

Diafiltration for Desalting or Buffer Exchange

Larry Schwartz

Diafiltration is an ultrafiltration membrane technique for completely removing, replacing, or lowering the concentration of salts or solvents from solutions containing proteins, peptides, nucleic acids, and other biomolecules. The process selectively uses permeable (porous) membrane filters to separate the components of solutions and suspensions based on their molecular size. Smaller molecules such as salts, solvents, and water pass freely through the ultrafiltration membrane, which retains the larger molecules.

CONCENTRATION

The solution retained by a membrane is known as concentrate or retentate. The solution that passes through a membrane is known as the filtrate or permeate. A membrane for concentration is selected based on its rejection

PRODUCT FOCUS: ALL BIOLOGICALS

PROCESS FOCUS: DOWNSTREAM PROCESSING

WHO SHOULD READ: PROCESS DEVELOPMENT, MANUFACTURING

KEYWORDS: ULTRAFILTRATION, DIAFILTRATION, BUFFER EXCHANGE, DESALTING, PROTEIN CONCENTRATION

LEVEL: INTERMEDIATE



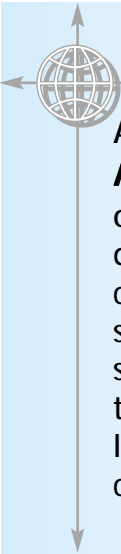
A typical development laboratory system comprising a Centramate holder with a peristaltic pump and pressure gauges, valves, and tubing. PALL CORPORATION
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characteristics for the mixture that is to be concentrated. As a general rule, the molecular weight cut-off (MWCO) of a membrane should be a third to a sixth the molecular weight of the molecule to be retained. This is known as the 3–6× Rule, which ensures complete retention. The closer the MWCO is to that of the sample, the greater the risk for some product loss occurring during concentration. That risk increases if diafiltration will also be used because the relative loss depends on the total volume of filtrate generated.

Membrane flux rate (defined as the filtrate flow rate per unit area of membrane) is related to pore size. The smaller the pores, the lower the flux rate for the same applied

pressure. Therefore, when selecting a membrane for concentration or diafiltration, consider the time factor versus product recovery. In most biological applications, recovery outweighs the time consideration. Increasing the amount of membrane area used can also reduce the process time.

Figure 1 provides an example of concentration. The sample is placed in a device containing a suitable ultrafiltration membrane that will retain the large molecules. Pressure is applied until half the volume has passed through that membrane. Large molecules are retained in half the original volume (concentrate), which also contains half of the salt molecules. The filtrate contains the other half of the salt molecules but



Another

ADVANTAGE

of using
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none of the large molecules. Therefore, the large molecules are concentrated as liquid and salt are removed. The salt-molecule-to-volume ratio in the concentrate remains constant, so the ionic strength of the concentrated solution remains relatively constant.

Diafiltration, the process of “washing” the remaining salt out with water, can subsequently reduce the ionic strength of a concentrate (retentate) solution. This is essentially a dilution process performed in conjunction with concentration. Water is added while filtrate is removed. If the washing solution is another buffer instead of water, the new buffer salt will replace the initial salt in the sample. For simplicity, the examples herein use a direct-flow filtration device such as a centrifugal concentrator. But the same principles apply to cross-flow filtration devices such as cassettes and hollow fibers.

BENEFITS OF DIAFILTRATION

Other techniques used for salt removal or buffer exchange (such as membrane dialysis and column-based gel filtration) can be effective but have certain limitations. Dialysis procedures can take several days, require large volumes of water for equilibration, and risk product loss through manual manipulation of dialysis bags. Gel filtration dilutes the sample and often requires an additional ultrafiltration step to concentrate it back. Adding steps to

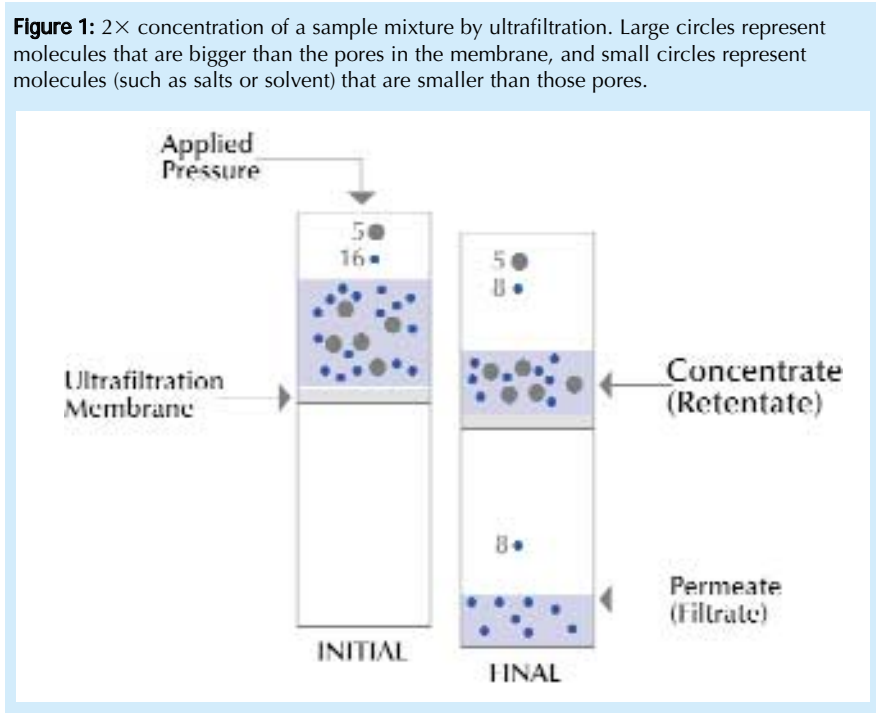


Table 1: Continuous (constant volume) diafiltration

Diafiltration Volumes	Removal of Small Molecules	
	Permeability 100% Rejection Coefficient = 0	Permeability 75% Rejection Coefficient = 0.25
1	63%	53%
2	86%	77%
3	95%	89%
4	98.2%	95%
5	99.3%	97.6%
6	99.7%	98.9%
7	99.9%	99.4%
8		99.7%
9		99.9%

Note: 0% rejection salts, solvents, buffers, etc.; 25% rejection molecules lower in molecular weight than the molecular weight cutoff of the membrane, but bigger than salts

a process can lead to the possibility of sample loss or contamination.

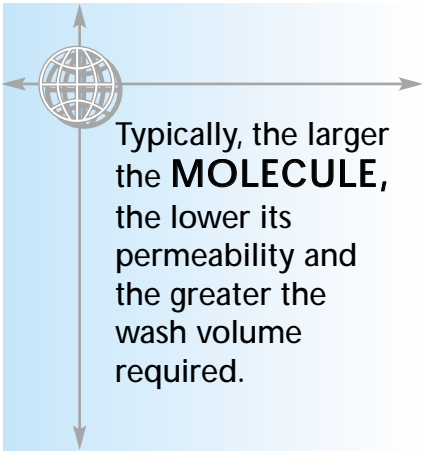
With diafiltration, salt or solvent removal and buffer exchange can be performed quickly and conveniently. Another advantage of using diafiltration is concentration of samples on one system, minimizing the risk of sample loss or contamination. Diafiltration is performed in three main ways: by continuous diafiltration and by discontinuous diafiltration through sequential dilution or volume reduction. Although the end result may be the same overall, the time and volume required to complete the process varies considerably. It is important to understand the different methods used and when to choose one over the other.

CONTINUOUS DIAFILTRATION

The technique of continuous diafiltration (also referred to as constant volume diafiltration) involves washing out the original buffer salts (or other low-molecular-weight species) in the retentate (sample) by adding water or a new buffer to it at the same rate filtrate is generated. As a result, the



A Minimate TFF Capsule device for development and processing applications using small sample volumes. Samples can be concentrated down to about 5 mL and diafiltered.
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retentate volume and product concentration do not change during the diafiltration process. If water is used for diafiltering, salts will be washed out and the retentate conductivity lowered.

If a buffer is used for diafiltering, the new buffer's salt concentration will increase at a rate inversely proportional to that of the molecular species being removed. The amount of salt removed is related to the filtrate volume generated relative to the retentate volume. The filtrate volume generated is usually referred to in terms of diafiltration volumes (DVs).

A single DV is the volume of retentate when diafiltration begins.

Table 2: Salt reduction from sample using volume reduction or constant volume diafiltration

Diafiltration Volumes	2× Volume Reduction		Constant Volume	
	100% Permeability 0%	75% Retention 25%	100% Permeability 0%	75% Retention 25%
1	50%	41%	63%	53%
2	75%	65%	86%	77%
3	88%	79%	95%	89%
4	94%	88%	98.2%	95%
5	96.9%	93%	99.3%	97.6%
6	98.4%	95.6%	99.7%	98.7%
7	99.2%	97.4%	99.9%	99.4%
8	99.6%	98.4%		99.7%
9	99.9%	99.4%		99.9%
10	99.9%	99.4%		

Note: 0% rejection salts, solvents, buffers, etc.; 25% rejection molecules lower in molecular weight than the molecular weight cutoff of the membrane, but bigger than salts

Liquid is added at the same rate as filtrate is generated, and when the volume of filtrate collected equals the starting retentate volume, 1 DV has been processed. Using continuous diafiltration, over 99.5% of a 100% permeable solute can be removed by washing through six volumes (6 DV) with the buffer of choice. Molecules that are larger than salts and solvents but smaller than the pores in the membrane also can be washed out. The permeability of such molecules, however, may be less than 100%. It takes more liquid (more DVs) to completely wash a partially permeable molecule through the membrane than it does to remove a 100% permeable molecule from a mixture. Typically, the larger the molecule, the lower its permeability and the greater the wash volume required. The permeability of a particular molecule through a specific membrane can be determined by measuring the concentration of that molecule in the filtrate compared to its concentration in the retentate under specified conditions (Equation 1).

Permeability is often described in terms of the rejection coefficient of the membrane (its ability to hold back or reject a given molecule from passing through). Thus, see Equation 2.

A rejection coefficient of 1 equals 0% permeability. A rejection coefficient of 0 equals 100% permeability. Permeability is affected by such factors as transmembrane pressure (TMP), crossflow rate, retentate concentration, pH, ionic strength, and gel layer formation (concentration polarization). Therefore, the permeability may change over the course of the diafiltration process.

Table 1 shows the relationship between permeability through a membrane and the number of diafiltration volumes required for removal of permeating species. As noted above, greater volumes of buffer are required to remove molecules that are partially retained. To remove 99.9% of a molecule that is 25% permeable to the membrane requires 9 DVs, whereas for a 100% permeable species, only 7 DVs are required to achieve the same

Equation 1

$$\% \text{ Permeability} = (\text{Concentration}_{\text{FILTRATE}} \div \text{Concentration}_{\text{RETENTATE}}) \times 100$$

Equation 2

$$\text{Rejection Coefficient} = 1 - (\text{Concentration}_{\text{FILTRATE}} \div \text{Concentration}_{\text{RETENTATE}})$$

Figure 2: Discontinuous diafiltration (sequential dilution). Large circles represent molecules that are bigger than the pores in the membrane, and small circles represent molecules (such as salts or solvent) that are smaller than those pores.

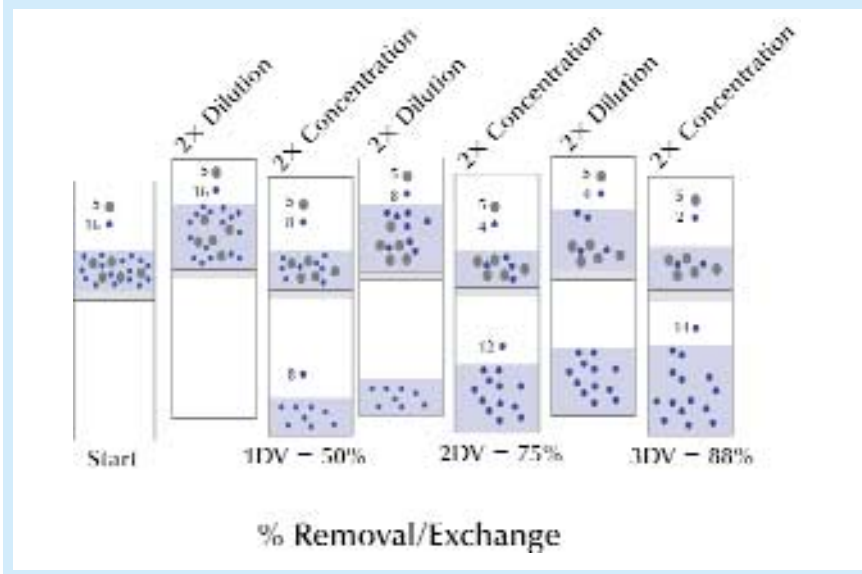
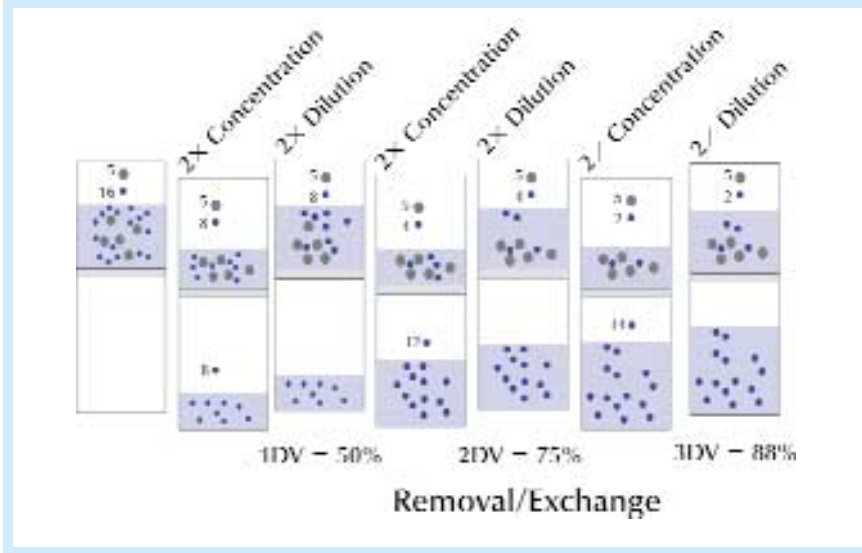


Figure 3: Discontinuous diafiltration (sequential dilution). Large circles represent molecules that are bigger than the pores in the membrane, and small circles represent molecules (such as salts or solvent) that are smaller than those pores.



removal.

DISCONTINUOUS DIAFILTRATION Sequential Dilution. Discontinuous diafiltration by sequential dilution involves first diluting a sample with water or a replacement buffer to a predetermined volume. The diluted sample is then concentrated back to its original volume by ultrafiltration. The process is repeated until the unwanted salts, solvents, or smaller molecules are removed. Each subsequent dilution and concentration step removes more

small molecules. As shown in Figure 2, the sample is generally diluted with an equal volume of buffer (1 DV). Adding volumes >1 DV will be less effective. For example, diluting the sample with 5 DVs and concentrating back will reduce the salt by only 80% compared with 88% for 2× volume reduction or sequential dilution. Diluting the sample usually lowers viscosity, which may increase the filtrate flux rate.

Volume Reduction. Discontinuous diafiltration by volume reduction

DIAFILTRATION DEFINITIONS

Diafiltration: Diafiltration is a technique that uses ultrafiltration membranes to completely remove or to lower the concentration of salt or solvent — or to replace buffer salts from solutions containing proteins and other large molecules.

Diafiltration Volume: One diafiltration volume equals the initial volume in which the molecule of interest is suspended. The number of diafiltration volumes required depends on whether the permeating molecular species is freely passing (salts, buffers, solvents) or partially retained.

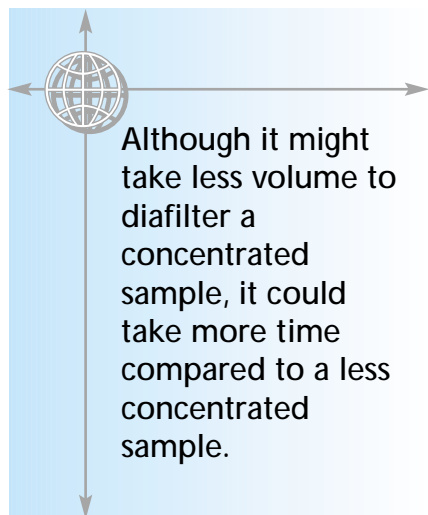
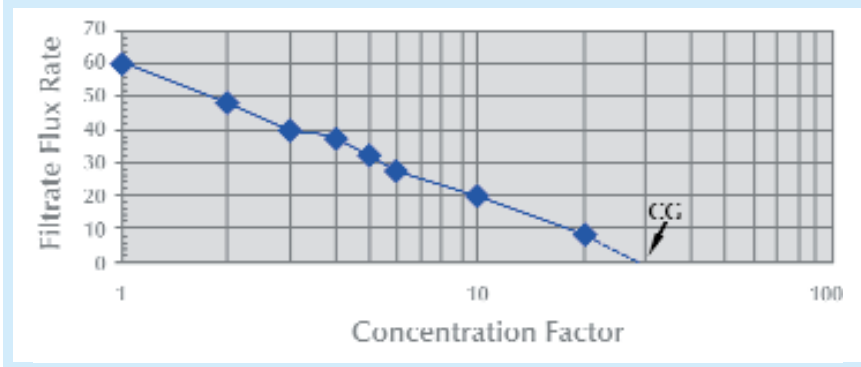
Continuous Diafiltration: The technique of continuous diafiltration (also referred to as constant volume diafiltration) involves washing out the original buffer salts (or other low-molecular-weight species) in the retentate (sample) by adding water or a new buffer to the retentate at the same rate as filtrate is being generated.

Discontinuous Diafiltration by Sequential Dilution involves first diluting the sample to a predetermined volume, and then concentrating that sample back to its original volume with water or replacement buffer. This is repeated until the unwanted salts, solvents, or smaller molecules are removed. Each subsequent dilution removes more of them.

Discontinuous Diafiltration by Volume Reduction involves first concentrating the sample to a predetermined volume, then diluting that sample back to its original volume with water or replacement buffer. This is repeated until the unwanted salts, solvents, or smaller molecules are removed. Each subsequent concentration and dilution removes more of them.

reverses the sequential dilution

Figure 4: Determination of the CG value for a product.



Although it might take less volume to diafilter a concentrated sample, it could take more time compared to a less concentrated sample.

procedure. A mixture is first concentrated to a predetermined volume and then diluted back to its original volume with water or a replacement buffer. This is repeated until the unwanted salts, solvents, or smaller molecules are removed. Each subsequent concentration and dilution removes more small molecules (Figure 3).

After the last buffer addition, the sample is generally concentrated before analysis or before the next purification step is performed. After diafiltration by either method (discontinuous $2\times$ volume reduction or sequential dilution), the final product is at the same volume and concentration as when diafiltration started. The salt concentration has been equally reduced in both examples. However, the volume of diafiltration buffer used by the volume reduction method reduced by the initial concentration step was half that used in sequential dilution. A DV is

equal to the volume where dilution occurs. Therefore, half the volume was required.

That being the case, it would seem that concentrating before diafiltration — by either discontinuous sequential dilution or constant volume diafiltration — should reduce the required diafiltration buffer volume and save time. And in most cases this is true. The factor we have not accounted for is filtrate flux rate, which equates to process time. As the product becomes concentrated, viscosity increases, and the filtrate flux rate decreases. The filtrate flux rate varies inversely as the log of the concentration factor:

$$J = k \ln (CG \div CB)$$

where J = filtrate flux rate, k = constant, \ln = natural log, CG = gel layer concentration, and CB = retentate (bulk flow) concentration. This becomes very significant as the CB increases above a few percent and is dependent on the characteristics of the specific molecules making up the sample mixture. Although it might take significantly less volume to diafilter a concentrated sample, it could take considerably more time compared to a less concentrated sample. Simple protocols are available to

find optimum conditions that maximize productivity.

WHICH TECHNIQUE SHOULD BE USED?

When deciding which technique to use and where in the downstream process to perform diafiltration, consider the following factors:

- Initial sample volume, concentration, and viscosity
- Required final sample concentration
- Stability of the molecule of interest at various concentrations
- Volume of buffer required for diafiltration
- Total processing time
- Reservoir size available
- Economics.

The choice of which method to use must be based on several criteria. Scale is an important consideration. What we do at laboratory scale may be very different than at process scale, especially if the process is automated. At lab scale, discontinuous diafiltration is often used for its simplicity. Continuous diafiltration requires a pump or special equipment to add the diafiltration solution at a constant rate. Both techniques can be automated for process applications.

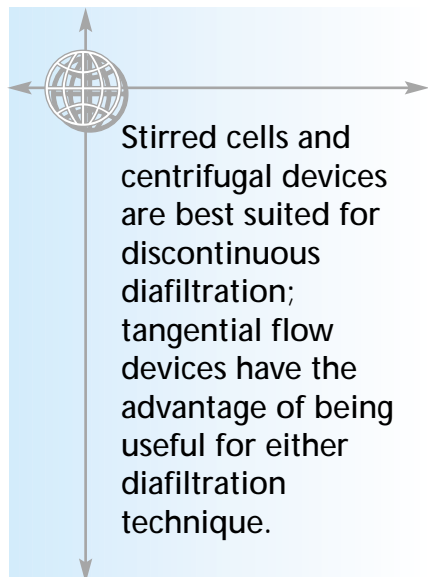
If we eliminate the equipment issue and focus on the process itself, we can compare the differences. Ionic strength, buffer composition, and stabilizer concentration can affect stability of the sample. Diafiltration may remove salts or stabilizing molecules, resulting in protein product denaturation and aggregation. The process of concentrating and diluting a protein solution can affect molecular interactions to cause denaturation or aggregation, subsequent precipitation, and product loss. It is necessary to evaluate the effect of

Equation 3

$$\text{Process Time} = \text{Filtrate Flow Rate} \times \text{Volume}$$

Equation 4

$$\ln (CG \div CR) = 1 \text{ or } CR_{\text{OPTIMUM}} = CG \div e = 0.37 \times CG$$



concentration on the product itself to determine where diafiltration is best performed relative to concentration effects. Continuous diafiltration offers an advantage over discontinuous diafiltration in that the retentate concentration remains constant. It is often seen as a more gentle process.

BEFORE OR AFTER CONCENTRATION?
We have already seen that concentrating a sample first can significantly reduce the volume of diafiltration solution required. We have also seen that continuous diafiltration takes less volume than discontinuous diafiltration with sequential dilution. Therefore, if the sample is first concentrated to its required final concentration and then continuous diafiltration performed, acceptable results should be obtained.

However, above a certain concentration, filtrate flux rates may become prohibitively slow. It may actually take longer to diafilter a concentrated sample than it would if the sample were first diluted to reduce its concentration. In this situation, even though continuous diafiltration of the diluted sample requires a greater diafiltration volume, the total processing time would be lower because of the faster filtrate flux rate (Equation 3).

In general, the optimum retentate concentration for

performing (continuous) diafiltration can be determined as in Equation 4, where CG = gel layer concentration, CR = retentate concentration, and CR_{OPTIMUM} = highest retentate concentration where diafiltration should be performed (1).

The CG value for a sample mixture can be determined from experimentation by concentrating a sample on a membrane while recording and plotting data for filtrate flux rate compared with log concentration (concentration factor). Then a curve can be extrapolated to filtrate flux rate = 0. The CG value will be the same for this particular product regardless of the starting concentration or filtrate flux rate.

In Figure 4's example, the CG value is a concentration factor of approximately $33\times$. Therefore, the optimal concentration factor to perform diafiltration would be $0.37 \times CG = 12.2\times$. If the starting product concentration is 5 mg/mL, then diafiltration should be performed when that concentration reaches 61 mg/mL. If the final concentration will be less than 61 mg/mL, then diafiltration should be performed after concentration unless it is necessary to remove a specific molecule first.

The ultrafiltration product selected may dictate your choice of continuous or discontinuous diafiltration. Stirred cells and centrifugal devices are best suited for discontinuous diafiltration because of their mode of operation. Tangential flow devices have the advantage of being useful for either diafiltration technique.

A USEFUL STEP IN DOWNSTREAM PROCESSING
Diafiltration is a fast and effective technique for desalting or buffer exchange of solutions. It can be performed in a continuous or discontinuous mode. Continuous diafiltration usually takes less volume to achieve the same degree of salt reduction as discontinuous diafiltration with sequential dilution and can be easier to perform.

Continuous diafiltration is also perceived as a "kinder and gentler" process on active biomolecules.

On the other hand, discontinuous diafiltration with volume reduction requires less volume than continuous diafiltration. Concentrating a sample mixture before diafiltration usually reduces the required filtrate volume and saves time. However, if sample viscosity becomes too great, the filtrate flux rate will decrease, which can increase process times substantially. Determining the CG for your sample can help answer the question: *At what concentration should I perform diafiltration?*

Highly selective polyethersulfone (PES) microfiltration and ultrafiltration membranes with narrow pore size distribution and high flow rates provide fast processing time and efficient separation for diafiltration. Initial volumes from a few milliliters up to thousands of liters can be processed using tangential flow filtration. TFF systems allow easy addition of membrane surface area, providing the flexibility to reduce processing times or scale up your process. The range of devices and systems available provides scalability as well as the flexibility to work with almost any sample volume.

REFERENCE

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