 ºC for no more than one week or for immediate use.

i. Refer to Section C below for use instructions.

C. Heparin Affinity-based Pre-fractionation

One of the following approaches can be used:

- Varying the binding conditions: 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: 1) specific elution with a heparin solution; 2) use of a pH gradient from 8-10; 3) use of a NaCl gradient up to 2-3 M, 4) use of chaotropic agents such as urea up to 8 M; 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 purification strategy and are summarized in Table 4.22. Either strategy or a combination of both can be applied to purifiy components of a complex sample, such as plasma or serum in one of the following formats.

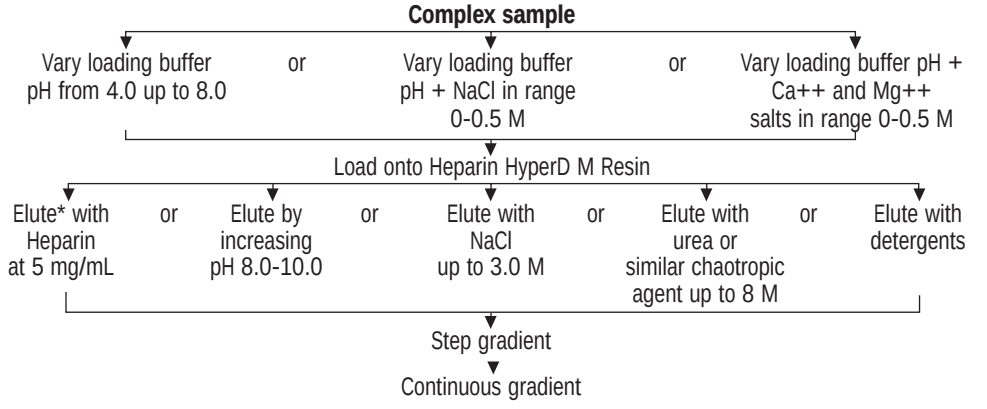
1. Gravity flow column format

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

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 4.22.*

Table 4.22
Summary of Purification 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.*

c. Collect the column effluent in 1 mL fractions. Measure the 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).*

d. After unretained protein has been eluted, the column should be washed with 5 column volumes (CV) of loading buffer before elution of retained material is attempted.

e. Retained fractions can then be eluted by biospecific elution with heparin (5 mg/mL); b) pH gradient from 8.0-10.0, serial steps up to 3.0 M salt, 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. 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).*

f. 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 SDR detergent removal (see Section 2.3, page 141). After an SDS detergent elution, the column should be discarded.

2. Chromatography glass column format

a. Prepare a 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 effluent at 280 nm.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 4.22.*

c. Collect the column effluent in 1 mL fractions. Measure the A_{280} 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® or Macrosep® centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).*

d. Retained fractions can then be eluted by biospecific elution with heparin (5 mg/mL), pH gradient from 8.0-10.0, serial steps up to 3.0 M salt, 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. The volume of the gradient should be at least 10 CV. Fractions of 1 mL should be collected.

e. After protein has eluted, the column can be regenerated by 5 CV of 3.0 M NaCl followed by equilibration back to initial buffer conditions. Some slight dilution of the sample will occur during elution. If necessary these fractions can be re-concentrated with a centrifugal UF spin filter, such as a Nanosep or Macrosep centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).

Ordering Information for Heparin HyperD® 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 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.

4.2.8 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 4.23.

Table 4.23
Macromolecules Purified or Show Affinity for a Lysine Affinity Ligand

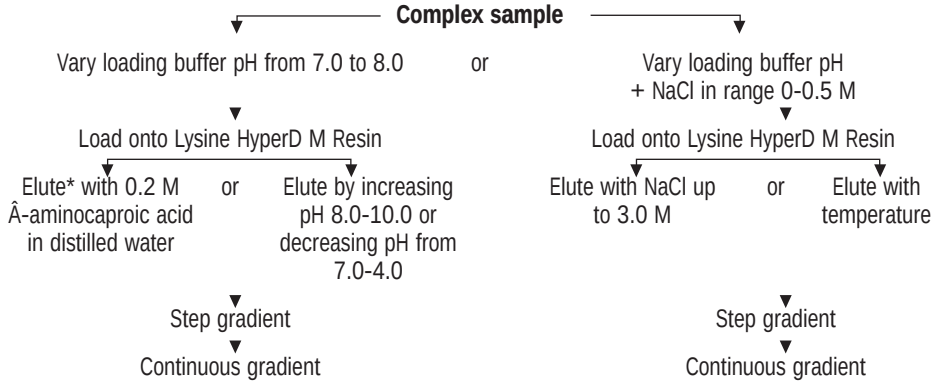
Macromolecule	Reference
Cytochrome C Oxidase	*Protein Expr Purif.* 1992 Feb;3(1):36-40
Fibrinogen from Human Plasma	*Prep Biochem Biotechnol.* 2003 Nov;33(4):239-52
Escherichia coli-derived Endotoxins	*J Chromatogr A.* 1995 Sep 8;711(1):81-92
105 kDa Protein Kinase from Rat Liver Nuclei	*Biochim Biophys 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 e-amino and a-carboxyl groups free to interact with sample substances during chromatography. The mechanism by which separation occurs appears to be both electrostatic and stereo-specific, 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. Properties of the Lysine HyperD M resin are summarized in Table 4.24. Recommendations for how the binding and elution conditions can be varied to bring about pre-fractionation are summarized in Table 4.25.

Table 4.24
Properties of Lysine HyperD M 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)

Table 4.25
Summary of Purification Options for Lysine HyperD® M Resin



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

Lysine HyperD M resin provides high binding capacity and high flow rates, ideal for small- and large-scale applications. In this section, the focus will be on small-scale purification in gravity flow and packed glass columns for use on a liquid chromatography instrument. Methods development and scouting protocols employing small-scale single and multi-well devices are described in small-scale protein pre-fractionation in Section 2.2.3.2, page 80.

Protocol for Lysine HyperD Resin

A. Materials Required

1. Choose one of the following:
   - **a.** Empty, plastic, small-volume column with porous PE frits (disposable polypropylene column, e.g., Pierce PN 29922); or
   - **b.** Glass column 6.6 mm ID x 10 cm length, 1-2 mL volume (e.g., Omnifit PN 006CC-06-10-AF)
2. Degassed 50% (v/v) slurry of the Lysine HyperD M resin
3. Degassed buffer, such as 50 mM phosphate buffer, pH 7.5

Tips on Handling Lysine HyperD 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. 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 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 Lysine HyperD® M 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/water to remove any gel that may have remained along the sides during packing.
- **k.** Packed column is now ready for storage at 4 ºC for no more than one week or for immediate use.
- **i.** Refer to Section C on page 368 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. Glass chromatography column format

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

b. Attach the bottom end fitting onto 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 ºC for no more than one week or for immediate use.

i. Refer to Section C below for use instructions.

C. Lysine Affinity-based Pre-fractionation

One of 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: 1) specific elution with 0.2 M Â-aminocaproic acid in distilled water for plasma plasminogen; 2) use of an up and down pH gradient; 3) use of a NaCl gradient up to 2-3 M; and 4) use of a temperature gradient.

The affinity, for example, of 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 4.25. 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 as described above.

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 4.25.*

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 unretained protein has been eluted, the column should be washed with 5 column volumes (CV) of loading buffer before elution of retained fractions 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 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).*

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

2. Chromatography glass column format

a. Prepare a 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 effluent at 280 nm.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 4.25.*

c. Collect the column effluent in 1 mL fractions. Measure the 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).*

d. 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.

e. The volume of the gradient should be at least 10 CV. Fractions of 1 mL should be collected. After protein has eluted, the column can be regenerated by 5 CV of 3.0 M NaCl or 1% (v/v) Triton X-100 in water warmed to 37 ºC followed by equilibration back to initial buffer conditions. Some slight dilution of the sample will occur during elution. If necessary, these fractions can be concentrated with a centrifugal UF spin filter, such as a Nanosep or Macrosep® centrifugal device, with a 10K MWCO UF membrane (see Section 2.4, page 152).

4.2 – Section 4.2.8

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.

4.2.9 Ultrogel® AcA Size Exclusion Chromatography Resin

Purification and desalting of components isolated from complex samples, such as cell lysates or plasma, by size exclusion or gel filtration chromatography can be used as a final polishing step after earlier chromatographic steps. This process can be used to remove aggregates or contaminants that can be resolved from the main product peak by size.

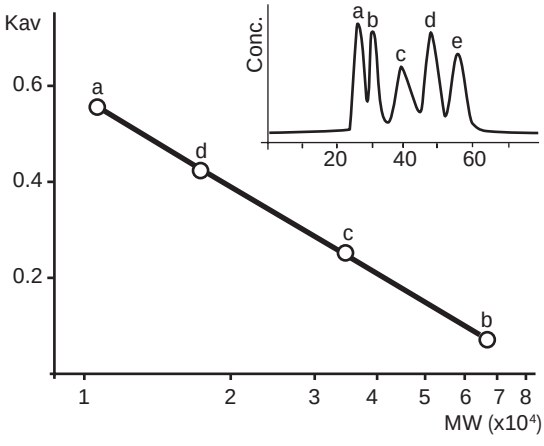
The molecular sieving process takes place as a solute passes through a packed bed stationary phase. The separation depends on the different abilities of the various sample molecules to enter the pores of the bead-based stationary phase. Large molecules, which cannot enter the pores, are excluded and pass through the column in the interstitial phase, eluting early in the void volume V_o. Smaller molecules, which can enter the pores are retarded and move through the column more slowly. Molecules are, therefore, eluted in a volume V_e, 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 volume of a peak of interest can be used to estimate its molecular weight. The term Kav, which represents the fraction of the stationary gel volume that 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 4.24 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 4.24

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 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, which 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 eluent 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 and 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 4.26.

Table 4.26
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 configuratio

In this section, the application focus will be on larger-scale protocols (> 10 mL) for size exclusion and desalting applications employing Ultrogel resins on a liquid chromatography workstation in glass columns. Smaller-volume size-based pre-fractionation (0.5-10 mL volume) of complex samples in packed glass columns gravity fed or pumped with a liquid chromatography workstation or peristaltic pump are described in Section 2.2.5, page 132.

Protocol for Ultrogel® AcA Size Exclusion Chromatography Resin

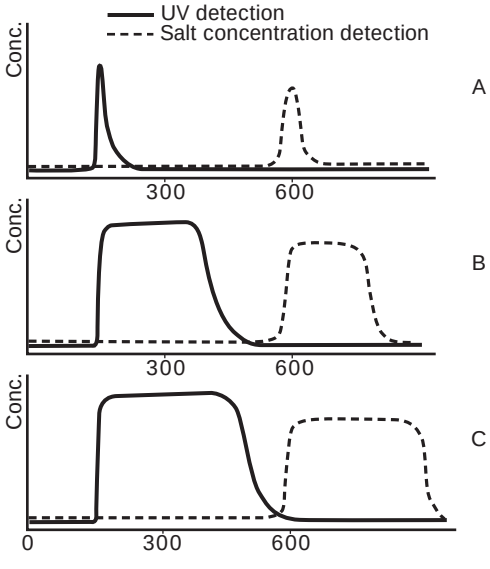
A. Materials Required

1. Glass column where the column diameter/length ratio is > 20 to provide the optimal geometry for peak resolution in size exclusion chromatography. Two examples are described in this protocol section to cover the 10-60 mL sample volume range. Other column configurations are possible as long as the above diameter/length ratio is met when size based fractionation is the intended application. In the case of desalting with Ultrogel AcA 202 resin, it is possible to use lower column diameter/length ratio due to the unique nature of this media and its application. In this process, the sample of interest is excluded from entering the gel-based media and rapidly passes through the packed media bed (see Figure 4.25).

a. Glass column 1.5 cm ID x 50 cm length, max 83 mL bed volume (e.g., Omnifit PN 006CC-15-50-AF) for a 2-10 mL sample volume load.

b. Glass column 3.5 cm ID x 100 cm length, max 693 mL bed volume (e.g., Omnifit PN 006CC-35-75-AF) for a 10-60 mL sample volume load.

Figure 4.25
Large-Scale Desalting on Ultrogel AcA 202 Resin



Column: 5 x 37 cm; volume: 730 mL; Sample: bovine serum albumin (5 mg/mL) containing NaCl (6.5 mg/mL); Flow rate: 7 cm/h (i.e. 140 mL/h). Sample volumes: A = 36 mL (5 % of sorbent volume); B = 220 mL (30 % of sorbent volume); C = 327 mL (45 % of sorbent volume); 280 nm UV detection.

2. Column packing reservoir for 1.5 cm x 50 cm column (e.g. Omnifit PN 006-PS-15 and empty glass column PN 006-RG-15-50).

3. Column packing reservoir for 3.5 cm x 75 cm column (e.g. Omnifit PN 006-PS-35 and empty glass column PN 006-RG-35-75).

4. Suitable gravity flow system of adjustable height to achieve flow in the above columns.

5. Peristaltic pump or liquid chromatography workstation able to provide buffer linear velocities in 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 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 Resin

For successful size exclusion chromatography, it is very important to prepare a properly packed Ultrogel AcA media 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.

1. Packed glass column format
   - **a.** Equilibrate column, packing accessories, degassed 50% (v/v) 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 form underneath the bottom support frit. Drain down to about 20% of the expected bed height. Stop flow.
   - **e.** Add sufficient volume of 50% (v/v) degassed gel slurry to obtain the desired settled gel volume of 69-83 mL (15 x 50 cm column) or 616-693 mL (35 x 75 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 that can lead to reduced flow rates.

k. The bottom outlet should then be connected to pump and buffer flowed up through the column at 0.5 mL/min to displace air from the top fittings.

k. Packed column is now ready for storage at 4 ºC for no more than one week or for immediate use.

i. Refer to Section C below for use instructions.

Tip: *Size exclusion chromatography can be carried out at a range of temperatures, including 4 ºC. The column must reach the desired temperature and equilibrate for 8-16 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 Purification

One of the following approaches can be used:

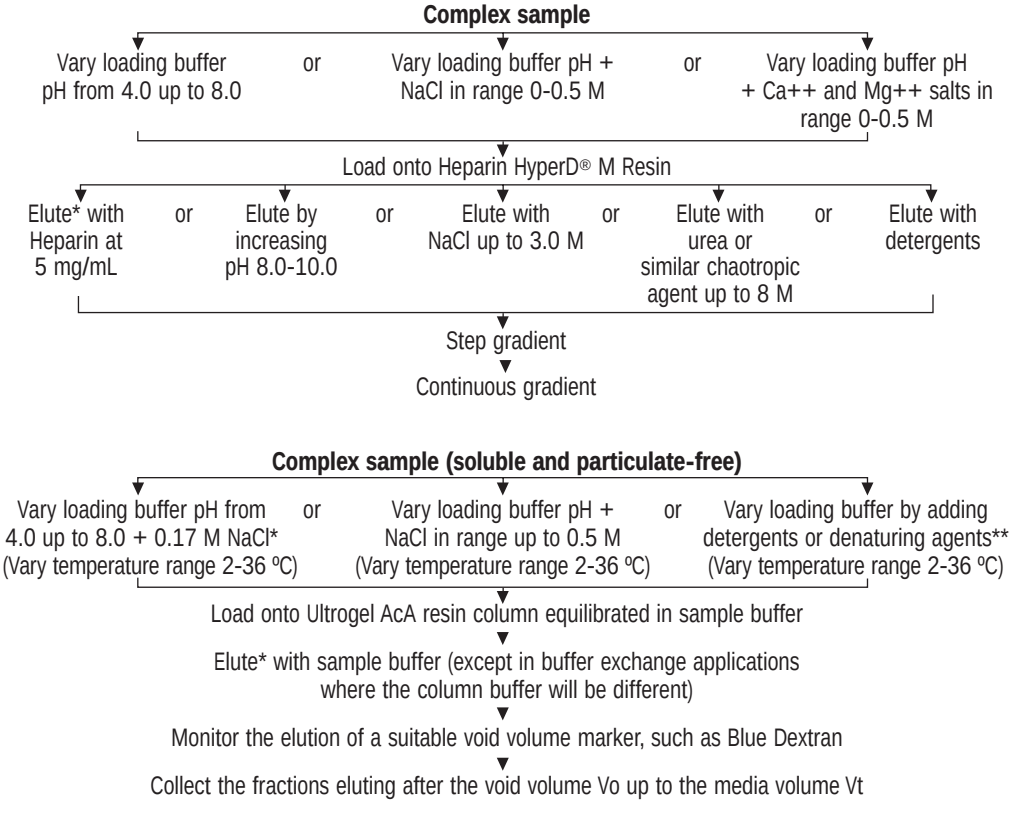
- Varying the binding conditions, such as pH and salt concentration of the sample to influence the size or shape of the proteins in the complex sample.
- Size exclusion can be carried out in the presence of limited amounts of detergents, such as sodium dodecyl sulfate (SDS), Triton* X-100 and sodium 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 media; or 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 size exclusion purification polishing step and are summarized in

Table 4.27. Either strategy or a combination of both can be applied to purify a component isolated from a complex sample. During size exclusion chromatography there will be some dilution of the samples fractionated on the media due to peak diffusion. If necessary, the sample (10-60 mL) can be concentrated with a centrifugal UF devices, such as a Pall Microsep™, Macrosep®, or Jumbosep™ with 10 K MWCO UF membrane (see Section 4.4, page 391). Or for larger volumes (25 mL-1 L) Minimate™ Tangential Flow Filtration Systems with a 10K MWCO UF membrane (contact Pall for more information). In the case of desalting with Ultrogel AcA 202, the excluded volume fraction will only experience slight dilution (peak volume may increase by 100-150% of the sample volume loaded) and may not required re-concentration.

Table 4.27

Summary of Pre-Fractionation Options for Ultrogel® AcA Size Exclusion Resin



**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 size of molecules in the complex sample that may impact the size 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, page 141).

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. In the case of desalting with Ultrogel® AcA 202 resin, this volume can be up to 45% due to the unique uniformity of the bead shape and pore size distribution. See Figure 4.25 for an example of this application.

a. Prepare a size exclusion column as described above and equilibrate with a suitable buffer of the same composition as the sample. Exception is in the case of a buffer exchange application (see Section 2.4, page 152) with Ultrogel AcA 202 resin, where the column equilibration buffer will be different.

b. Prepare a sample in the appropriate buffer (see Table 4.27 for options) and prefilter with a suitable volume 0.45 µm pore size microfiltration device (see Section 2.4, page 152). 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 the microfiltration, 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 the adjustable plunger method which requires the use of a three-way valve attached as close to the top fitting as possible. The column is set up and the top fitting 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 or a peristaltic pump via the three-way valve without disturbing the packed column bed.

d. For fractionation applications, load a sample volume 5-10% of the column volume; then elute with column buffer and collect the effluent in 1 mL fractions. Measure absorbance at 280 nm to locate eluting 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 or the media volume, fraction collection should be stopped as these fractions should be only very low molecular weight. Peaks can either be pooled or processed singly and some dilution of the sample will occur during elution. If necessary, the sample can be concentrated.

e. For desalting or buffer exchange applications, load up to 45% of the column volume then elute with column buffer (in the case of buffer exchange this will be a different buffer to the sample) and collect fractions. Monitor the A_{280} to locate eluting peaks.

f. The peak eluting with the void volume V_o will be the desalted sample.

g. If a high loading is used (up to 45% of the column volume), the desalted sample will continue eluting and should return to baseline absorbance before the low molecular weight fraction elutes (see Figure 4.25, Panel C).

h. If the desalted sample elutes after the low molecular weight fraction, the column was overloaded. Fractions containing low molecular weight material should be pooled separately and re-run on the column to achieve complete desalting.

i. After protein has eluted, the column can be washed with 2 CV (5 CV in the case of desalting to remove the low molecular weight fraction from the column bed) of loading buffer before re-use is attempted.

j. Packed column is now ready for storage at 4 ºC for no more than one week or for immediate use.

Tip: *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 ºC, the packed bed column should be re-equilibrated overnight. Before re-use, any sodium azide in the column that may be present should be removed by washing with 2 CV of buffer.*

Application Data for Ultrogel® AcA Size Exclusion Chromatography Resin

Selectivity Curve for Size-based Pre-fractionation Using Ultrogel AcA 54 Resin

Molecular weights of proteins may be reliably determined by choosing an Ultrogel AcA media which has the required fractionation range. The first step in the process consists in the creation of a selectivity curve from the elution curves of known standard proteins for the media and column configuration (see Figure 4.24). If the media or column configuration is changed, a new selectivity curve will need to be established. The second step consists of the size exclusion chromatography of the protein of interest and the comparison of its elution volume or calculated Kav with the selectivity curve to obtain an estimate of the molecular weight.

Ultrogel AcA resin provides excellent separation efficiency, as demonstrated by the low HETP (Height Equivalent to a Theoretical Plate) values in Table 4.26. The HETP of approximately 0.15 mm corresponds to over 1,500 theoretical plates per meter; except for Ultrogel AcA 202 resin where the HETP is 0.3 mm (eq. 3,000 plates per meter). There are only moderate HETP variations as a function of flow rate.

Desalting with Ultrogel AcA 202 Resin

In desalting applications, the separation of proteins from salts can be achieved with Ultrogel AcA 202 resin. When optimal column geometry is used, very large volumes may be processed. The sample volume must theoretically correspond to the difference between the total media volume and the void volume (generally 60% of the media volume). For traditional media, the recommended sample volume loaded is only 30% of the column volume. Because peak broadening is reduced with Ultrogel AcA 202 resin, as a result of the uniformity of the particles and the pores, the sample volume with this media may be as high as 40% of the media volume, or even 45% under certain circumstances. An example of a loading study on Ultrogel AcA 202 resin is shown in Figure 4.25. The data clearly show that loading up to 327 mL of 5 mg/mL BSA in Panel C (45% of the column volume) still achieved elution of the desalted protein before the conductivity due to salt increased.

The concentration of sample may be as high as 40 g/L without affecting the desalting quality. Peak fractions eluting after the void volume V_o, showed minimal dilution and may not require further re-concentration processing. In addition to the removal of salts, desalting can be extended to:

- Removal of low molecular weight sugars, such as lactose from whey.
- Removal of small aromatic compounds, such as phenol during the purification of nucleic acids.
- Removal of certain agents used for the solubilizing proteins, such as urea and guanidine salts.

Detergents, such as SDS, CHAPS and ASB-14, used above their critical micellar concentration form large micelles in aqueous systems. These micelles co-elute with the protein in the void volume V_o and cannot be desalted on an Ultrogel® AcA 202 column. At lower than their critical micellar concentration, where equilibrium favors the free-detergent state, it may be possible to use this bead-based desalting approach. At these concentrations, proteins may be less soluble, which can lead to aggregation or precipitation in the column. Trial experiments are recommended to screen for sample stability at these reduced detergent levels. As an alternative, removal of these detergents up to and above their critical micellar concentration limit can be achieved with SDR HyperD® resin (see Section 2.3, page 141).

Ordering Information for Ultrogel AcA Size Exclusion Chromatography Resin

Part Number	Description	Pkg
23013-014	Ultrogel AcA 22	1000 mL
23015-019	Ultrogel AcA 34	1000 mL
23022-015	Ultrogel AcA 44	1000 mL
23019-011	Ultrogel AcA 54	1000 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., et al. (2001). *J. 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.

4.3.1 Introduction

Chromatography continues to be an essential technology for the purification of biomolecules. Pall offers a line of chromatography resins ideal for protein purification applications. This broad line of chromatography products exhibits superior performance and is useful for affinity, ion exchange, size exclusion, and hydrophobic interaction chromatography (HIC). Unique mixed-mode BioSepra® products also exist to provide solutions to current sample preparation challenges.

The resins Pall offers for small-scale applications are the same ones offered to our customers currently manufacturing biopharmaceuticals. The ability to scale up is essential for those working in drug discovery, development, and manufacturing. These resins can be used in varying size chromatography columns, as well as in batch mode for single or high throughput mode. This is ideal for quick preps or in situations where optimizing purification conditions is required.

Pall also offers recommendations and services for streamlining initial scouting of protein purification conditions through our Process Proteomic Service Centers. The identification and optimization of protein purification parameters can be a tedious task. Process proteomic methodologies can be used to streamline the initial scouting of protein purification conditions, as well as for some of the optimization steps. Process proteomics is performed using common chromatographic chemistries (e.g., anion exchange, cation exchange, IMAC) on a protein chip, in the wells of a multi-well filter plate, or using small columns. Using this approach, multiple binding, washing, and elution conditions can be tested on your sample simultaneously. Successful scale-up from these small-scale experiments to traditional chromatography columns has proven to be quite useful.

4.3.2 Screening of Chromatographic Resin in an AcroPrep™ Multi-Well Plate Format

The development of a protein purification process is an empirical process employing a large number of experiments, which often represent a bottleneck for protein process development. To overcome this limitation, a multi-well, plate-based platform can be used to support the rapid performance mapping of chromatographic media. This multi-well plate process can lead to higher throughput methods development, avoiding the preparation of numerous packed columns and evaluation runs on liquid chromatography systems. This parallel screening format can facilitate more rapid selection of media and development of optimal binding and elution conditions.

This section will cover the application of AcroPrep 96 filter plates with 0.45 µm pore size GHP (0.35 and 1.0 mL volume) and Supor® (0.35 mL volume) membranes for screening applications with bead-based media. A range of beaded Pall purification media are available for:

- Ion exchange (see Section 4.2.2, page 310)
- Dye-based affinity (see Section 4.2.3, page 325)
- IMAC (see Section 4.2.4, page 332)
- Hydroxyapatite (see Section 4.2.5, page 341)
- Mixed mode affinity media (see Section 4.2.6, page 350)
- Specific affinity media based on Heparin (see Section 4.2.7, page 359), Lysine (see Section 4.2.8, page 365), and Protein A (see Section 4.2.9, page 371)

The specifications of these plates are summarized in Table 4.28 and a guide to rapid purification methods development is presented in Table 4.29.

Table 4.28
Specifications of the AcroPrep™ 96 Multi-Well Filter Plates

	Parameter	
Specification	96-Well, 350 µL	96-Well, 1 mL
Membranes	GHP (hydrophilic polypropylene) Supor® (hydrophilic polyethersulfone)	
Device	Polypropylene	
Effective Membrane Area	0.25 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)	1.66 cm (0.655 in.)	3.35 cm (1.32 in.)
Height (without Lid)	1.44 cm (0.565 in.)	2.87 cm (1.13 in.)
Tip Length	0.53 cm (0.21 in.)	0.53 cm (0.21 in.)
Capacities		
Maximum Well Volume	0.35 mL	1.0 mL
Recommended Volume	0.30 mL	0.9 mL
Hold-up Volume (Membrane/Support)	< 0.014 mL	< 0.018 mL
Maximum Centrifugal Force	3,000 x g	
Centrifuge Rotor	Swinging bucket with adapters	
Operating Vacuum	25.4 cm Hg (10 in. Hg)	

Table 4.29
Guide to Rapid Purification Development and Scouting Applications of Pall Chromatography Resin

Media	Mode of Action	Binding Variables	Elution Variables
Ion Exchange–Anion	Ion exchange interaction based on anionic charge state of protein	pH 7.5-9.0 ionic strength up to 0.15 M, non-ionic denaturants, such as urea, up to 5 M	Lower pH to 4.5, salt gradient up to 1.0 M NaCl ionic detergents, such as SDS
Ion Exchange–Cation	Ion exchange interaction based on cationic charge state of protein	pH 4.5-6.0 ionic strength up to 0.15 M, non-ionic denaturants, such as urea, up to 5 M	Raise pH to 9.0, salt gradient up to 1.0 M NaCl ionic detergents, such as SDS
Blue Trisacryl® M Resin	Blue dye-based mixed mode interaction	pH 6.0-8.0, ionic strength up to 0.075 M	Biospecific elution with ADP, NAD, NADP, NADH, NADPH at 1 mg/mL, increase pH 8.0-10.0, ionic strength up to 3 M NaCl, urea up to 8 M, ionic detergents, such as SDS
IMAC HyperCel™ Resin	Metal ion-based affinity interaction	Metal ion pH, ionic strength, presence of denaturants, such as Guanidine HCL	Imidazole up to 0.1 M, imidazole + NaCl up to 0.5 M, 1 M Phosphate pH 3.5 + 0.14 M NaCl, ionic detergents, such as SDS EDTA or EGTA, up to 50 mM
Ultrogel® Hydroxyapatite Resin	Mixed mode interaction with Ca Phosphate	pH 6.0-8.0, ionic strength up to 0.15 M NaCl, preload surface with Fe (III), Cu (II), Zn (II), or Co (II) metal ions	Phosphate gradient elution up to 0.5 M above + NaCl up to 1.5 M, urea up to 8 M, detergents imidazole up to 0.3 M, EDTA up to 50 mM
MEP HyperCel Resin	Hydrophobic Charge Induction Chromatography (HCIC)	pH 7.0-9.0	Add 25 mM Na Caprylate, reduce pH to 4.0-5.0
Heparin HyperD® F Resin	Specific affinity	pH 4.0-8.0, above pH range + NaCl up to 0.5 M, add Ca++ and Mg++ salts up to 0.5 M	Heparin up to 5 mg/mL, increase pH 8.0-10.0, NaCl up to 3 M, urea up to 8 M, ionic detergents, such as SDS

Table 4.29 (continued)
Guide to Rapid Purification Development and Scouting Applications of Pall Chromatography Resin

Media	Mode of Action	Binding Variables	Elution Variables
Lysine HyperD® F Resin	Specific affinity	pH 7.0-8.0, above pH range + NaCl up to 0.5 M	0.2 M aminocaproic acid in water, increase pH 8.0-10.0, decrease pH 7.0-4.0, NaCl up to 3 M temperature gradient, ionic detergents, such as SDS
Protein A HyperD F Resin	Immobilized Fc binding receptor for IgG	pH 7.0-9.0, presence of salts such as Na Citrate or Sulfate, up to 3 M	Decrease pH down to 2.5, strong chaotropic agent, such as KCNS, Ethylene glycol + pH decrease, ionic detergents, such as SDS
Anti-HSA Fractionation Resin	Immunochemical	pH, ionic strength, non-ionic denaturants, such as urea, up to 5 M	Decrease pH down to 2.5, strong chaotropic agent, such as KCNS, Ethylene glycol + pH decrease, ionic detergents, such as SDS
Anti-IgG Fractionation Resin	Immunochemical	pH, ionic strength, non-ionic denaturants, such as urea, up to 5 M	Decrease pH down to 2.5, strong chaotropic agent, such as KCNS, Ethylene glycol + pH decrease, ionic detergents

Protocol for Screening of Chromatographic Resin in an AcroPrep™ Multi-Well Plate Format

A. Materials Required

1. AcroPrep 96 filter plate, 350 µL well, 0.45 µm GHP membrane (PN 5030); AcroPrep 96 filter plate, 350 µL well, 1.2 µm Supor® membrane (PN 5039); and/or AcroPrep 96 filter plate, 1 mL well, 0.45 µm GHP membrane (PN 5054)
2. Collection plates [e.g., Axygen 96-well polypropylene V bottom 0.5 mL (Axygen PN P96450V) or round bottom 1.64 mL (Axygen PN PDW20)]
3. Self adhesive plate sealing sheets (e.g., Sigma-Aldrich EASYseal* A 5596 or equivalent), or cap mat (PN 5230)
4. 50% (v/v) slurry of the chromatographic media
5. Suitable buffer for equilibration of chromatographic media

6. 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

Tips on Handling BioSepra® Resin:
Some 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 50% (v/v) slurry.

For packed mini-columns in a multi-well format, 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 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 Bead-based Resin in a Multi-well Plate Format

In this plate 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 0.35 mL plate is 0.175 mL packed bed volume (0.35 mL of a 50% slurry) and up to 0.525 mL packed bed volume (3 x 0.35 mL of 50% slurry) for the 1.0 mL plate. Higher amounts are difficult to mix with sample and material will be lost from the device when spinning. For larger volumes (> 0.2 mL packed bed volume), the AcroPrep™ 1 mL volume plate is recommended. The Supor® membrane may offer higher flow rates than the GHP membrane plates but is only available in the 0.35 mL volume size.

1. Wash the chromatographic media with 5 column volumes (CV) of buffer to remove the storage buffer. Adjust to a final 50% (V/V) slurry with the loading buffer as described above.
2. Place a multi-well plate (0.35 or 1.0 mL volume) on a suitable vacuum manifold (PN 5017) with a collection plate underneath matched to the volume of the multi-well plate.
3. Mix the 50% media slurry and quickly pipette 0.05-0.35 mL to the 0.35 mL volume 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. The resin loaded in the 1.0 mL plate can be in the range of 0.05-0.525 mL packed bed volume.*

4. After final addition, allow the vacuum to remove the excess liquid from the resin bed which should partially fill the wells of plate.

5. The plate is ready for immediate use. To store the plate, add approximately 0.2 mL of buffer per well, then cover. To stop buffer leakage and loss due to evaporation, use an adhesive plate sealer on the top of the plate and store in a moisture-resistant plastic bag or container. Do not allow the bottom of the plate to come in contact with dry adsorbent surfaces as this may lead to moisture "wicking" from the wells and dehydration of the resin. 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. If storage is required > 1 week at 4 ºC, the storage buffer in the column should contain 0.02% sodium azide or equivalent as a preservative.*

C. Screening of Chromatographic Resin in a Multi-well Format

Multi-well plates can be used to assemble parallel mini-columns for high-throughput screening of binding and elution conditions on chromatographic media to develop purification processes. In this approach, it is possible to set up a matrix of binding conditions, which themselves can then be screened with a set of elution reagents. A summary of the potential screening matrices utilizing chromatographic media available from Pall can be found in Table 4.29. In this table, the binding and elution variables are listed and can be used to create a multi-well screening matrix on the layout of an AcroPrep™ 96 filter plate. The following protocol describes the processing of samples in this multi-well format.

1. Prepare the packed multi-well plate as described above.
2. Centrifuge the multi-well plate and collection plate in a suitable swinging bucket rotor at 500 x g for 2 minutes to remove excess fluid from the packed bed.
3. Vacuum can also be used if preferred, but the performance is not as consistent. See Appendix, Section 6.3.
4. Remove the filtrate from the collection plate.
5. Very carefully pipette the sample (0.125-0.25 mL for the 0.35 mL volume plate and 0.375-0.85 mL for the 1.0 mL volume plate) onto the top of the dry packed bed in the multi-well filter plate.

Tip: *At this stage, sample loading conditions should be optimized following recommendations in Table 4.29. The amount of sample loaded should be < 50% of the static binding capacity of the resin (see specification in Table 4.28). Overloading with sample should be avoided.*

6. To minimize buffer leakage and loss due to evaporation, use an adhesive plate sealer on the top of the plate and incubate in a moisture-resistant plastic bag or container.
7. The sample and well contents should be thoroughly mixed on a suitable shaker table and kept in contact for a minimum of 30 minutes at room temperature to improve binding.

Tip: *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. High-density media, such as HyperD® resin, can be more difficult to keep suspended in the well. They will require optimized agitation conditions, which should be determined in pilot experiments.*

8. 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 remove the top sheet slowly to avoid any contamination between wells.
9. Centrifuge the multi-well plate and collection plate in a swinging bucket rotor at 500 x g for 2 minutes to pass the sample through the resin bed. Or apply vacuum sufficient to pass the sample through the bed in 2 minutes.
10. Filtrate recovered from the collection plate will be the unretained fraction for the binding conditions being evaluated.
11. After unbound proteins are collected, the resin should be washed with 5x 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 interactions. These conditions should be controlled carefully to minimize elution of weak binding specific interactions with the chromatography support.*

12. Retained fractions can then be eluted according to the mode of adsorption of the resins being screened (see Table 4.29 for elution variables). 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. Steps C7-C8 should be followed to ensure that adequate contact time and thorough mixing of sample and resin occur for efficient elution of bound material.

Tip: *If samples are too diluted for further analysis at this stage, concentration can be carried out in an AcroPrep™ 96 filter plate with a 10K MWCO membrane (see Section 2.4.2.2, page 179).*

13. After the last elution, tightly bound material can be removed with 1% (w/v) SDS in buffer and recovered by acetone precipitation or SDR detergent removal using SDR HyperD® resin (see Section 2.3.1, page 141). After an SDS detergent elution, the multi-well plate and packed mini-columns should be discarded.

Application Data for Screening of Chromatographic Resin in an AcroPrep Multi-Well Plate Format

High throughput sample processing in proteomic research requires that protein recovery be consistent from well-to-well in the filter plates. As shown in Figure 4.26, the elution from 96 identical samples processed as mini packed chromatography beds in a single AcroPrep filter plate was consistent from well-to-well as judged by the intensity of filtrate protein bands in SDS PAGE gels. In addition, the protein recovery of each eluted sample was quantified by BCA assay, giving a CV of 9.3% for the whole plate. This result shows that there is high well-to-well reproducibility of packed mini-columns in the AcroPrep 96 filter plate format for rapid purification and methods development applications.

Figure 4.26
Well-to-Well Reproducibility in Packed Mini-Column Format with Multi-Well Processing

Ninety-six 0.02 mL aliquots of Ni-NTA resin (Qiagen) were mixed with* E. coli *inclusion body lysate containing a His-tagged TEV protease construct. Place the AcroPrep™ 96 filter plate on the vacuum manifold and load the above samples. Apply vacuum at 25.4 cm Hg (15 in. Hg) for 1 minute. Collect the flowthrough in a 96-well collection plate. Wash the resin with 0.2 mL of washing buffer (100 mM NaPi, 300 mM NaCl, 20 mM Imidazole, pH 8.0). Apply vacuum at 25.4 cm Hg (10 in. Hg) for 1 minute. Collect the wash in fresh 96-well collection plates. Repeat twice. Elute with 0.1 mL of elution buffer (100 mM NaPi, 300 mM NaCl, 250 mM Imidazole, pH 8.0). Apply vacuum at 25.4 cm Hg (10 in. Hg) for 1 minute. Collect the elution in a fresh 96-well collection plates. Repeat twice. The eluted samples from each well were analyzed using SDS-PAGE. The protein recovery in the elution from separate wells was measured by BCA assay. The coefficient of variation was 9.3%, indicating excellent well-to-well consistency.*

Ordering Information for Screening of Chromatographic Resin in an AcroPrep Multi-Well Plate Format

AcroPrep 96 Filter Plates, 350 µL well

Part Number	Description	Pkg
5030	0.45 µm GHP membrane, natural	10/pkg
5043	0.45 µm GHP membrane, white	10/pkg
5029	0.45 µm Supor® membrane, natural	10/pkg
5039	1.2 µm Supor membrane, natural	10/pkg

AcroPrep 96 Filter Plates, 1 mL well

Part Number	Description	Pkg
5054	0.45 µm GHP membrane, natural	5/pkg

4.3.3 Column Scale-Up to Pilot Process Applications

Scaleable Product

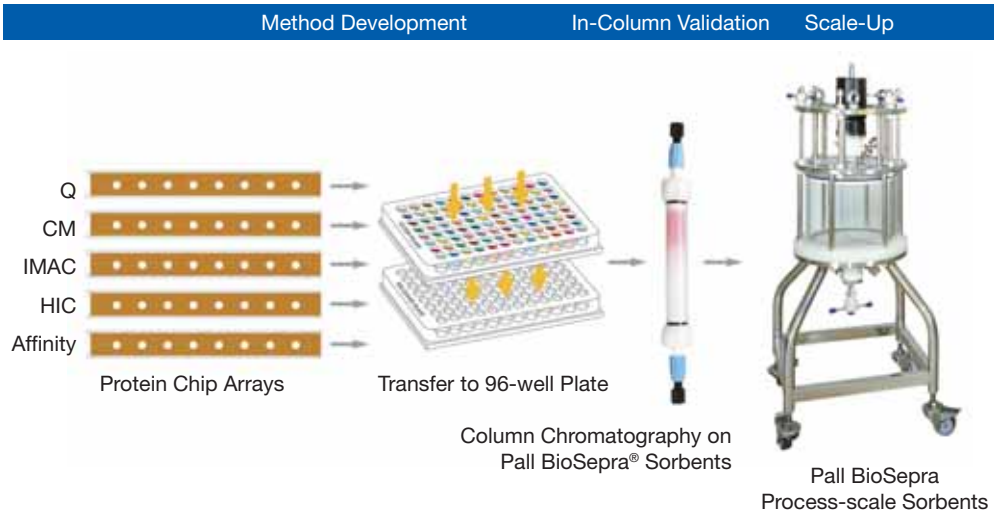
Pall offers chromatography products to facilitate research needs, scale-up, and polishing. The resin Pall offers for small-scale discovery applications are the same ones offered to our customers currently manufacturing biopharmaceuticals. The ability to scale-up is essential to those working in drug discovery, development, and manufacturing. These resins can be used in varying size chromatography columns.

Unique Optimization Techniques

The identification and optimization of protein purification parameters can be a tedious task. Process proteomics methodologies can be used to streamline the initial scouting of protein purification conditions as well as for some of the optimization steps. Process proteomics is performed using common chromatographic chemistries (e.g., anion exchange, cation exchange, IMAC, etc.) on a protein chip, in the wells of a multi-well filter plate, or using small columns. Using this approach, multiple binding, washing, and elution conditions can be tested on your sample simultaneously. Successful scale-up from these small-scale experiments to tradtional column chromatography has proven to be quite useful.

Pall's Process Proteomics Service Centers assist customers in selecting and optimizing resins and membranes for the purification of proteins used in the scale-up and production of therapeutic proteins and other bioprocess applications. With access to a large portfolio of both resin and media technologies, Pall can provide highly integrated solutions for our customers.

Figure 4.27
Process Proteomics Scale-up Process



4.4.1 Introduction

Biomolecule purification involves a complex series of steps where targets are selectively separated through a series of manipulations. The process 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-based spin filters, ultrafiltration-based multi-well filtration plates, and bottled gel filtration media. Refer to Table 4.30 for a selection of ultrafiltration-based products available.

Table 4.30
Ultrafiltration 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

Compared to other methods, ultrafiltration-based 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 ultrafiltration-based products eliminates problems with gel beds.
- Versatility–ultrafiltration 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 4.31 for general properties.

Table 4.31
Properties of Ultrogel AcA and Trisacryl GF-05 Resin Specifications

	Parameter	
Specification	Ultrogel AcA 202 Resin	Trisacryl GF-05 M 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	1000-15,000	200-2,500
Resolving Power (plates/m)	3,000	2,500
Working pH Range	3-10	1-11

Figure 4.28
Centrifugal Devices

4.4.2 Microsep™ UF Spin Filter for Samples 1-3.5 mL

Ultrafiltration (UF) is a membrane separation technique based on selection by molecular size, although other factors, such as molecule 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 leads to concentration of the protein solute in the retained fraction (termed the retentate) and can be recovered from above the membrane. There are three classic applications of UF membranes in purification processes:

1. Buffer exchange to transition a sample from one step in a purification process to another requiring different buffer conditions, such as pH or ionic strength.
2. Desalting to remove low molecular weight contaminants, such as salt or an elution reagent, that will interfere with detection or subsequent steps of the purification process.
3. Concentration of diluted protein or peptide samples that are generated during elution or washing steps in a process.

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 diluted 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 Table 4.32, the retention characteristics of Omega™ UF membranes for proteins are summarized. For proteins, it is recommended that an MWCO be selected that is 3-6 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 purification application in proteomics.

Table 4.32
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	1.8	-
Chymotrypsinogen A	25,000	% Recovery	-	97	94	2.1	-
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 Microsep™ UF Spin Filter for Samples 1-3.5 mL

A simple guide to choosing the appropriate MWCO UF membrane in the Microsep device for a range of purification applications is summarized in Table 4.33. Full specifications of the Microsep UF device are summarized in Table 4.34 and a diagram in Figure 4.29. If low recovery of retentate samples is seen with these devices, an optional pre-treatment process to reduce potential non-specific binding to the membrane and device surfaces is recommended.

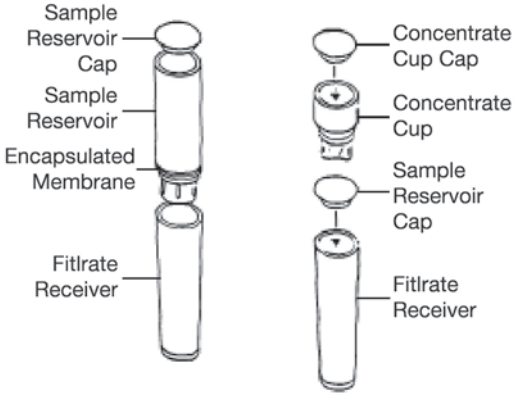
Table 4.33
Purification Application Guidelines on MWCO Selection

	MWCO UF Membrane		
Application	10K	30K	100K
Buffer exchange or salt removal of chromatography eluates, gradient fractions	✓		
Concentrating dilute samples to enhance sensitivity for biological assay	✓		
Recovery of antibodies from cell culture			✓ (IgM)
Recovery of low molecular weight compounds from fermentation broth	✓	✓	
Natural products screening for medicinal chemistry	✓	✓	✓
Virus concentration or removal			✓

Table 4.34
Specifications of the Microsep™ Spin Filter

Specification	**Parameter**
UF Membrane	Omega™ membrane (low protein-binding, modified polyethersulfone on polyethylene substrate)
Materials of Construction	
Device	Styrene acrylonitrile (SANS)
Collection Tube	Polypropylene
Effective Membrane Area	0.46 cm^2
Dimensions	
Diameter	1.7 cm
Overall Length (with Cap)	9.9 cm
Capacities	
Maximum Sample Volume	3.5 mL
Final Retentate Volume	0.030-0.05 mL
Final Receiver Volume	3.5 mL
Hold-up Volume (Membrane/Support)	0.02 mL
Operating Temperature Range	0-40 °C (32-104 °F)
pH Range	1-14
Maximum Centrifugal Force	7,500 x g
Centrifuge	Rotor accepting 1.7 x 10 cm tubes
Sanitization	70% ethanol

Figure 4.29
Components of the Microsep™ Centrifugal UF Device



Protocol for Microsep UF Spin Filter for Samples 1-3.5 mL

Omega™ membranes in Microsep devices contain trace amounts of glycerin and sodium azide (0.65 to 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 spin filters with Omega MWCO UF membrane and a collection tube. For specifications, see Table 4.34 and Figure 4.29.
2. Extra collection tubes for the Microsep UF spin filter
3. Degassed high purity water or buffer, such as phosphate buffered saline (PBS)

B. Basic Instructions for Use

1. Attach the filtrate receiver to bottom of sample reservoir. Pipettete 1.0 to 3.5 mL sample into upper reservoir. Place cap on reservoir to prevent evaporation.

Tip: *If pre-flushing to remove glycerin and sodium azide are required, add 3 mL of high purity water into the sample cup and process. Discard the filtrate and repeat with sample.*

2. Place device into fixed angle centrifuge rotor that accepts 17 x 100 mm tubes.

Tip: *Always counterbalance the rotor with another Microsep spin filter containing equivalent sample volume.*

3. Spin at 3,000-7,500 x g for the required length of time, typically 30-90 minutes to achieve desired concentrate volume. For optimal performance, it is recommended that spin time and g-force be determined for each application. See Table 4.35 and Table 4.36 to determine appropriate protocol.

Table 4.35
Typical Protein Recovery/Passage with Omega™ UF Membranes in a Microsep™ Centrifugal UF Device

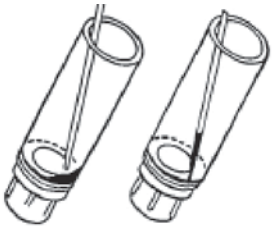
Microsep Device MWCO	Recommended g-force	MWt. Range
1K, yellow	5,000-7,000 x g	3K-10K
3K, gray	5,000-7,500 x g	10K-0K
10K, blue	5,000-7,500 x g	30K-90K
30K, red	5,000-7,500 x g	90K-180K
50K, green	1,000 x g	150K-300K
100K, clear	1,000 x g	300K-900K
300K, orange	1,000 x g	900K-1,800K
1000K, purple	1,000 x g	> 3000K

Table 4.36
Processing Times for Microsep UF Devices

		Time to "Dead Stop" (min)		
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	—

4. At the end of spin time, stop centrifugation 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 (See Figure 4.30).

Figure 4.30
Recovery of Retentate from Microsep™ UF Device



5. Cap storage cup containing concentrated sample, and store. Filtrate collected in filtrate receiver can also be stored for further analysis.
6. For application-specific protocol, see Section 2.4.2.1, page 154.

C. Buffer Exchange of Purification Samples (1-3.5 mL)

In purification it is a common occurrence that serial process steps are not always compatible and require buffer exchange to adjust pH or ionic strength without loss of sample. Using a suitable MWCO UF membrane (see Tables 4.35 and 4.36) to retain the molecule of interest, buffer exchange can be achieved within 2-3 cycles of processing in the Microsep spin filter.

1. Select the Microsep spin filter with an MWCO three times smaller than the MWt. of the protein to be retained.
2. If the devices have been pre-treated, proceed directly to the 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 Table 4.36). Discard the filtrate.
4. Add up to 3.5 mL of the sample and centrifuge as described in step B3. At this stage, it is important to achieve concentration of the sample to < 0.05 mL to achieve efficient buffer exchange. 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 buffer exchange.*

5. Commence buffer exchange by adding 3.5 mL of the second buffer to the retentate cup. Mix using a pipette (cycle up and down) to thoroughly mix the retentate with the new buffer solution. Re-centrifuge as described in Steps B3-B4.
6. Usually 2-3 cycles of buffer exchange will remove over 99% of the original components of the sample and achieve buffer exchange. Monitor pH and conductivity after each step to follow the progress of buffer exchange.

Tip: *Multiple buffer exchange steps can decrease overall yields.*

7. Recover the retained sample with a pipette tip (see Figure 4.30). To maximize recovery, rinse the retentate cup twice with 0.01-0.02 mL new buffer.

D. Desalting of Purification Samples (1-3.5 mL)

During purification steps, samples are frequently eluted from chromatography surfaces with high salt (up to 3 M NaCl) or biospecific eluates, such as 200 mM imidazole (see IMAC HyperCel™ resin), or 5 mg/mL heparin resin (see Heparin HyperD® F resin). These samples need to be desalted to remove reagents that can interfere with later purification steps or may inhibit biological activity in an assay. Detergents at concentrations above their critical micelle concentrations (CMC), such as Triton*-X100, Tween-20, CHAPS, or SDS, are more difficult to remove by size exclusion since they are present in solution as large micelles. The micellar state of these detergents prevents then from being easily resolved from the molecule of interest. For this application, SDR HyperD F resin (see Section 2.3.1, page 141) is highly recommended. If the detergent to be desalted is present lower than its CMC, then it may be possible to remove these low molecular weight materials by UF-based desalting. On removal of a detergent, sample solubility can change and may lead to aggregation or precipitation. It may be necessary to carry out exchange (see Section 2.4, page 152) to place the sample into a new buffer system, to maintain sample solubility. It is highly recommended to carry out some pilot experiments to confirm that detergent in its non-micellar state can be removed from the sample without compromising its solubility.

1. Select the Microsep™ spin filter with an MWCO three times smaller than the MWt. of the protein to be retained.
2. If the devices have been pre-treated, 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 Table 4.36). Discard the filtrate.
4. Add up to 3.5 mL of the sample and centrifuge as described in Step B3. At this stage, it is important to achieve concentration of the sample to < 0.05 mL to achieve efficient desalting. 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 desalting.*

5. Commence the desalting process by adding 3.5 mL of high purity water to the retentate cup. Mix using a pipette (cycle up and down) to thoroughly mix the retentate with the new buffer solution. Re-centrifuge as described in Steps B3-B4.
6. Usually 2-3 cycles of desalting will remove over 99% of the salt from the sample.

Tip: *Multiple desalting steps can decrease overall yields.*

7. Recover the retained sample with a pipette tip (see Figure 4.30). To maximize recovery, rinse the retentate cup twice with 0.01-0.02 mL high purity water.

E. Concentration of Samples (1-3.5 mL)

Samples eluting from chromatographic processes are frequently more dilute than the original starting sample. In many cases, the samples recovered are too dilute for the next step of processing or for detection in a biological assay. Re-concentrating dilute samples is a key application for UF membrane devices in purification processes. This process can efficiently remove solvent and retain samples of interest up to very high protein concentrations (> 20 mg/mL have been achieved with BSA). At these high levels, some protein-protein aggregation and, in extreme cases, precipitation can occur. It is highly recommended that some pilot studies be carried out to ascertain whether there are any protein-protein interactions and set some limits on the concentration target for the filtrate in this UF membrane-based process.

1. Select the Microsep™ spin filter with an MWCO three times smaller than the MWt. of the protein to be retained (see Tables 4.35 and 4.36).
2. If the devices have been pre-treated, proceed directly to Step E3.
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 in the MWCO membrane used (see Table 4.36). Discard the filtrate.
4. Add up to 3.5 mL of the sample and centrifuge as described in Step B3. 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 4.30). To maximize recovery, rinse the retentate cup twice with 0.01-0.02 mL new buffer or water.

Application Data for Microsep UF Spin Filter for Samples 1-3.5 mL

Pall centrifugal UF spin filters 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. The results for the removal of 0.5 M NaCl from a 5 mg/mL human serum albumin (HSA) solution are summarized in Table 4.37 for a Microsep spin filter. 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 elapsed time was 90 minutes for the Microsep spin filter. After one 10-30 minute spin, > 98% of the NaCl was removed in the filtrate. Two more spins achieved complete desalting.

Table 4.37
Diafiltration to Remove NaCl from a BSA Solution in Microsep™ Spin Filters Using a 10K MWCO Omega™ Membrane

	Starting Sample (0.5 M NaCl)	Cycle #1 (% Desalting)	Cycle #2 (% Desalting)	Cycle #3 (% Desalting)
Conductivity of Retentate (mS/cm)	9.25	0.69	0.048	0.008
% Desalted	100%	98.4%	99.8%	99.9%
Protein Recovery				94.3%

A BSA sample (5 mg/mL) in 0.5 M NaCl was processed for desalting. 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-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 Microsep UF spin filter. The retentate was then re-mixed with a pipette as described above. After the final cycle of desalting, 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 then used to rinse the device with a pipette, then removed and added to the original retentate. The pooled volume was then measured by aspiration up into a 1 mL pipette and the protein content measured by absorbance at 280 nm.

Troubleshooting for Microsep UF Spin Filter for Samples 1-3.5 mL

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. (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 Microsep™ UF Spin Filter for Samples 1-3.5 mL

Microsep Centrifugal Devices, Omega™ Membranes

Part Number	Description	Pkg
OD001C41	1K, yellow	24/pkg
OD001C46	1K, yellow	100/pkg
OD003C41	3K, grey	24/pkg
OD003C46	3K, grey	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
M 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

References for Microsep UF Spin Filter for Samples 1-3.5 mL

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.

4.4.3 Macrosep® UF Spin Filter for Samples 3.5-15 mL

Ultrafiltration (UF) is a membrane separation technique based on selection by molecular size, although other factors, such as molecule 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 leads to concentration of the protein solute in the retained fraction (termed the retentate) and can be recovered from above the membrane. There are three classic applications of UF membranes in purification processes:

1. Buffer exchange to transition a sample from one step in a purification process to another requiring different buffer conditions, such as pH or ionic strength.
2. Desalting to remove low molecular weight contaminants, such as salt or an elution reagent, that will interfere with detection or subsequent steps of the purification process.
3. Concentration of dilute protein or peptide samples that are generated during elution or washing steps in a process.

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 Table 4.38, the retention characteristics of Omega™ UF membranes for proteins is summarized. For proteins, it is recommended that an MWCO be selected that is 3-6 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 purification application in proteomics.

Table 4.38
Typical Protein Recovery/Passage with Omega™ UF Membranes in a Nanosep® Centrifugal UF Spin Filter

		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	1.8	-
Chymotrypsinogen A	25,000	% Recovery	-	97	94	2.1	-
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 Macrosep® UF Spin Filter for Samples 3.5-15 mL

A simple guide to choosing the appropriate MWCO UF membrane in the Macrosep device for a range of purification applications is summarized in Table 4.39. Full specifications of the Macrosep UF device is summarized in Table 4.40 and a diagram in Figure 4.31. If low recovery of retentate samples is seen with these devices, an optional pre-treatment process to reduce potential non-specific binding to the membrane and device surfaces is recommended.

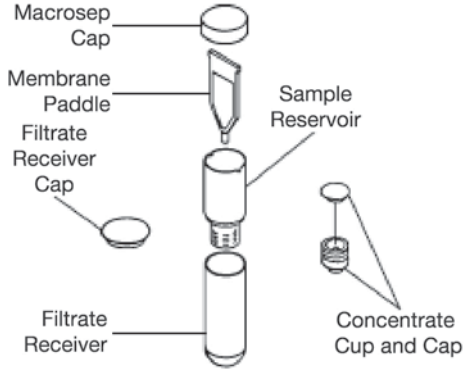
Table 4.39
Purification Application Guidelines on MWCO Selection

	MWCO UF Membrane		
Application	10K	30K	100K
Buffer exchange or salt removal of chromatography eluates, gradient fractions	✓		
Concentrating dilute samples to enhance sensitivity for biological assay	✓		
Recovery of antibodies from cell culture			✓ (IgM)
Recovery of low molecular weight compounds from fermentation broth	✓	✓	
Natural products screening for medicinal chemistry	✓	✓	✓
Virus concentration or removal			✓

Table 4.40
Specifications of the Macrosep® UF Spin Filter

Specification	**Parameter**
UF Membrane	Omega™ membrane (low protein binding, modified polyethersulfone on polyethylene substrate)
Materials of Construction	
Device	Polypropylene and polyethylene
Collection Tube	Polypropylene
Effective Membrane Area	10 cm^2
Dimensions	
Diameter	2.9 cm
Overall Length (with Cap)	10.9 cm
Capacities	
Maximum Sample Volume	15.0 mL
Final Retentate Volume	0.5-1.5 mL
Final Receiver Volume	15.0 mL
Hold-up Volume (Membrane/Support)	0.03 mL
Operating Temperature Range	0-40 °C (32-104 °F)
pH Range	1-14
Maximum Centrifugal Force	5,000 x g
Centrifuge	Rotor accepting 50 mL volume tubes
Sanitization	70% ethanol

Figure 4.31
Components of a Macrosep® Centrifugal UF Device



Protocol for Macrosep UF Spin Filter for Samples 3.5-15 mL

Each Macrosep spin filter 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.

A. Materials Required

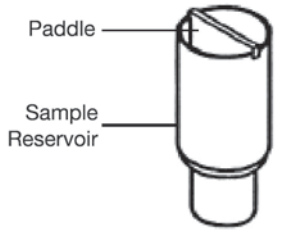
1. Macrosep UF spin filters with Omega MWCO UF membrane and a collection tube. For specifications, see Table 4.40 and Figure 4.31.
2. Extra collection tubes for the Macrosep UF device
3. Degassed high purity water or buffer, such as phosphate buffered saline (PBS)

B. Basic Instructions for Use

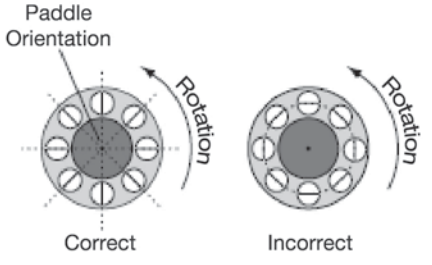
1. Insert the paddle firmly into the bottom of the sample reservoir. The “hooks” on the top part of the paddle must rest firmly in the notches on top of the sample reservoir (see Figure 4.32, Panel A). For best alignment, turn the reservoir upside down on the bench top and gently press the paddle into 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 4.32
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
Correct Versus Incorrect Device Placement in Centrifuge



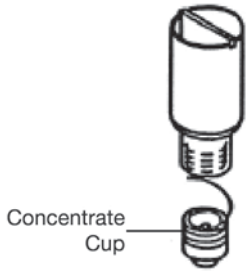
Tip: *If pre-flushing to remove glycerin and sodium azide are required, add 15 mL of high purity water into the sample tube and process. Discard the filtrate and repeat with sample.*

2. Attach the filtrate receiver to the bottom of the sample reservoir.
3. Pipette 5-15 mL of sample into the non-membrane side of the sample reservoir. Place cap on reservoir.

Tip: *If introducing a volume of 20 mL into the sample reservoir, make sure that the optional 20 mL filtrate receiver is attached.*

4. Place the Macrosep spin filter into a bucket or rotor which accepts standard 50 mL tubes.
5. In a fixed-angle rotor, align the Macrosep spin filter so that one of the "hooks" faces the center of the centrifuge rotor (see Figure 4.32, Panel B). This prevents a buildup 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 to 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, remove devices from the centrifuge. 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 4.31). 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 4.33).

Figure 4.33
Concentrate Recovery in Macrosep® UF 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 to 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 B7a above.

(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 maximum concentrate recovery.

C. Buffer Exchange of Purification Samples (3.5-15 mL)

In purification it is a common occurrence that serial process steps are not always compatible and require buffer exchange to adjust pH or ionic strength without loss of sample. Using a suitable MWCO UF membrane (see Tables 4.39 and 4.40) to retain the molecule of interest, buffer exchange can be achieved within 2-3 cycles of processing in the Macrosep spin filter.

1. Select the Macrosep spin filter with a MWCO three times smaller than the MWt. of the protein to be retained.

2. If the devices have been pre-treated, proceed directly to Step C4.

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 B5-B6 above. Discard the filtrate.

4. Add up to 15 mL of the sample and centrifuge as described in Steps B5-B6. 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 buffer exchange.*

5. Commence buffer exchange by adding 15 mL of new buffer to the non-membrane side of the sample reservoir. Mix using a pipette (cycle up and down) to thoroughly

mix the retentate with the new buffer solution. Place cap on reservoir. Re-centrifuge as described in Steps B5-B6.

6. Usually three cycles will achieve optimal buffer exchange. Monitor pH and conductivity after each step to follow the progress of buffer exchange.

Tip: *Multiple buffer exchange steps can decrease overall yields.*

7. Recover the retained sample as described in Steps B7a-B7b.

D. Desalting of Purification Samples (3.5-15 mL)

During purification steps, samples are frequently eluted from chromatography surfaces with high salt (up to 3 M NaCl) or biospecific eluates, such as 200 mM imidazole (see IMAC HyperCel™ resin), or 5 mg/mL heparin resin (see Heparin HyperD® F resin). These samples need to be desalted to remove reagents which can interfere with later purification steps or may inhibit biological activity in an assay. Detergents at concentrations above their critical micelle points (CMC), such as Triton*-X100, Tween-20, CHAPS, or SDS, are more difficult to remove by size exclusion since they are present in solution as large micelles. The micellar state of these detergents prevents them from being easily resolved from the molecule of interest. For this application, SDR HyperD F resin (see Section 2.3.1, page 141) is highly recommended. If the detergent to be desalted is present lower than its CMC, then it may be possible to remove these low molecular weight materials by UF-based desalting. On removal of a detergent, sample solubility can change and may lead to aggregation or precipitation. It may be necessary to carry out exchange (see Section 2.4, page 152) to place the sample into a new buffer system, to maintain sample solubility. It is highly recommended to carry out some pilot experiments to confirm that detergent in its non-micellar state can be removed from the sample without compromising its solubility.

1. Select the Macrosep® spin filter with an MWCO three times smaller than the MWt. of the protein to be retained.
2. If the devices have been pre-treated, proceed directly to Step D4.
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 B5-B6 above. Discard the filtrate.
4. Add up to 15 mL of the sample and centrifuge as described in Steps B5-B6. At this stage it is important to achieve concentration of the sample to < 1 mL to achieve efficient desalting. 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 desalting.*

5. Commence desalting by adding 15 mL of high purity water to the non-membrane side of the sample reservoir. Mix using a pipette (cycle up and down) to thoroughly mix the retentate with the water. Place cap on reservoir. Re-centrifuge as described in Steps B5-B6.
6. Usually three cycles will achieve optimal desalting.

Tip: *Multiple desalting steps can decrease overall yields.*

7. Recover the retained sample as described in Steps B7a-B7b.

E. Concentration of Samples (3.5-15 mL)

Samples eluting from chromatographic processes are frequently more dilute than the original starting sample. In many cases, the samples recovered are too dilute for the next step of processing or for detection in a biological assay. Re-concentrating dilute samples is a key application for UF membrane devices in purification processes. This process can efficiently remove solvent and retain samples of interest up to very high protein concentrations (> 20 mg/mL have been achieved with BSA). At these high levels, some protein-protein aggregation and, in extreme cases, precipitation can occur. It is highly recommended that some pilot studies be carried out to ascertain whether there are any protein-protein interactions and set some limits on the concentration target for the filtrate in this UF membrane-based process.

1. Select the Macrosep® spin filter with an MWCO three times smaller than the MWt. of the protein to be retained.
2. If the devices have been pre-treated, proceed directly to Step E4.
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 B5-B6 above. Discard the filtrate.
4. Add up to 15 mL of the sample and centrifuge as described in Steps B5-B6. 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 B7a-B7b.

Troubleshooting for Macrosep UF Spin Filter for Samples 3.5-15 mL

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. (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 Macrosep® UF Spin Filter for Samples 3.5-15 mL

Macrosep Centrifugal Devices, Omega™ Membrane

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

References for Macrosep® UF Spin Filter for Samples 3.5-15 mL

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.

4.4.4 Jumbosep™ UF Spin Filter for Samples 15-60 mL

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 leads to concentration of the protein solute in the retained fraction (termed the retentate) and can be recovered from above the membrane. There are three classic applications of UF membranes in purification processes:

- Buffer exchange to transition a sample from one step in a purification process to another requiring different buffer conditions, such as pH or ionic strength.
- Desalting to remove low molecular weight contaminants, such as salt or an elution reagent, that will interfere with detection or subsequent steps of the purification process.
- Concentration of diluted protein or peptide samples that are generated during elution or washing steps in a process.

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 Table 4.41, the retention characteristics of Omega™ UF membranes for proteins are summarized. For proteins, it is recommended that an MWCO be selected that is 3-6 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 purification application in proteomics.

Table 4.41
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	1.8	-
Chymotrypsinogen A	25,000	% Recovery	-	97	94	2.1	-
Ovalbumin	45,000	% Recovery	-	97	92	3	-
BSA	67,000	% Recovery	-	-	100	26	1.5
Phosphorylase B	97,400	% Recovery	-	-	95	91	1
IgG	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 Jumbosep™ UF Spin Filter for Samples 15-60 mL

A simple guide to choosing the appropriate MWCO UF membrane in the Jumbosep device for a range of purification applications is summarized in Table 4.34. Full specifications of the Jumbosep UF device are summarized in Table 4.43 and a diagram in Figure 4.34. If low recovery of retentate samples is seen with these devices, an optional pre-treatment process to reduce potential non-specific binding to the membrane and device surfaces is recommended.

Table 4.42
Purification Application Guidelines on MWCO Selection

	MWCO UF Membrane		
Application	10K	30K	100K
Buffer exchange or salt removal of chromatography eluates, gradient fractions	✓		
Concentrating dilute samples to enhance sensitivity for biological assay	✓		
Recovery of antibodies from cell culture			✓ (IgM)
Recovery of low molecular weight compounds from fermentation broth	✓	✓	
Natural products screening for medicinal chemistry	✓	✓	✓
Virus concentration or removal			✓

Table 4.43
Specifications of the UF Jumbosep™ Device

Specification	Parameter
UF Membrane	Omega™ membrane (low protein-binding, modified polyethersulfone on polyethylene substrate)
Materials of Construction	
Device	Polysulfone and polyethylene
Collection Tube	Polypropylene
Effective Membrane Area	15.2 cm^2
Dimensions	
Diameter	6.0 cm
Overall Length (with Cap)	11.3 cm
Capacities	
Maximum Sample Volume	60.0 mL
Final Retentate Volume	3.5-4.0 mL
Final Receiver Volume	60.0 mL
Hold-up Volume (Membrane/Support)	0.2 mL
Operating Temperature Range	0-40 °C (32-104 °F)
pH Range	1-14
Maximum Centrifugal Force	3,000 x g
Centrifuge	Rotor accepting 250 mL bottles
Sanitization	70% ethanol

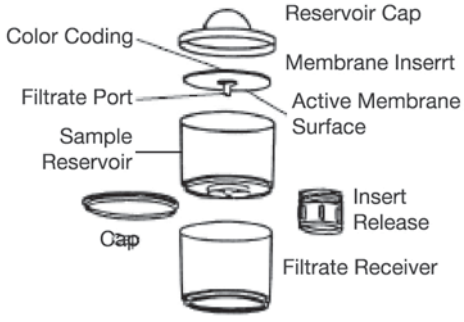
Protocol for Jumbosep UF Spin Filter for Samples 15-60 mL

Centrifugation up to 3,000 x g provides the driving force for filtration, moving samples toward 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 4.34 for a diagram showing the components of the Jumbosep centrifugal UF device.

A. Materials Required

1. Jumbosep UF devices with Omega MWCO UF membrane and a collection tube. For specifications, see Table 4.43 and Figure 4.34.
2. Extra filtrate receiver bottles for the Jumbosep UF device
3. Degassed high purity water or buffer, such as phosphate buffered saline (PBS)

Figure 4.34
Components of Jumbosep™ Centrifugal UF Device



B. Basic 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 top of 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 to 40 minutes, to achieve the desired retentate volume. It is recommended that spin times and g-force be determined experimentally for each application.

Tip: *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 Jumbosep 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 the retentate 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.

12. For application specific protocol, see Section 2.4.2.1, page 154.

C. Buffer Exchange of Purification Samples (15-60 mL)

In purification it is a common occurrence that serial process steps are not always compatible and require buffer exchange to adjust pH or ionic strength without loss of sample. Using a suitable MWCO UF membrane (see Table 4.41) to retain the molecule of interest, buffer exchange can be achieved within 2-3 cycles of processing in the Jumbosep™ device.

1. Select the Jumbosep device with an MWCO three times smaller than the MWt. of the protein to be retained.

2. If the devices have been pre-treated, proceed directly to Step C4

3. Add 15-60 mL of high purity water into the sample reservoir. Centrifuge as described in Steps B7-B8 above. Discard the filtrate.

4. Add up to 15-60 mL of the sample and centrifuge as described in Steps B7-B8. 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 that the retentate volume be < 4.0 mL to achieve efficient buffer exchange. 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 buffer exchange.*

5. Commence buffer exchange by adding 15-60 mL of new buffer to the sample reservoir. Mix using a pipette (cycle up and down) to thoroughly mix the retentate with the new buffer. Place cap on reservoir. Re-centrifuge as described in Steps B7-B8.

6. Usually three cycles of buffer exchange will remove over 99% of the original components and achieve buffer exchange.

Tip: *Multiple buffer exchange steps can decrease overall yields.*

7. Recover the retained sample as described in Step B11.

D. Desalting of Purification Samples (15-60 mL)

During purification steps, samples are frequently eluted from chromatography surfaces with high salt (up to 3 M NaCl) or biospecific eluates, such as 200 mM imidazole (see IMAC HyperCel™) or 5 mg/mL heparin (see Heparin HyperD® F). These samples need to be desalted to remove reagents that can interfere with later purification steps or may inhibit biological activity in an assay. Detergents at concentrations above their critical micelle concentrations (CMC), such as Triton*-X100, Tween-20, CHAPS, or SDS, are more difficult to remove by size exclusion since they are present in solution as large micelles. The micellar state of these detergents prevents them from being easily resolved from the molecule of interest. For this application, SDR HyperD F resin (see Section 2.3.1, page 141) is highly recommended. If the detergent to be desalted is present lower than its CMC, then it may be possible to remove these low molecular weight materials by UF-based desalting. On removal of a detergent, sample solubility

can change and may lead to aggregation or precipitation. It may be necessary to carry out exchange (see Section 2.4, page 152) to place the sample into a new buffer system, to maintain sample solubility. It is highly recommended to carry out some pilot experiments to confirm that detergent in its non-micellar state can be removed from the sample without compromising its solubility.

1. Select the Jumbosep™ device with an MWCO three times smaller than the MWt. of the protein to be retained.
2. If the devices have been pre-treated, proceed directly to Step D4.
3. Add 15-60 mL of high purity water into the sample reservoir. Centrifuge as described in Steps B7-B8 above. Discard the filtrate.
4. Add up to 15-60 mL of the sample and centrifuge as described in Steps B7-B8. 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 that the retentate volume be < 4.0 mL to achieve efficient desalting. 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 desalting.*

5. Commence desalting by adding 15-60 mL of high purity water to the sample reservoir. Mix using a pipette (cycle up and down) to thoroughly mix the retentate with the water. Place cap on reservoir. Re-centrifuge as described in Steps B7-B8.
6. Usually three cycles of desalting will remove over 99% of the original low molecular weight salts and achieve the necessary desalting.

Tip: *Multiple desalting steps can decrease overall yields.*

7. Recover the retained sample as described in Step B11.

E. Concentration of Samples (15-60 mL)

Samples eluting from chromatographic processes are frequently more dilute than the original starting sample. In many cases, the samples recovered are too dilute for the next step of processing or for detection in a biological assay. Re-concentrating dilute samples is a key application for UF membrane devices in purification processes. This process can efficiently remove solvent and retain samples of interest up to very high protein concentrations (> 20 mg/mL have been achieved with BSA). At these high levels, some protein-protein aggregation and, in extreme cases, precipitation can occur. It is highly recommended that some pilot studies be carried out to ascertain whether there are any protein-protein interactions and set some limits on the concentration target for the filtrate in this UF membrane-based process.

1. Select the Jumbosep device with a MWCO three times smaller than the MWt. of the protein to be retained.
2. If the devices have been pre-treated, proceed directly to Step E4.
3. Add 15-60 mL of high purity water into the sample reservoir. Place cap on reservoir. Centrifuge as described in Steps B7-B8 above. Discard the filtrate.
4. Add up to 15-60 mL of the sample and centrifuge as described in Steps B7-B8. 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 B11.

Troubleshooting for Jumbosep™ UF Spin Filter for Samples 15-60 mL

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. (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-subunit 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 Jumbosep™ UF Spin Filter for Samples 15-60 mL

Jumbosep Centrifugal Devices

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 for Jumbosep UF Spin Filter for Samples 15-60 mL

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.

4.5.1 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 4.35
Choose the Best Clarification Device for Your Needs

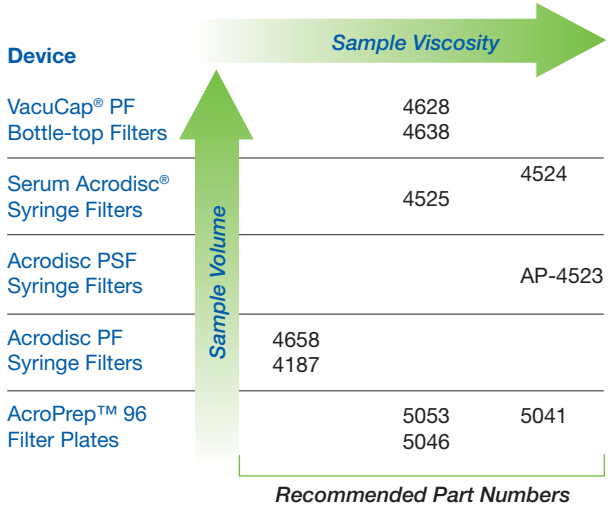
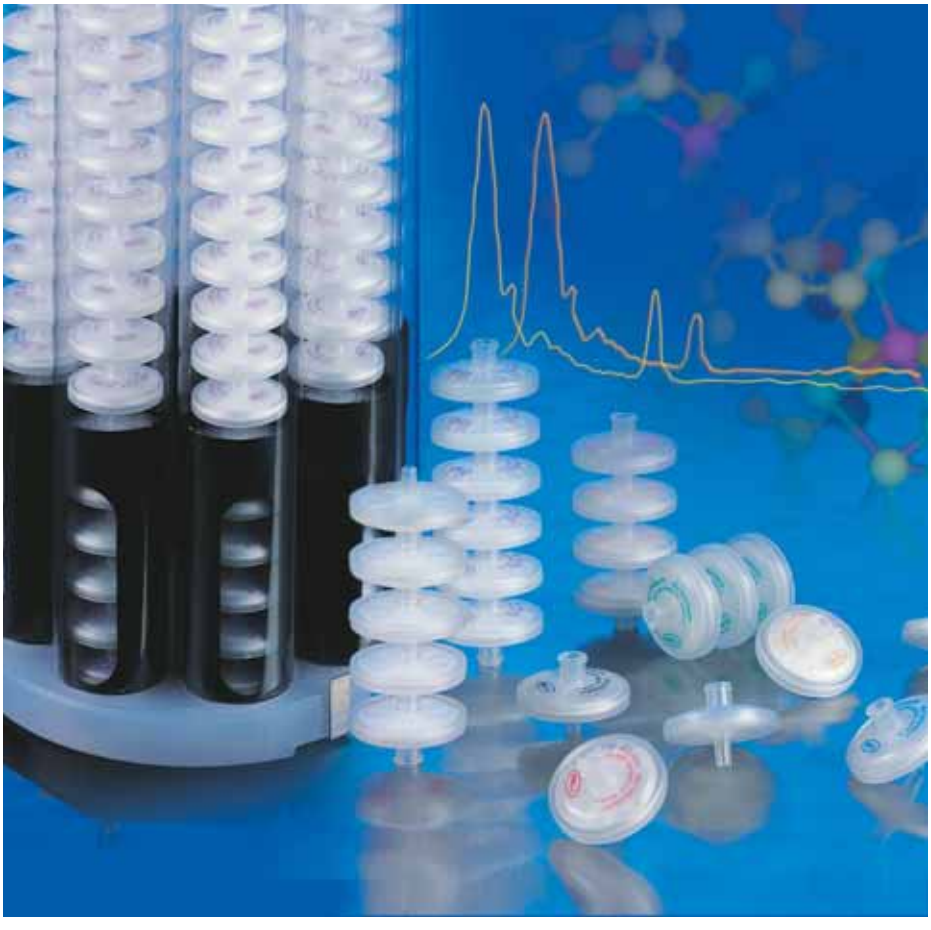


Figure 4.36
Acrodisc® Syringe Filters for Analytical Sample Preparation



4.5.2 Clarification of Samples (< 1 mL) by MF Filtration in Nanosep® Spin Filters

Uncharged microporous membrane filters remove particles from a fluid stream by a 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 pre-filters or clarifying filters.

Application Guidelines for Clarification of Samples (< 1 mL) by MF Filtration in Nanosep Spin Filters

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, in two different membrane chemistries; a) Bio-Inert®, a modified low protein binding nylon surface, and b) GHP, a hydrophilic modified polypropylene surface. The centrifugal MF device properties are summarized in Table 4.44. 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.

Table 4.44
Properties of the Nanosep® MF Centrifugal Spin Devices

Specification	Parameter
MF Filtration Membrane	Bio-Inert® (modified nylon) GHP (hydrophilic polypropylene)
Sample Reservoir, Membrane Support Base, Filtrate Receiver, and Concentrate Cup	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 Working With Nanosep® MF samples < 1 mL

A. Materials Required

1. Nanosep MF devices with Bio-Inert® or GHP membranes and a collection tube. For specifications, see Table 4.44.
2. Extra collection tubes for the Nanosep MF device
3. Degassed high purity water or buffer, such as phosphate buffered saline (PBS)

B. MF Filtration of Samples

For MF filtration with 0.45 µm or 0.2 µm pore size, 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 µm or 0.2 µm centrifugal MF filters.

1. Select the Nanosep device with a 0.45 µm 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 as described in Sections 2.5.6, page 226.
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) by MF Filtration in Nanosep® Spin Filters

Nanosep MF Centrifugal Devices, 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, GHP Membrane

Part Number	Description	Pkg
ODGHPC34	0.45 µm, clear	100/pkg
ODGHPC35	0.45 µm, clear	500/pkg

4.5.3 Filter Sterilization of Samples (1-100 mL) in Sterile Acrodisc® Syringe Filters

Final product sterility in purification is crucial to guarantee their use in later stage testing in cell-based assays or *in vivo*. However, not all protein products can be sterilized by heat and pressure in an autoclave. When heat-labile products or additives for cell culture, such as insulin or antibiotics, need to be sterilized, microfiltration provides a good non-invasive alternative. Currently, filters are challenge tested with one of the smallest known bacteria, *Brevundimonas diminuta* ATCC 19146. The test allows the detection of 1 CFU per filtrate. For 0.2 µm pore size sterilizing-grade membranes, the filtrate should contain no challenge test organisms, according to regulatory guidelines.[1]

Acrodisc syringe filter devices with Supor® hydrophilic, modified, polyethersulfone membranes in 0.1, 0.2 and 0.45 µm pore sizes can be used for filter sterilization of heat labile samples. In bacterial challenge testing, 0.2 µm membranes showed no passage of test microorganisms while the 0.45 µm showed 5.5-6.8 log fold reduction of bacteria in the filtrate.[2] The Pall sterile Acrodisc MF filters have the following attributes for this application:

- Superior flow rates and higher throughputs than competitive devices.
- Low protein binding to minimize sample loss.
- Sterilized by gamma irradiation to eliminate potential contamination by EtO residuals.
- A range of sizes (13 to 32 mm) to accommodate sample volumes from < 10-100 mL.
- Acrodisc PF and Serum Acrodisc syringe filters feature built-in prefilters for increased throughput of difficult-to-filter liquids.
- Reduce mycoplasma with the use of 0.1 µm pore size.

The sterile Acrodisc MF device properties are summarized in Table 4.45 for 13, 25, and 32 mm diameter devices.

Table 4.45
Properties of the Acrodisc® MF Sterile Syringe Filter

Specification	Parameter
Membranes	
Supor® PES	Hydrophilic polyethersulfone
GF Prefilter	Binder free borosilicate glass fiber
Device	
13 mm	Polypropylene
25/32 mm	Modified acrylic
Serum Acrodisc	ABS
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 µm
Acrodisc PF	0.8/0.2 µm
Serum Acrodisc	GF/0.2 µm
Sample Volume	
13 mm	< 10 mL
25 mm	< 25 mL
32 mm	< 50 mL
Hold-up Volumes	
13 mm	0.028 mL
25 mm	0.07 mL
32 mm	0.100 mL
Maximum Temperature	55 °C
Maximum Pressure Limit	
13 mm	6.3 bar (630 kPa, 91 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 (0.1 µm) at 45 psi	35 (PES)
13 mm (0.2 µm) at 10-45 (PES) psi	17-35 (PES)
13 mm (0.45 µm) at 10-45 (PES) psi	28-35 (PES)
25 mm (0.1 µm) at 30-45 (PES) psi	35 (PES)-175
25 mm (0.2 µm) at 30-45 (PES) psi	175 (PES)-195
25 mm (0.45 µm) at 30-45 (PES) psi	195-300 (PES)
25 mm (0.8/0.2 µm) at 30-45 (PES) psi	145

Table 4.45 (continued)
Properties of the Acrodisc® MF Sterile Syringe Filter

Specification	Parameter
Typical Water Flow Rate (mL/min)	
32 mm (0.1 µm) at 45 psi	100
32 mm (0.2 µm) at 45 psi	490
32 mm (0.45 µm) at 45 psi	700
32 mm (0.8/0.2 µm) at 45 psi	440
Inlet/Outlet Connectors	
13 mm	Female luer-lok inlet, minispike outlet
25 mm	Female luer-lok inlet, male slip luer outlet
32 mm	Female luer-lok inlet, male slip luer outlet
Endotoxin	< 0.25 EU/mL using Limulus Amoebocyte Lysate (LAL) test
Biological Safety	Passes United States Pharmacopeia (USP) Biological Reactivity Test, *In Vivo*
Sterilization	Sterilized by gamma irradiation and individually blister packed

Application Guidelines for Filter Sterilization of Samples (1-100 mL) in Sterile Acrodisc Syringe Filters

Syringe adapted sterile filtration devices, such as Acrodisc syringe filters, have a wide variety of applications and are key to analytical and screening processes in bioscience. Typical applications of Acrodisc MF filter sterilization in proteomics include:

- Preparation of cell culture media with heat-labile additives, such as insulin or antibiotics.
- Filter sterilization of samples that may be used for raising antibodies *in vivo* mixed with a suitable adjuvant.
- Filter sterilization of samples for cell-based assays.
- Filter sterilization of samples for toxicity testing.
- Filter sterilization of products for *in vivo* clinical testing.
- Filter sterilization of water used in clinical analyses.

Protocol for Filter Sterilization of Samples (1-100 mL) in Sterile Acrodisc® Syringe Filters

A. Materials Required

1. Acrodisc sterile syringe filter devices with MF membranes. For specifications, see Table 4.45.
2. Empty syringes (10-30 mL volume)
3. Sterile container to receive filtrate for 1-100 mL volume
4. Access to a sterile laminar flow hood and equipment to maintain aseptic handling conditions

B. MF Acrodisc Filter Sterilization in Syringe Adapted Format

To maintain sterility of the filtrate, handling of the syringe and filter when removed from its protective packaging should be carried out under aseptic handling conditions. This process should be carried out under a suitable laminar flow hood taking precautions, such as the use of gloves and a face mask barrier, to minimize personal contamination of the final filtrate.

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 sterile filtered.

Tip: *Use of syringes smaller than 10 mL can generate excessive pressure on the filter, which may exceed maximum operating pressure.*

3. Examine the outer packaging on the pre-sterilized Acrodisc MF filter and confirm that the device sterility has been maintained. Open packing under aseptic handling and expose the top female luer fitting on the device.
4. 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.
5. Remove the device and syringe from the packaging material and open a filtrate receiving container (sterile) under aseptic conditions.
6. Place the male luer outlet over the filtrate container.
7. Apply gentle pressure to begin filtration. (A gentle pressure helps assure maximum throughput.)

Tip: *As the filter removes particulates, filtration will become more difficult. The syringe plunger will be harder to push. Pressure will rapidly increase on the filter. If it becomes necessary to change filters due to the resistance, repeat Steps B3-B7.*

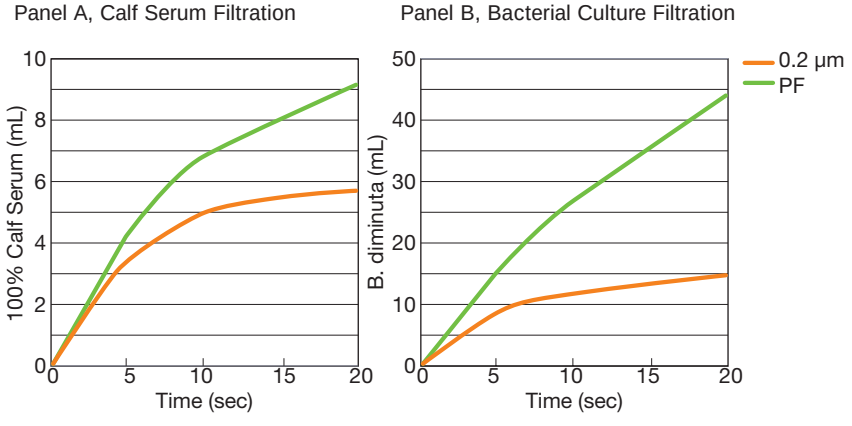
These filters are for SINGLE USE ONLY.

Application Data for Filter Sterilization of Samples (1-100 mL) in Sterile Acrodisc® Syringe Filters

The addition of a 0.8 µm PES prefilter to a 0.2 µm PES sterilization membrane considerably improves the filter sterilization throughput with viscous, particulate-laden, and 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 and a bacterial culture with a 0.8/0.2 µm Supor® PES Acrodisc syringe filter is summarized in Figure 4.37. The prefilter considerably extends the filter life of these samples beyond 10-20 seconds when the 0.2 µm PES filter reached a plateau of throughput.

Figure 4.37

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 syringe filters with Supor membrane 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 Filter Sterilization of Samples (1-100 mL) in Sterile Acrodisc® Syringe Filters

Acrodisc Syringe Filters, Supor® Membrane

Part Number	Description	Pkg
4602	0.2 µm, 13 mm, sterile	75/pkg
4604	0.45 µm, 13 mm, sterile	75/pkg
4611	0.1 µm, 25 mm, sterile	50/pkg
4612	0.2 µm, 25 mm, sterile	50/pkg
4614	0.45 µm, 25 mm, sterile	50/pkg
4651	0.1 µm, 32 mm, sterile	50/pkg
4652	0.2 µm, 32 mm, sterile	50/pkg
4654	0.45 µm, 32 mm, sterile	50/pkg

Acrodisc PF Syringe Filters, Supor Membrane

Part Number	Description	Pkg
4187	0.8/0.2 µm, 25 mm, sterile	50/pkg
4658	0.8/0.2 µm, 32 mm, sterile	50/pkg

Serum Acrodisc Syringe Filter, Supor Membrane

Part Number	Description	Pkg
4525	GF/0.2 µm, 37 mm, sterile	20/pkg

References for Filter Sterilization of Samples (1-100 mL) in a Sterile Acrodisc Syringe Filters

1. (1982). Microbial evaluation of filters for sterilizing liquids. Health Industry Manufacturers Association, 4, Washington, D.C.
2. Griffiths, M.H., Andrew, P.W., Ball, P.R., & Hall G.M. (2000). Rapid methods for testing the efficacy of sterilization-grade filter membranes. *Applied and Environmental Microbiology*, (66), 3432–3437.

4.5 – Section 4.5.3

Notes