



## Protein Purification and Handling

- Background
- Protein Concentration from Dilute Solutions
- Improving the Recovery of Proteins from Dilute Solutions (Passivation)
- Protein Desalting or Buffer Exchange (Diafiltration)
- Gross Fractionation of Complex Protein Solutions
- Concentration of Monoclonal Antibodies from Culture Supernatants

### Background

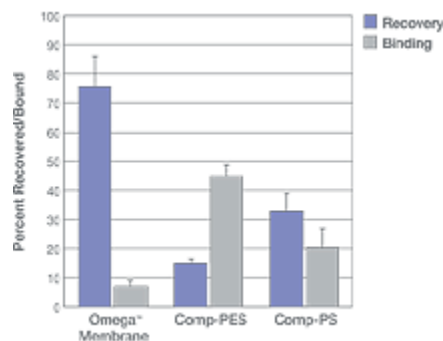
Ultrafiltration has been used successfully for years and is an excellent and gentle method to purify and concentrate protein samples. Ultrafiltration can be used to replace precipitation, evaporation, dialysis, lyophilization, and gel filtration to concentrate and desalt protein without significant protein loss (Figure 16).

Advantages of ultrafiltration:

- the most gentle method for concentrating proteins
- minimal denaturation of protein compared to precipitation
- maintains ionic strength, hence does not result in hyperconcentration of salts
- produces high yields
- much faster than dialysis and less expensive than lyophilization

Ultrafiltration, however, is a separation technique rather than a fractionation technique. With very few exceptions, the use of ultrafiltration for protein fractionation remains impractical unless the two proteins to be separated have at least a ten-fold difference in their molecular weights.

**Figure 16**  
Protein Binding and Recovery



[Click graph to enlarge.](#)

*Nanosep devices (Omega membrane) and competitive devices (Comp-PES, Comp-PS) were used to filter and recover radioactively-labeled BSA ( $^{125}\text{I}$ -BSA). A 1  $\mu\text{g}$  sample of labeled BSA was diluted into 500  $\mu\text{L}$  phosphate buffered saline and centrifuged in a fixed angle rotor using instructions from the respective device manufacturers for speed and spin duration. The resulting retentate was recovered in 40  $\mu\text{L}$  PBS and placed directly into counting vials containing scintillation solution. After recovering the retentate, the upper receivers were submerged in*

*separate vials containing scintillation solution. The graph contains the results of counting (PerkinElmer, Gaithersburg, MD, USA) of two independent experiments where each device was analyzed in triplicate.*

## Protein Concentration from Dilute Solutions

### Description

One of the most popular and successful UF applications is concentration of dilute protein solutions containing antibodies, enzymes, growth factors, etc. Protein purification schemes include cell disruption, followed by initial fractionation, then a secondary fractionation, and finally a polishing step.

After these steps are completed, the dilute protein-containing solutions need to be concentrated prior to use in downstream applications. Ultrafiltration is an efficient method of protein concentration and desalting under gentle conditions without significant loss of biological activity (Table 4).

### Step-by-Step Procedure

1. Choose the appropriate device depending on the sample volume (Table 1).
2. Place dilute protein samples into the sample reservoir of the device with the appropriate MWCO membrane.
3. Note: Increased solute concentration in the sample can decrease the passage of a specific protein and the use of a higher MWCO device may be required. If larger sample volumes are to be concentrated, a pilot experiment is suggested using 100  $\mu$ L of the sample in a Nanosep device to determine which membrane is best suited for the specific large-scale application.
4. Centrifuge the device at the recommended g-force; longer spin times will be required for solute-laden samples or lower MWCO devices.
5. After concentration, the protein sample can be pipetted from the retentate chamber. Higher yields can be achieved if a small volume of buffer (20  $\mu$ L for a Nanosep device) is used to rinse the membrane surface for remaining protein samples.

**Table 4**

Typical Protein Retentate Recovery/Passage

Solute	Solute MW (Kd)	MWCO	3K	10K	30K	100K	300K
		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 (1 mg/mL)	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 10 to 60  $\mu$ L.*

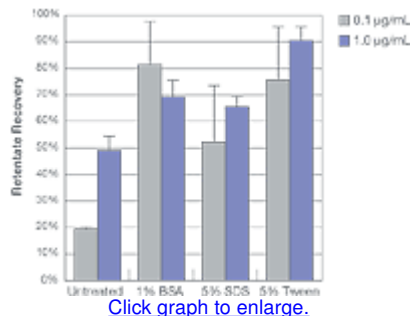
## Improving the Recovery of Proteins from Dilute Solutions (Passivation)

### Description

For very dilute protein solutions (< 10  $\mu$ g/mL), concentrate recovery in UF devices is often not quantitative (Figure 1). Pall centrifugal devices have been specifically constructed of materials that minimize nonspecific binding. However, certain proteins, particularly when dilute, can be problematic. The extent of nonspecific binding varies with the structure of the individual protein. Proteins containing charged or hydrophobic domains tend to show a high affinity toward various surfaces that may lead to irreversible binding.

Strategies that reduce adsorptive loss of proteins on surfaces are either based on pretreatment of the surface to fill the exposed binding sites or by changes in the composition of the solution, usually by addition of protein (often albumin), detergents, or salts. In most cases, pretreating (passivating) the device before concentration of dilute protein solutions can improve recovery (Figure 17).

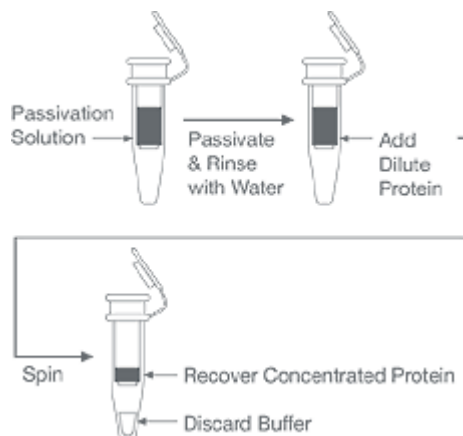
**Figure 17**  
Use of Passivation to Increase Protein Recoveries



*0.1 µg/mL and 1.0 µg/mL bovine serum albumin (BSA) solution were centrifuged in pretreated 30K Nanosep devices. Pretreatment involved incubating the devices filled with either 1% BSA, 5% Sodium Dodecyl Sulfate (SDS), or 5% Tween 20 for 1 hour. The device was then rinsed and used immediately. Passivation increased the recovery of protein, particularly for the most dilute samples.*

### Step-by-Step Procedure

1. Add 500 µL of the sterile passivation solution to the Nanosep device (see below). Close the cap and incubate the solution in the device for at least one hour at room temperature. Passivation solutions:
  - 1% BSA in PBS
  - 5% SDS in distilled water
  - 5% Tween-20 in distilled water
  - 5% Triton-X in distilled water
  - 5% PEG compound in distilled water
  - 1% IgG in PBS
1. Discard the passivation solution by either pouring or pipetting it out of the device, and rinse the Nanosep device thoroughly with sterile distilled water.
2. To ensure that residual passivation solution is removed, add 500 µL of distilled water to the device and centrifuge at 14,000 x g for 5 to 10 minutes. Discard the filtrate.
3. The device can be used immediately or stored for later use. If the device is to be used later, add 100 µL sterile distilled water to the sample reservoir and store at 4 °C to retard bacterial growth. Important: *Do not allow the membrane to dry out once the device has been passivated.*



### Protein Desalting or Buffer Exchange (Diafiltration)

#### Description

Centrifugal concentrators are ideal for the removal or exchange of salts. Desalting by dialysis is time-consuming and works best when the concentration differential between the two solutions is large. Dialysis does not concentrate dilute samples and may result in even further dilution.

A single round of protein concentration using ultrafiltration results in a sample with essentially the same buffer 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/replaced (Table 5).

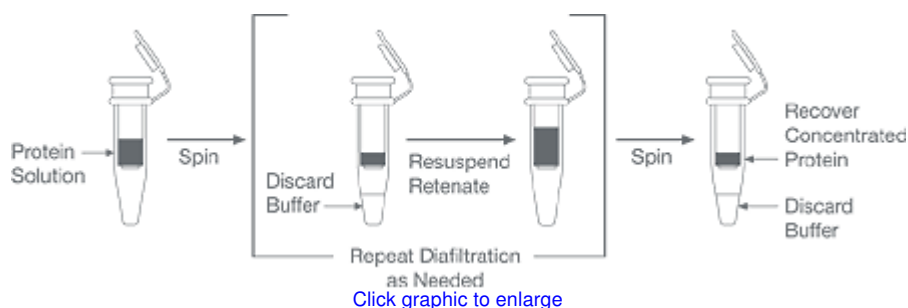
**Table 5**  
Desalting Estimates

Spin No.	Salt Concentration After Spin	Percent Salt Removal
1	500 mM	95%
2	25 mM	99.75%
3	1.25 mM	99.99%
4	0.06 mM	100%

*Estimates are made for concentrating 500  $\mu$ L to 25  $\mu$ L each spin.*

### Step-by-Step Procedure

1. Select the Nanosep device with a MWCO three times smaller than the MW of the protein to be retained (Table 3).
- 2a. To concentrate the sample without changing salt concentration: pipette 500  $\mu$ L of sample into the sample reservoir and centrifuge at the speed and time needed to concentrate the sample. Spin times will depend on sample concentration and MWCO.
- 2b. To desalt or exchange buffer, first concentrate the sample (step 2) then add new buffer or water to dilute the concentrated sample to 500  $\mu$ L. Centrifuge again. This process can be repeated to achieve a lower salt concentration. Usually two cycles of dilution and concentration will remove over 99% of salts and over 90% of small molecular weight contaminants. If a higher level of purity is desired, repeat the dilution and concentration steps for a third time. Multiple diafiltration steps will decrease overall yields; therefore, quality versus yield considerations must be made.
3. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20  $\mu$ L new buffer or water.



### Gross Fractionation of Complex Protein Solutions

#### Description

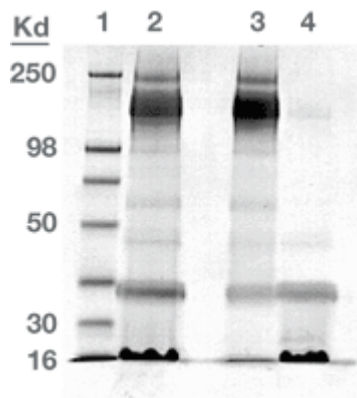
Although ultrafiltration is primarily a separation technique, under some conditions it can be used for the gross fractionation of proteins that differ significantly in size (Figure 18). In order to fractionate or enrich for a particular protein, the following must be considered:

- The proteins must have at least 10-fold difference in MW.
- The MW of retained protein should be at least 3 times the MWCO of membrane.
- The passing protein should be at least 3 times smaller than the MWCO of membrane.
- The sample concentration should be 5 mg/mL or less.
- Centrifugation should be performed at lower than maximum recommended g-force (500 to

1,000 x g).

It is important to remember that separation is rarely absolute and is better described as an enrichment.

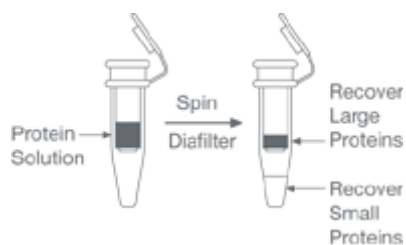
**Figure 18**  
Gross Fractionation of Proteins



A 500  $\mu$ L sample of a 5.0 mg/mL protein solution containing IgG (156 kD) and Cytochrome C (12.4 kD) was centrifuged at 1,000 x g for 30 minutes in a Nanosep 100K device. The retentate was recovered in 500  $\mu$ L; 15  $\mu$ L samples of the retentate and filtrate were analyzed on 10% NuPAGE Bis-Tris polyacrylamide gel. Lane 1 = NOVEX SeeBlue Pre-Stained Protein Standards. Lane 2 = Mixture of IgG and Cytochrome C. Lane 3 = Retentate. Lane 4 = Filtrate. After two spins (not shown), over 95% of Cytochrome C was found in filtrate while more than 85% of the IgG was retained by the membrane.

### Step-by-Step Procedure

1. Fill the Nanosep device (usually 100K or 300K) with 500  $\mu$ L of the protein mixture.
2. Centrifuge at low speed (500 to 1,000 x g) for approximately 20 minutes.
3. Transfer the filtrate from the bottom receiver to a new tube for storage. The filtrate can be concentrated in a lower MWCO device if needed.
4. To diafilter the retentate, fill the sample reservoir to 500  $\mu$ L with appropriate buffer. Mix briefly, centrifuge, and collect filtrate as before.
5. Remove or resuspend the retentate in the appropriate buffer and transfer it to a fresh tube for storage.



### Concentration of Monoclonal Antibodies from Culture Supernatants

#### Description

The concentration of monoclonal antibodies (mAbs) in culture supernatants is often too low to be useful for most in vitro and in vivo immunological techniques. Concentrated mAbs are obtained from ascites fluid induced in hybridoma cells. However, development of ascites from hybridoma cells is a complicated and time-consuming procedure, and antibodies still need to be purified prior to use.

Ultrafiltration can be used to rapidly purify and recover concentrated mAbs directly from culture supernatants. Use of a 100K MWCO membrane allows for the concentration of antibodies while at the same time removing serum albumin and other low molecular weight proteins present in the culture media.

**Step-by-Step Procedure**

1. Add 0.5 mL (Nanosep 100K device), 3 mL (Microsep 100K device), or up to 15 mL (Macrosep 100K device) of the culture supernatant to the centrifugal device.
2. Centrifuge at 5,000 x g for 30 minutes.
3. Recover mAbs by pipetting the retentate from the sample reservoir.
4. Optional: To perform discontinuous diafiltration, add sufficient amount of buffer to bring the sample volume to 3 mL (Microsep device) or 12 mL (Macrosep device) and centrifuge again. Usually two cycles of dilution and concentration will remove over 99% of salts and over 90% of small molecular weight contaminants. If a higher level of purity is desired, repeat the dilution and concentration steps for a third time. Multiple diafiltration steps will decrease overall yields; therefore, quality versus yield considerations must be made.

