



Nanosep® Centrifugal Devices - Protocols for Use

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Ultrafiltration Fundamentals

Background

Ultrafiltration (UF) is a membrane separation technique used to separate extremely small particles and dissolved molecules in fluids. The primary basis for separation is molecular size, although other factors such as molecule shape and charge can also play a role. Molecules larger than the membrane pores will be retained at the surface of the membrane (not in the polymer matrix as they are retained in microporous membranes) and concentrated during the ultrafiltration process.

Compared to non-membrane processes ([chromatography](#), dialysis, solvent extraction, or centrifugation), ultrafiltration:

- ▶ Is far gentler to the molecules being processed.
- ▶ Does not require an organic extraction which may denature labile proteins.
- ▶ Maintains the ionic and pH milieu.
- ▶ Is fast and relatively inexpensive.
- ▶ Can be performed at low temperatures (for example, in the cold room).
- ▶ Is very efficient and can simultaneously concentrate and purify molecules.

The retention properties of ultrafiltration membranes are expressed as Molecular Weight Cutoff (MWCO). This value refers to the approximate molecular weight (MW) of a dilute globular solute (i.e., a typical protein) which is 90% retained by the membrane. However, a molecule's shape can have a direct effect on its retention by a membrane. For example, linear molecules like DNA may find their way through pores that will retain a globular species of the same molecular weight.

There are three generic applications for ultrafiltration:

1. **Concentration.** Ultrafiltration is a very convenient method for the concentration of dilute protein or DNA/RNA samples. It is gentle (does not shear DNA as large as 100 Kb or cause loss of enzymatic activity in proteins) and is very efficient (usually over 90% recovery).
2. **Desalting and Buffer Exchange (Diafiltration).** Ultrafiltration provides a very convenient and efficient way to remove or exchange salts, remove detergents, separate free from bound molecules, remove low molecular weight materials, or rapidly change the ionic or pH environment.
3. **Fractionation.** Ultrafiltration will not accomplish a sharp separation of two molecules with similar molecular weights. The molecules to be separated should differ by at least one order of magnitude (10X) in size for effective separation. Fractionation using ultrafiltration is effective in applications such as the preparation of protein-free filtrates, separation of unbound or unincorporated label from DNA and protein samples, and the purification of PCR products from synthesis reactions.

Choosing the Correct Device

Devices are available in a range of sizes that accommodate the following sample volumes (Table 1):

Table 1

Device Selection Based on Volume Filtered

Sample Volume	Use
50 µL to 500 µL	Nanosep Device
500 µL to 3.5 mL	Microsep™ Device
3 mL to 15 mL	Macrosep® Device
15 mL to 60 mL	Jumbosep™ Device

Choosing the Correct MWCO

Once sample volume is determined, the next step is to select the appropriate MWCO (for ultrafiltration) or pore size (for microfiltration). MWCOs are nominal ratings based on the ability to retain > 90% of a solute of a known molecular weight (in Kilodaltons). Tables 2 and 3 provide retention characteristics of different MWCO membranes for some solutes. For proteins, it is recommended that a MWCO be selected that is 3 to 6 times smaller than the molecular weight of the solute being retained. If flow rate is a consideration, choose a membrane with a MWCO at the lower end of this range (3X); if the main concern is retention, choose a tighter membrane (6X).

It is important to recognize that retention of a molecule by a UF membrane is determined by a variety of factors, among which its molecular weight serves only as a general indicator. Therefore, choosing the appropriate MWCO for a specific application requires the consideration of a number of factors including molecular shape, electrical charge, sample concentration, sample composition, and operating conditions. Because different manufacturers use different molecules to define the MWCO of their membranes, it is important to perform pilot experiments to verify membrane performance in a particular application.

Common variables that increase molecule passage:

- ▶ Sample concentration less than 1 mg/mL (Figure 1).
- ▶ Linear versus globular molecules.
- ▶ High transmembrane pressure created by g-force in centrifugal concentrators. (This is especially important in the case of linear molecules, for example DNA fragments. Decreasing the g-force can increase retention of molecules by a membrane.)
- ▶ Buffer composition that favors breakup of molecules.
- ▶ pH and ionic conditions that change the molecule (for example, cause conformational changes or aggregation).

Common variables that decrease molecule passage:

- ▶ Sample concentration higher than 1 mg/mL.
- ▶ Buffer conditions that permit molecules to aggregate.
- ▶ Presence of other molecules that increase sample concentration.
- ▶ Lower transmembrane pressure. (In the case of centrifugal concentrators, lower g-force.)
- ▶ Adsorption to the membrane or device.
- ▶ Low temperature (4 °C versus 24 °C).

Table 2
MWCO Selection for Nucleic Acid Applications

MWCO	Base Pairs (DS)	Bases (SS)
1K	5-16 Bp	9-32 Bs
3K	16-32 Bp	32-65 Bs
5K	25-50 Bp	50-95 Bs
10K	50-145 Bp	95-285 Bs
30K	145-285 Bp	285-570 Bs
50K	240-475 Bp	475-950 Bs
100K	475-1,450 Bp	950-2,900 Bs

300K	1,450-2,900 Bp	2,900-5,700 Bs
1,000K	4,800-9,500 Bp	> 9,500 Bs

Table 3
MWCO Selection for Protein Applications

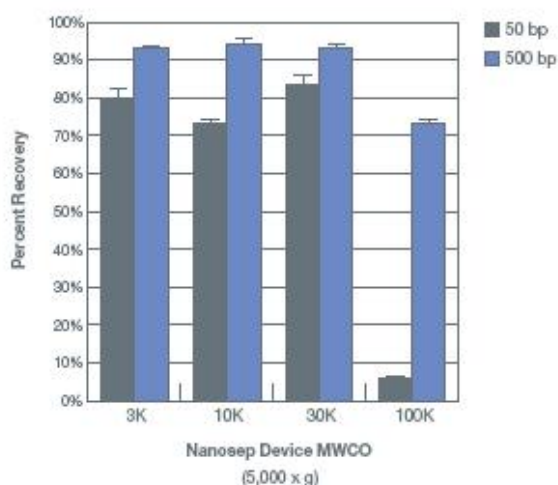
MWCO	Membrane Nominal Pore Size	Biomolecule Size	Biomolecule Molecular Weight
1K			3K-10K
3K			10K-20K
5K			15K-30K
10K			30K-90K
30K			90K-180K
50K	5 nm	15-30 nm	150K-300K
100K	10 nm	30-90 nm	300K-900K
300K	35 nm	90-200 nm	900K-1,800K
1,000K	100 nm	300-600 nm	> 3,000K

Figure 1
Retention and Passage Dilute Protein Samples

□

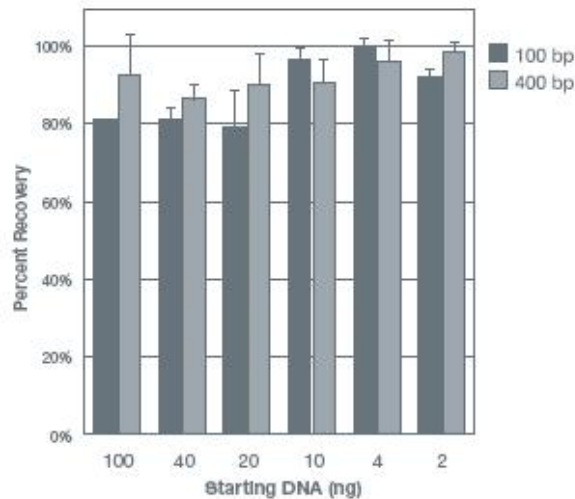
Ten 400 μ L samples of a dilute solution of BSA (100 ng/mL) were centrifuged in the indicated MWCO Nanosep devices. Retentate was recovered in 40 μ L of phosphate buffered saline (10X concentrated relative to the original sample). 20 μ L aliquots of the retentate and filtrate from each device were denatured and electrophoresed on a 4 to 20% polyacrylamide gel and stained with coomassie blue. At higher initial concentrations, some BSA would normally be retained by the 100K device (data not shown) while at an initial concentration of 100 ng/mL, no BSA was detected in the retentate.

Figure 2a
Retention and Passage of Dilute Protein Samples



A 500 μ L sample of a 100 μ g/mL DNA solution containing 50 bp and 500 bp double-stranded DNA fragments was centrifuged at 5,000 x g in Nanosep devices to a final volume of 50 μ L. Recovered samples were quantitated using absorbance at 260 nm. We found that the 100K device is able to separate these fragments; the 50 bp fragment was recovered in the filtrate, and the 500 bp fragment was recovered in the retentate.

Figure 2b
Recovery of Dilute DNA Samples



Nanosep 30K devices were used to filter dilute radioactive DNA fragments. In order to accurately quantify DNA recovery from dilute samples, PCR products (100 and 400 bp) were dual labeled to low-specific activity with ^{32}P -dCTP and ^{32}P -dATP and prepared for filtration. After synthesis, unincorporated nucleotides as well as termination products were removed by ultrafiltration using a 30K Nanosep device. The resulting retentate was checked for size and quantitated using gel electrophoresis. Labeled DNA in quantities ranging from 100 ng all the way down to 2 ng per device was diluted to 500 μL using TE. The samples (in triplicate) were centrifuged at 5,000 $\times g$ for 10 minutes (spun to dryness) and recovered in two washes of 20 μL water. The resulting retentate was added to a counting vial containing scintillation solution and counted. Nanosep devices reliably recover small, dilute DNA samples.

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Purification and Handling of DNA Fragments

Background

From a biochemical standpoint, DNA is a rather simple molecule and, unlike proteins, chemical extraction methods cannot separate one similarly-sized DNA molecule from another. Gel electrophoresis separates DNA molecules at a very high resolution based on size. However, the majority of scientists need to do much more than simply analyze restriction digest DNA fragments on agarose gels. For these applications, DNA molecules are cut, ligated, sequenced, transformed, electrophoresed, and labeled. In addition, desalting, buffer exchange, or elution from agarose may be required during any of a number of procedures. The handling of these sensitive and often very small and dilute biomolecules requires specific proactive laboratory procedures to prevent the loss or contamination of valuable samples.

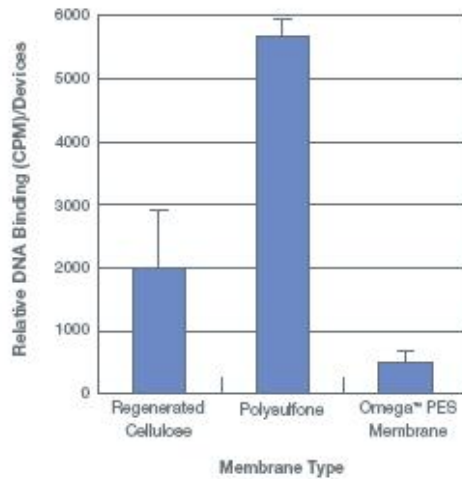
Purification and recovery of DNA molecules from either agarose gels or other complex reaction solutions can be accomplished in several ways.

1. Electroelution, using an electric charge with either dialysis bags or a specialized electrophoresis device to capture DNA migrating out of a gel fragment. Often this method requires vigilance, and the recovered DNA sample requires a concentration/desalting step.
2. [Affinity Chromatography](#), using either glass beads, resins, or glass fiber membranes to bind DNA and elute off after washing. This method is labor intensive, can cause shearing, and leaves glass particles, ethanol, and salts in the DNA sample.
3. Precipitation, using differential solubility to selectively separate DNA from other alcohol-soluble components. This method results in poor recoveries from dilute DNA solutions. It is also time consuming, and gel and buffer contaminants can be co-precipitated. In addition, over-dried pellets may not redissolve properly.
4. Column [Chromatography](#) relies on size separation in a gel bed. This technique often requires the addition of carrier tRNA to achieve high yields. Column chromatography requires significant handling and dilutes the original sample.
5. Microfiltration/Ultrafiltration allows the rapid purification and concentration of the DNA using Nanosep devices. With this method there is very little handling, yields are high, DNA binding is low (Figure 3), DNA is undamaged, and the concentrated DNA is free of contaminants and ready for further reactions.

In general, DNA molecular weights do not correspond well with the MWCO because DNA is a long linear molecule (Table 2).

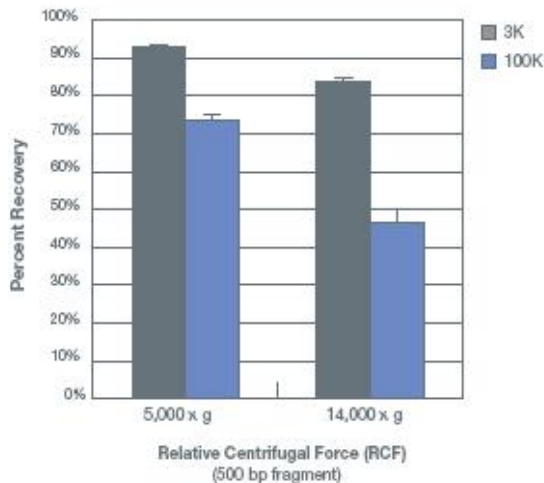
Effective retention of DNA by an ultrafiltration membrane requires a reduction in g-force, otherwise DNA can be forced through many MWCO membranes regardless of size (Figure 4).

Figure 3
DNA Binding to Centrifugal Devices



A 500 μ L sample of a 32 P-labeled PCR product (400 bp, 50 ng/mL) was centrifuged at 5,000 x g in a 100K Nanosep or competitive device. The retained DNA was recovered in 40 μ L TE (10 mM Tris, 1 mM EDTA pH 8). The remaining radioactivity in the device was counted in a scintillation counter. A value at 1000 CPM roughly corresponds to 1% of the total sample.

Figure 4
Effect of RCF on DNA Recovery



A 500 μ L sample of a 100 μ g/mL solution of a 500 bp double-stranded DNA fragment was centrifuged in a 3K or 100K Nanosep device for 5 to 25 minutes. Recovered samples were quantitated using absorbance at 260 nm. Increased loss of the 500 bp fragment was seen at high speed for the 100K device.

Removal of Free Nucleotides from Labeling Reactions

Description

Synthesis of radioactively-labeled DNA is one of the most commonly used procedures in molecular biology labs. These sequence-specific labeled DNA fragments can be hybridized to specific DNA sequences bound to membranes, allowing them to be detected by autoradiography. Recently, a variety of non-radioactive methods for tagging and detecting biological markers have been developed. These methods use enzyme activity tags that can produce color, chemiluminescent, or fluorescent signals.

In all labeling methods, a target molecule is modified using enzymes that catalyze the addition of a molecular tag in the presence of reaction buffers and small molecule substrates. Despite efforts to increase labeling efficiencies, incorporation typically ranges between 10 and 80%, leaving a significant quantity of unincorporated label present after the reaction is complete. In order to reduce background noise on blots or to prevent problems with subsequent downstream reactions, purification steps are often required to remove the reaction buffer components and unincorporated label from the labeled DNA. Nanosep centrifugal devices are ideal for the rapid separation of labeled products from their building blocks (Figure 5).

For full details, see Pall Life Sciences technical report, "[Purification and Handling of DNA Fragments.](#)"

Figure 5
Removal of Free Nucleotides from Labeling Reactions



A PCR labeling reaction was set up using 100 ng pUC18, 20 nmole primers, and PCR Supermix (Life Technologies) supplemented with 10 uCi ^{32}P -dCTP resulting in 400 bp low-specific-activity PCR products with about 5% of the available label incorporated and 95% free nucleotides for each reaction. PCR reactions were centrifuged in a 30K Nanosep device at 5,000 x g for 15 minutes and recovered using two 20 μL TE (10 mM Tris, 1 mM EDTA, pH 8) rinses and equivalent loads were electrophoresed using a 10% Tris Borate polyacrylamide gel. RET= Retained DNA, FT= Flow-Through. The 30K Nanosep device is clearly capable of removing the large number of free nucleotides completely without losing the valuable 400 bp PCR product in the process.

Step-by-Step Procedure

1. Dilute the labeling reaction to 500 μL with TE (10 mM Tris/1 mM EDTA, pH 8).
2. Transfer the diluted sample into a separate, appropriate Nanosep device (Table 2). Note: Random hexamer synthesized labeling reactions generally produce smaller and more variably-sized DNA fragments and work best with a 30K device.
3. Centrifuge at 5,000 x g for 10 to 15 minutes; do not overspin.
4. Optional: To ensure small molecule removal, rinse retentate with 100 to 400 μL TE and repeat centrifugation.
5. Recover retained DNA from the membrane surface with water or TE by rinsing the membrane surface with a pipette tip. Highest yields result from two rinses of 20 μL each.
6. The labeled molecule's specific activity can be approximated by counting the radioactivity of the retentate and comparing this to the counts in the filtrate* or starting material.

*While developing your protocol, you should save the filtrate in the event that a different MWCO is needed.



Single-tube DNA Purification and Cloning Using Ultrafiltration Devices

Description

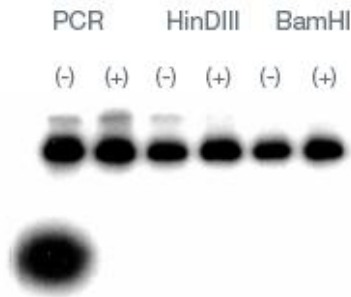
Traditional methods for subcloning DNA fragments or shuttling sequences between vectors involve the ligation of restriction digest fragments from chromosomes, plasmids, cosmids, or PCR products into specific sites on a selected cloning vector. In order to desalt, remove primers or adapters, or concentrate DNA fragments, researchers generally use gel electrophoresis and precipitation steps that are labor intensive and time consuming. Using Nanosep centrifugal ultrafiltration devices for subcloning PCR-generated fragments results in a rapid process that is more streamlined than traditional methods.

After optimizing the parameters for effective target DNA purification, PCR products were synthesized with terminal adapter sequences containing restriction sites. These adapters were digested, generating the appropriate cohesive ends for ligation (2). The resulting DNA products were then concentrated and desalted while simultaneously allowing the primers and adapter fragments to pass into the discarded filtrate during a five-minute centrifugation step. The resulting ligation-ready DNA fragments were ligated to digested vector directly within the ultrafiltration device, and then transformed into competent cells (1). Using Pall Nanosep ultrafiltration devices achieves significant savings in time, labor, and starting materials, while the resulting yield of transformed recombinants roughly equals that of the traditional techniques.

For full details, see Pall Life Sciences technical report, "[Single-tube DNA Purification and Cloning Using Ultrafiltration Devices.](#)"

Figure 6

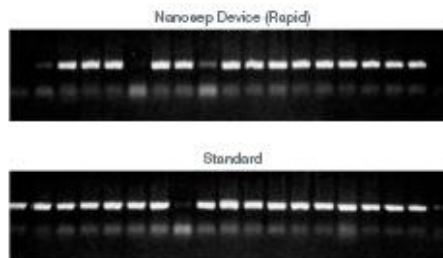
Effect of Terminal Restriction Digestion on Subsequent Ultrafiltration Retention



A radioactive, dilute (100 ng) PCR product amplified from a cloned fragment of *Ceratopteris* phytochrome DNA template was repeatedly purified using a Nanosep 100K device. The restriction digest products are shown for the first (*HinDIII*) and second (*BamHI*) restriction digests. (-) = sample prior to Nanosep device concentration, (+) = recovered concentrated sample after filtration. Equivalent band intensities approximate 100% recovery.

Figure 7

PCR Products Resulting from the Amplification of DNA Templates from Individual Recombinant Colonies



The 620 bp band visualized on an agarose gel indicates successful recombination of the amplified gene fragment into the vector. The absence of a 620-bp product indicates a spurious recombination event. The lengthy standard cloning procedure was used as a control.

Step-by-Step Procedure

PCR

1. Prepare PCR reaction (25 μ L) using an appropriate PCR Master Mix supplemented MgCl₂, 0.2 mM primers, and 1 μ L genomic template DNA.
2. Incubate PCR reactions in a preheated thermocycler at 90 °C for 1 minute, then subject sample to 35 cycles of 30 seconds at 94 °C, 30 seconds at 45 °C, and 60 seconds at 72 °C, followed by a postcycle extension for 3 minutes at 72 °C (or use your favorite conditions).
3. Remove a 5 μ L aliquot of each reaction; electrophorese to confirm the size and purity of the products and to quantitate the fragments for subsequent cloning.

Nanosep Device Sample Preparation

1. Digest 0.1 to 1 μ g of PCR product (either together or in separate tubes) and 0.2 μ g of vector with the appropriate restriction enzymes directly in a Nanosep 100K device for 2 hours at 37 °C.
2. Heat inactivate the enzyme at 95 °C for 5 minutes.
3. Centrifuge through the Nanosep device at 1,500 x g for 5 minutes, and wash the retentate twice by addition of 50 μ L sdH₂O.
4. If sequential digests are required due to buffer incompatibilities, repeat steps 1 and 2 with each enzyme.
5. Resuspend in 8.5 μ L sdH₂O, 1 μ L 10x ligation buffer with ATP, and 0.5 μ L ligase. Ligation was allowed to proceed directly in the Nanosep device at 17 °C overnight.
6. If digested in separate tubes, resuspend the vector in 8.5 μ L sdH₂O and transfer to the insert tube for ligation.

Transformation and Recombinant Analysis

Add 3 μ L aliquot of ligation product to transform competent cells. Follow standard transformation procedures.

Plate onto selective culture media to select for transformants.

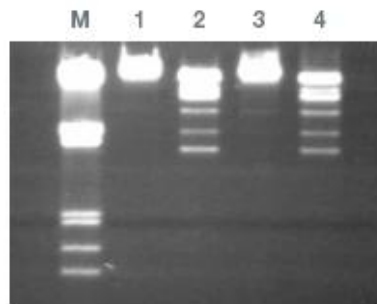
DNA Purification/Buffer Exchange for Restriction Digestions

Description

To be effectively digested or modified, DNA should be free of contaminants such as phenol, chloroform, alcohol, EDTA, detergents, drugs (like actinomycin and distamycin frequently used in DNA studies), or excessive salts. All of these compounds can interfere with restriction endonuclease activity causing either partial digestion or altered specificity (star activity).

Commonly-used protocols recommend dialysis or ethanol precipitation, followed by a wash-and-dry regimen prior to restriction digestion of the DNA sample. Very often, cleaving a DNA substrate with two or more enzymes requires the use of different buffers to satisfy the buffer requirements of each enzyme (Figure 8). Discontinuous diafiltration (a series of concentration and dilution steps) can be used for the easy and efficient removal of small molecular weight contaminants and salts from the DNA sample. While this protocol is written for serial restriction digests, this basic desalting/buffer exchange protocol can be modified to fit a variety of applications.

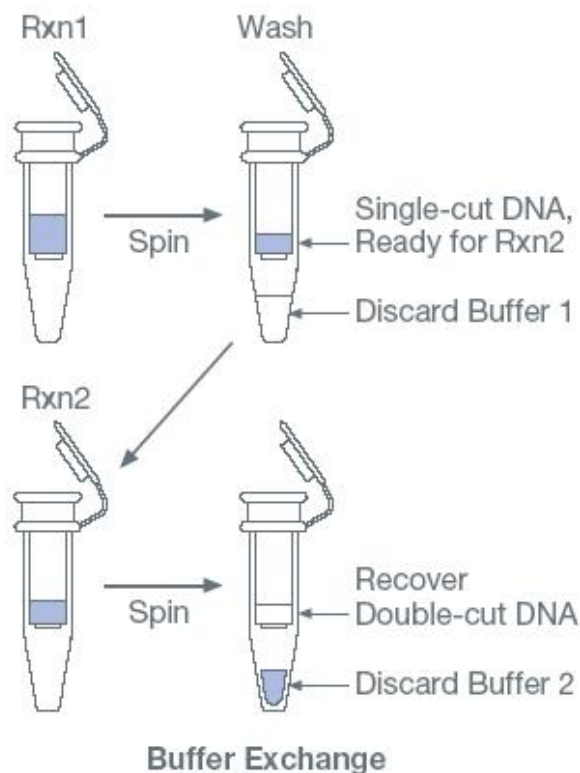
Figure 8
DNA Desalting and Enzyme Activity



The Nru I restriction enzyme requires a unique restriction buffer for the optimal activity. Lane M = size markers-Lambda DNA EcoR1 & Hind III digest, Lane 1 = (negative control) Lambda DNA digested with Nru I in NEBuffer 1, Lane 2 = (positive control) Lambda DNA in Nru I buffer incubated with Nru I, Lane 3 = Lambda DNA in NEBuffer 1 incubated with Nru I, Lane 4 = the same DNA sample from Lane 3, following buffer exchange and digestion with Nru I. Use of a 100K Nanosep device ensures Nru I activity following desalting.

Step-by-Step Procedure

1. Select the Nanosep device with the proper MWCO (Table 2). Note: Increased solutes in the solution will decrease the passage of DNA and may require the use of a higher MWCO device.
2. Dilute the digested DNA sample to 500 μ L with the single strength (1X) buffer to be used for the second digestion.
3. Centrifuge at 5,000 \times g for 5 (100K) to 30 (3K) minutes.
4. Optional: To perform discontinuous diafiltration: Add a sufficient amount of buffer to bring the retentate volume to 500 μ L 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.
5. Resuspend in appropriate 1X restriction digestion buffer, add the second enzyme, digest according to manufacturer's instructions (repeat step 3), and recover double-cut DNA in at least 20 μ L water or buffer.



Purification of DNA Fragments from Agarose Gels

Description

Numerous methods have been developed to purify electrophoretically separated DNA fragments away from the agarose gel matrix. These methods allow specific, electrophoretically separated DNA fragments to be used in further analysis and cloning procedures.

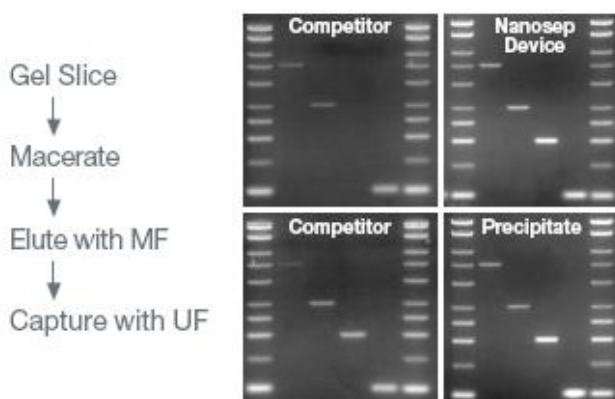
The isolation and purification of DNA fragments from a gel slice is done in two simple steps. First, the DNA is eluted from the agarose slurry. The agarose gel matrix is retained by a Nanosep MF device containing a 0.2 μm or 0.45 μm membrane, and the gel buffer (containing DNA) freely flows through. Second, DNA is concentrated and washed using a Nanosep device containing a MWCO membrane adequate to retain the DNA (see Table 2) and wash away contaminants. This method is more rapid and reliable than the standard precipitation or dialysis methods (Figure 9).

Our data show that the DNA recovery by Nanosep 100K devices was greater than 90% based on band intensity. The four different molecular weight bands represent independent experiments. Precipitated DNA bands also gave high yields but would contain many gel buffer contaminants not found in ultrafiltered DNA samples. DNA recoveries from the competitive devices were generally less reliable, ranging from 0 to 80%. One popular competitor regularly suffered from device seal failures in about 1 in 10 devices tested.

For full details, see Pall Life Sciences technical report, "[Purification and Handling of DNA Fragments.](#)"

Figure 9

Recovery of DNA Fragments from Agarose Gels

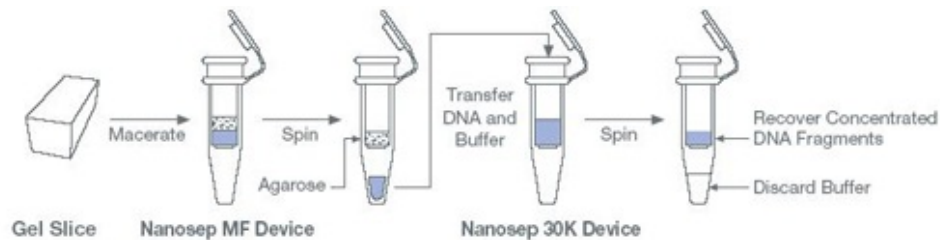


A 0.8% agarose TAE (Tris-acetate, EDTA) preparative gel (not shown) was electrophoresed with 4 μg total DNA from a commercially available 1Kb ladder (Life Technologies). Gel slices for 4 different molecular weight bands (MW) were excised, frozen, macerated and centrifuged using a 0.2 μm device. The DNA containing filtrate was diluted and aliquoted into four fractions. One 400 μL fraction was

precipitated using 1/10th volume sodium acetate and 2 volumes ethanol, incubated 2 hours at -20 °C, centrifuged at high speed for 30 minutes, dried and resuspended in 20 µL TE. The other 400 µL fractions were centrifuged in either a Nanosep 100K (10 minutes) or competitive 100K (20 minutes) device and recovered in 20 µL TE (two 10 µL rinses). The samples were electrophoresed in a 0.8% agarose TAE gel along with marker lanes containing 1 µg of a 1Kb ladder. Equal band intensities approximate 100% recovery.

Step-by-Step Procedure

1. Wear gloves, lab coat, and UV-resistant face protection to prevent UV burns from the transilluminator.
2. Minimize the UV exposure of the gel on the transilluminator to the sample to prevent DNA damage.
3. Cut out the desired DNA band and trim away excess agarose. Freeze at -20 °C (optional, for highest yields) and macerate the agar chunk with a pipette tip.
4. Add up to 400 µL of the agar slurry to a Nanosep MF device (0.45 µm or 0.2 µm); centrifuge at 5,000 x g for 5 minutes.
5. Collect the filtrate from the tube bottom and heat to 65 °C for 5 minutes to inactivate any DNases introduced during electrophoresis or gel handling.
6. Transfer the above filtrate to the appropriate MWCO device and centrifuge at 5,000 x g for 10 to 15 minutes (Table 2). Higher spin speeds will reduce DNA recovery.
7. Optional: To ensure small molecule removal, rinse retentate with 100 to 400 µL 10 mM Tris/1 mM EDTA (TE) pH 8 and repeat centrifugation.
8. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20 µL TE or water. Use a larger bore pipette tip to prevent shearing of fragments larger than 5 Kb.



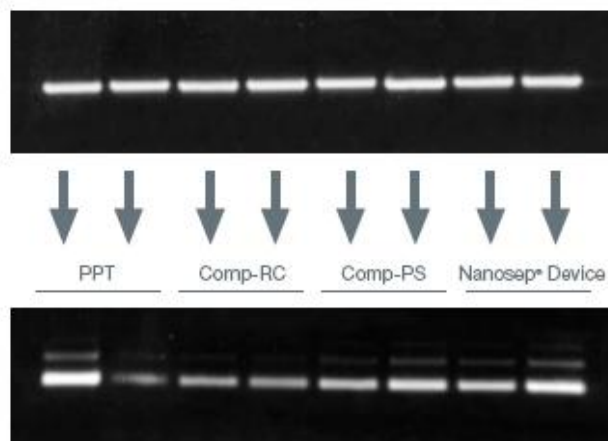
Purification of DNA Fragments from Enzyme-digested Agarose Gels

Description

Recently, enzymes that are able to digest agarose have been used to free DNA from gel slices. While the DNA is now free in solution, a number of soluble contaminants remain that can inhibit downstream handling and modification procedures. Because agarase-digested agarose is in a liquid state, it is now able to pass through a UF membrane as a small molecule. This allows DNA from the original sample to be purified and concentrated in one spin (Figure 10).

Figure 10

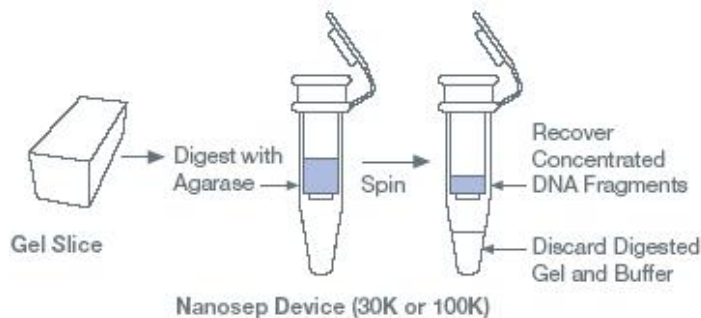
Recovery of DNA Fragments from Agarase Digested Gels



A 400 bp PCR product pool was electrophoresed in 8 aliquots in a 1% Low-Melting-Point (LMP) agarose TAE gel. The eight individual bands were excised and treated with agarase according to manufacturer's instructions (Life Technologies). After agarase digestion, two samples were precipitated with sodium acetate and ethanol according to the agarase manufacturer's protocol. The other six samples were centrifuged in Nanosep or competitive 30K UF centrifugal devices for 15 minutes (Nanosep device) to 30 minutes (competitor). The retentate was recovered in 30 µL TE. PPT = Ethanol Precipitation, Comp-RC = Competitor Regenerated Cellulose, Comp-PS = Competitor Polysulfone, Nanosep Device = high-flux polyethersulfone. The Nanosep devices gave the most reproducible and highest yields of all devices and techniques.

Step-by-Step Procedure

1. Wear gloves, lab coat, and UV-resistant face protection to prevent UV burns from the transilluminator.
2. Minimize the UV exposure of the gel on the transilluminator to the sample to prevent DNA damage.
3. Electrophorese the DNA sample(s) in Low-Melting-Point agarose.
4. Cut out the desired DNA band and trim away excess agarose. Take care handling the LMP gel as it tends to be softer than normal agarose.
5. Follow the manufacturer's instructions for agarase digestion. After digestion is complete, dilute the sample to 500 μ L with TE (10 mM Tris/1 mM EDTA, pH 8) or water.
6. Centrifuge the digested-diluted sample for 10 to 15 minutes at 5,000 x g in a 30K or 100K Nanosep UF device (Table 2).
7. Recover the retained DNA with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20 μ L TE or water. Take care not to pipette any remaining undissolved gel material adhering to the membrane.
8. Optional: To ensure complete small molecule removal, rinse retentate with 100 to 400 μ L TE and repeat centrifugation and recovery.



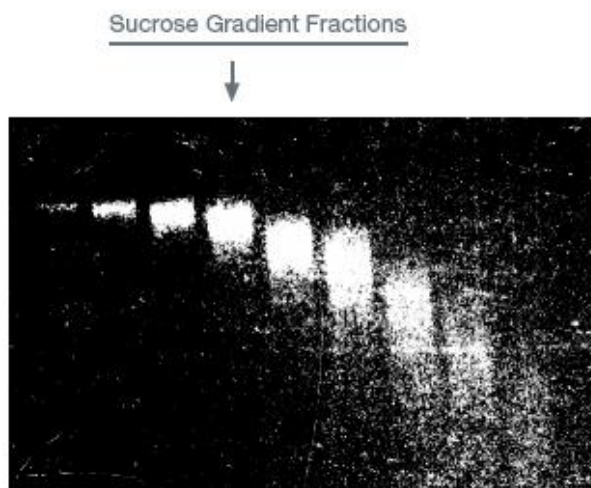
Purification of Partial-digest DNA Fragments from a Sucrose Gradient

Description

Isolating a population of large DNA fragments for use in the construction of gene libraries is still best accomplished by separating the DNA fragment pools using a sucrose gradient. Once the appropriate fraction has been identified, the next step is to purify the DNA from the sucrose and buffer mixture. Diafiltration may be needed to completely remove the sucrose prior to ligation. Partial digestion fragments are generally larger than 1 Kb, and a 100K Nanosep device is recommended (Figure 11).

Figure 11

Recovery of DNA Fragments from a Sucrose Gradient Fraction

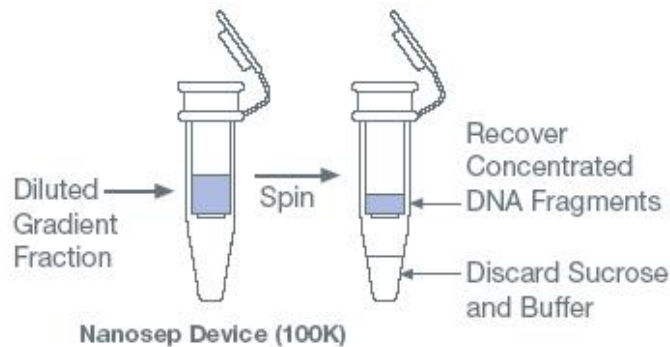


*A partial *Sau*3A digest of yeast genomic DNA was centrifuged on a sucrose gradient for 18 hours at 56,000 rpm. A series of 2 mL fractions was collected and 40 μ L was electrophoresed to check quality and fraction size range. Fraction #4 (see arrow) was diafiltered twice using a 100K device. This fraction was ligated into a cosmid vector and resulted in a 10,000 clone subgenomic library used successfully to clone a gene by chromosome walking.*

Step-by-Step Procedure

1. Dilute the appropriate fractions with at least one volume of TE (10 mM Tris/1 mM EDTA, pH 8) or water.
2. Aliquot each diluted fraction into a separate Nanosep 100K device (500 μ L each) or use a larger spin device for larger volumes (a Microsep™ or Macrosep® device).

3. Centrifuge at 5,000 x g for 10 minutes; discard filtrate.
4. Optional: To perform discontinuous diafiltration, add a sufficient amount of buffer to bring the sample volume to 500 μ L 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.
5. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20 μ L TE or water. Use a large-bore pipette tip to prevent shearing large DNA fragments.



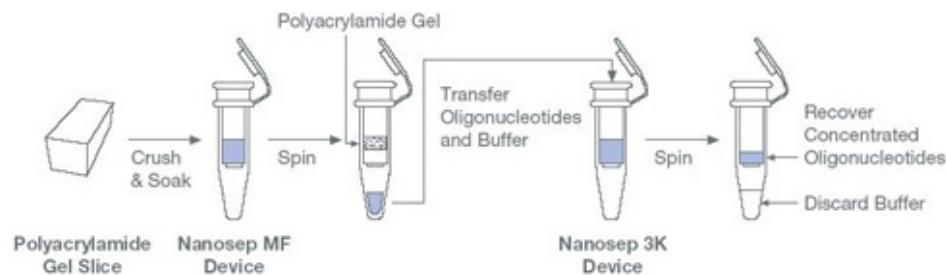
Purification of Oligonucleotides from Polyacrylamide Gels

Description

Oligonucleotides often require gel purification to separate shorter synthesis-failure sequences from full-length synthetic products. Once the desired band has been excised from the gel, it is necessary to recover the oligonucleotide from the gel matrix. The “crush and soak” technique is the method of choice for elution from polyacrylamide gels because it is inexpensive, easy, and can be accomplished without monitoring. Nanosep MF devices can be used to remove gel debris, followed by ultrafiltration using a Nanosep 3K device to concentrate and desalt the oligonucleotides.

Step-by-Step Procedure

1. Electrophorese oligonucleotides in a polyacrylamide gel. Use 20% acrylamide/8 M urea for oligonucleotides < 25 bases; 15% acrylamide/8 M urea for oligonucleotides 25 to 60 bases, and 12% acrylamide/8 M urea for 60 to 100 bases.
2. Visualize oligonucleotide bands by autoradiography (if radiolabeled). If unlabeled, detect using UV shadowing by placing the gel on a fluorescent TLC plate and using short-wavelength UV lamp to observe fluorescence quenching.
3. Excise each oligonucleotide band with a new razor blade.
4. Place the gel slice into a microcentrifuge tube and grind it into a fine powder with a microcentrifuge pestle. Add 0.5 mL of extraction buffer and vortex. Incubate the slurry at 37 °C for 2 hours or overnight at room temperature.
Extraction buffers:
 - Deionized water
 - 0.5 M NaCl, 2M triethylammonium acetate
 - 50 mM triethylammonium acetate
 - 0.5 M NaCl, 0.1 M Tris-HCl (pH 7.0), 1 mM EDTA
5. To remove acrylamide fragments, transfer the gel slurry to a Nanosep MF device and spin at 14,000 x g for 10 minutes. Rinse the upper chamber with 50 μ L TE (10 mM Tris/1 mM EDTA, pH 8) and spin again.
6. To simultaneously concentrate the oligonucleotides and remove dissolved urea and salts, transfer the filtrate to a Nanosep 3K device and centrifuge at 5,000 x g. Oligonucleotides larger than 30 bp may be centrifuged at 14,000 x g.
7. Optional: To perform discontinuous diafiltration, add a sufficient amount of buffer to bring the sample volume to 500 μ L 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.
8. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20 μ L TE or water.



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PCR: Before and After

Background

One of the most powerful and revolutionary procedures used today to analyze DNA sequences is based simply on the unique biochemistry of DNA replication and is called Polymerase Chain Reaction, or PCR.

This powerful technique allows researchers to “amplify” specific DNA sequences from samples that contain only a few individual DNA molecules with sequences complementary to the primers. However, PCR methods are not without problems. The extreme sensitivity of PCR increases the probability that false priming or DNA contamination will lead to the amplification of the wrong molecule.

One way to help prevent artifacts that arise from impurities is to work in an extremely controlled environment and to purify the reagents and primers prior to use. Even in a controlled environment, the sample itself can contain components that interfere with PCR or cause false priming. Using Nanosep devices to remove these contaminants prior to PCR can help to ensure correct results.

After the completion of a PCR reaction, the buffer (a mixture of primers and free nucleotides) and the newly synthesized DNA fragments need to be separated so that the synthesized DNA can be used for downstream applications.

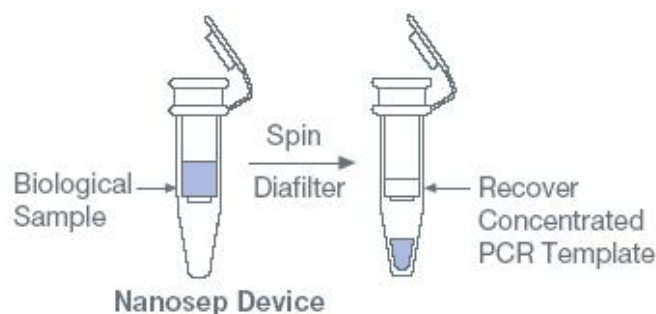
Preparation of Biological Samples for PCR

Description

Biological samples that contain DNA templates come from a variety of sources. Many of these sources contain small molecules that can inhibit the function and accuracy of PCR amplification. For example, heparin and EDTA (commonly used to preserve blood samples) can interfere with PCR reactions. Removal of small molecules while concentrating template DNA can be done by ultrafiltration.

Step-by-Step Procedure

1. Dilute the template containing sample to 500 μL with water or TE (10 mM Tris/1 mM EDTA, pH 8) and transfer to a suitable Nanosep UF device (Table 2).
2. Centrifuge at 5,000 \times g for 10 to 30 minutes.
3. Optional: To perform discontinuous diafiltration, add a sufficient amount of buffer to bring the sample volume to 500 μL 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.
4. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20 μL TE or water.



Preparation of Reagents and Solutions for PCR

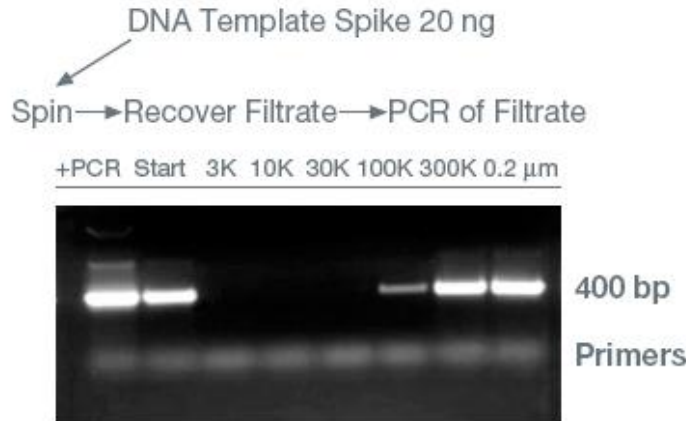
Description

The extreme sensitivity of PCR can result in amplification of minute amounts of contaminating DNA. This is a particular problem with ancient DNA or forensic human DNA samples because one contaminating molecule from a technician’s skin or hair cell can invalidate results.

Removal of contaminating DNA from stock reagents or master mixes can be done using ultrafiltration. To ensure that it is free of DNA, the final material synthesis buffer can be spun in a 3K or 10K Nanosep UF device prior to aliquoting into PCR tubes (Figure 12). Note: Never bring amplified samples into the same work area that is used to prepare reagents or set up future PCR reactions. Always use aerosol-resistant tips for pipetting.

For full details, see Pall Life Sciences technical report, "[Nanosep Centrifugal Ultrafiltration Devices and PCR: Before and After.](#)"

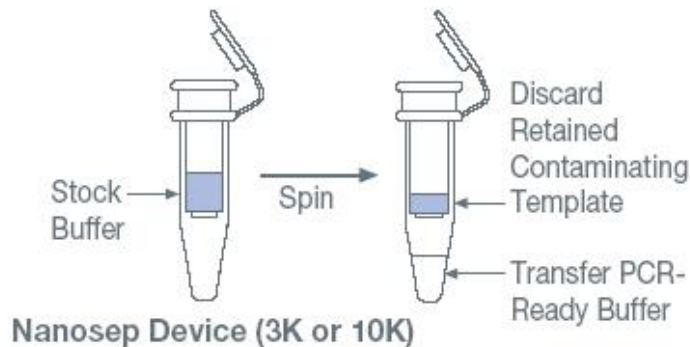
Figure 12
Removal of DNA Template from PCR Stock Solutions



DNA template sample of 500 ng plasmid pUC18 in TE was divided into seven 200 μL fractions, one fraction was kept as a control (START, 33 ng/μL) and the other fractions were centrifuged in Nanosep ultrafiltration devices. Following centrifugation, 3 μL was added to each PCR reaction containing 45 μL PCR mix (Life Technologies) and 2 μL of a 20 nmole pUC18-complementary primer mix. A PCR reaction of 25 cycles under standard conditions was performed and 25 μL of each reaction was electrophoresed and stained with ethidium bromide. No PCR product was detected using filtrate samples from the 3K, 10K, and 30K devices, demonstrating that they effectively removed the template spike. Filtrate from the 100K device gave a weak PCR product band, suggesting that some of the template was able to pass through this device. DNA template passed freely through the 0.2 μm and 300K devices.

Step-by-Step Procedure

1. Always use new or "set-aside" chemicals to make stock PCR reagents. Use care to avoid adding any new contaminants during preparation.
2. Prepare reagents away from the area used to process PCR products.
3. Centrifuge the stock reagents or master mixes (without primers, templates, or enzymes) in an appropriate 3K or 10K UF device (Table 2).
4. Transfer filtrate to sterile tubes. Sub-aliquotes are suggested to minimize future contamination of entire stocks.
5. For extra insurance, ultrafiltration can be used to filter the master mix or commercial buffer mix immediately prior to the start of PCR.

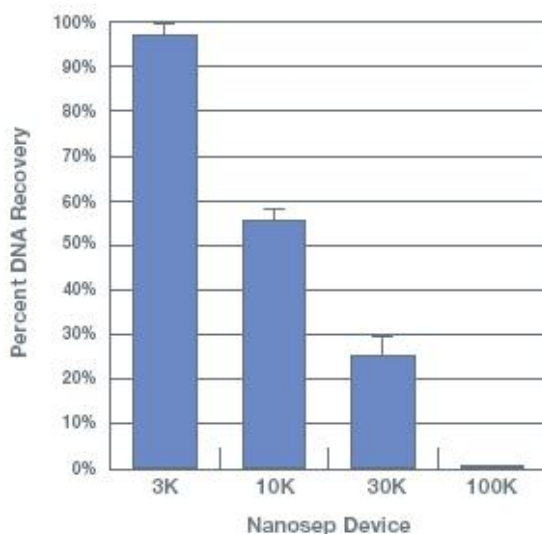


Purification and Recovery of PCR Primers

Description

Synthetic oligonucleotides are a critical component of the PCR reaction as they act as primers for the synthesis of DNA from DNA sequence-specific start points. Oligonucleotides are synthesized by chemical means. After synthesis, purification steps are required to purify the full-length oligonucleotides from the synthesis mixture. Prior to PCR, desalting is required to remove residual by-products from the synthesis, cleavage, and deprotection procedures. Ultrafiltration using centrifugal concentrators is an efficient, high-yield way to desalt and concentrate oligonucleotides (Figure 13).

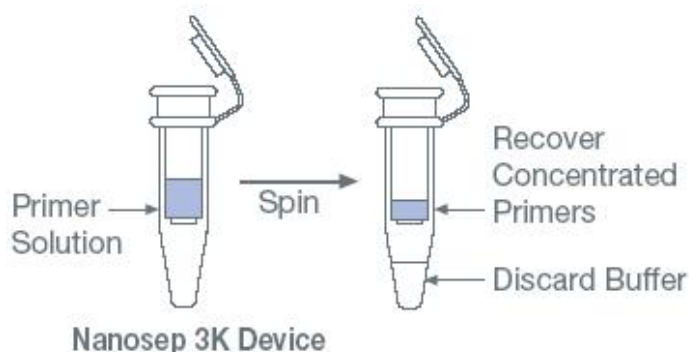
Figure 13
Purification and Recovery of 25 bp Oligonucleotides



A 400 μL solution containing 50 ng end-labeled 25 bp oligonucleotides was filtered, in duplicate, using different molecular weight cutoff (MWCO) devices. These devices were centrifuged for 10 minutes (100K, 30K, 10K) to 30 minutes (3K) at 5,000 $\times g$. The retained material was resuspended in 40 μL TE, diluted to 400 μL , and then recovered. Radiolabeled DNA was quantified using a scintillation counter. The Nanosep 3K device retained close to 90% of the radiolabeled oligonucleotide. The choice of a higher MWCO device allowed for a shorter spin time but resulted in decreased yields. If the intention was to pass all of the primers, then the 100K device would be the best choice.

Step-by-Step Procedure

1. For oligonucleotides up to 50 bases long, use a Nanosep 3K device. For longer oligonucleotides, the Nanosep 10K device can be used.
2. Place the oligonucleotide solution in the sample reservoir. Dilute the sample to a volume of 500 μL with TE (10 mM Tris/1 mM EDTA, pH 8) or water.
3. Optional: To perform discontinuous diafiltration, add a sufficient amount of buffer to bring the sample volume to 500 μL 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.
4. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20 μL TE or water.



Cleanup and Recovery of PCR Products

Description

The final PCR reaction may contain up to a microgram of amplified DNA that can be used for a variety of molecular biology applications. These applications may be more or less sensitive to the residual components of the PCR reaction mix. Certain restriction enzymes and particularly DNA ligase are very sensitive to the presence of contaminants in DNA samples. Because a PCR reaction mixture contains a variety of salts, free nucleotides, glycerol, proteins, and primers, most downstream applications will require some sort of cleanup of the PCR product.

Purification of the PCR product away from buffer components, free nucleotides, and primers can be performed in a variety of ways:

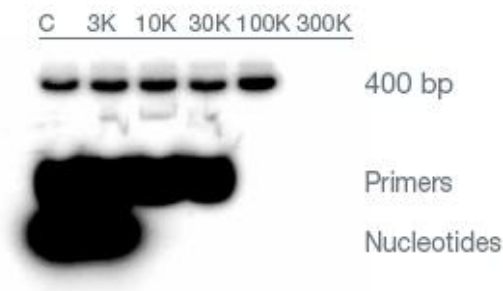
1. Precipitation, using chemical solubility properties to selectively separate DNA. The primary drawbacks for using this method to purify PCR products are the time involved and incomplete removal of co-precipitating buffer components and contaminants.
2. Chromatography, using size exclusion resin or affinity to glass to purify DNA from the PCR mixture components. This technique is costly, generally requires significant handling, and samples must be concentrated after elution from the matrix.
3. Ultrafiltration involves the isolation and concentration of PCR products using size exclusion membrane devices (Figure 14). It is rapid, there is very little handling, yields are high, DNA is undamaged, and the concentrated DNA is free of contaminants that may inhibit downstream reactions (Figure 15).

For full details, see Pall Life Sciences technical report, "[Nanosep® Centrifugal Ultrafiltration Devices and PCR: Before and After.](#)"

Step-by-Step Procedure

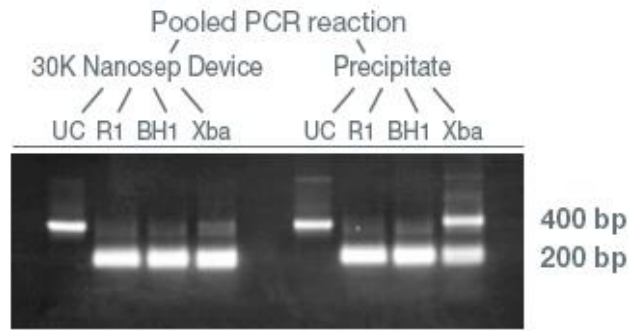
1. Avoid pipetting the oil or wax into the subsequent steps. Oil can be removed by freezing the sample at -70 °C and drawing off the surface liquid, or the sample can be successfully removed by careful pipetting with a narrow bore pipette tip from below the surface of the oil.
2. Dilute the reaction mixture to 500 µL with water or TE (10 mM Tris/1 mM EDTA, pH 8) and transfer to a Nanosep 30K or 100K UF device (Table 2).
3. Centrifuge at 5,000 x g for 10 minutes (greater speeds increase PCR product loss).
4. Optional: To perform discontinuous diafiltration, add a sufficient amount of buffer to bring the sample volume to 500 µL 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.
5. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20 µL TE or water.

Figure 14
Purification and Recovery of PCR Products



A small amount of ^{32}P -labeled dCTP was added to the PCR reaction mix (Life Technologies) containing ^{32}P -end labeled primers. The reaction was run for 30 cycles using standard conditions. Ten reactions were pooled and 100 µL aliquots were diluted to 500 µL and centrifuged in the indicated MWCO devices. The retained material was recovered in 20 µL TE, electrophoresed using a 10% polyacrylamide Tris Borate gel (BioRad) and analyzed by autoradiography. The 100K Nanosep device demonstrated the best combination of PCR product retention along with complete primer and nucleotide removal. If the desire is to ensure the removal of the buffer and free nucleotides but not primers, then the 30K or 10K devices will retain the PCR product while removing buffer components. Cleanup using the 10K device may be used if the PCR product is smaller than 200 bp and the presence of primers does not inhibit downstream applications.

Figure 15
Biological Activity of Purified PCR Products



A pooled mixture of PCR products was divided in half. One fraction was precipitated with 1/10th volume sodium acetate and two volumes ethanol, chilled at -20 °C for 2 hours, centrifuged at high speed for 30 minutes, rinsed with 70% ethanol and air dried prior to resuspension in 40 µL water. The second fraction was diluted to 500 µL and centrifuged for 15 minutes in a 30K Nanosep device. The retained material was resuspended in 40 µL water and divided into 10 µL aliquots for analysis. One fraction was electrophoresed as the uncut (UC) control and the others were diluted into a 20 µL restriction digest reaction according to manufacturer's instructions (EcoRI = RI, BamHI = BH1, Xba1 = Xba). These samples were digested for 30 minutes at 37 °C, electrophoresed in a 1.5% agarose gel, and DNA bands were visualized by staining with ethidium bromide. Our data show clearly that the XbaI enzyme is unable to digest to completion the 400 bp PCR fragment from a sample that has been precipitated. In contrast, the sample that was rapidly purified with the 30K device was completely digested using XbaI, indicating that use of this device removed contaminants that could interfere with digestion.

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Protein Purification and Handling

Background

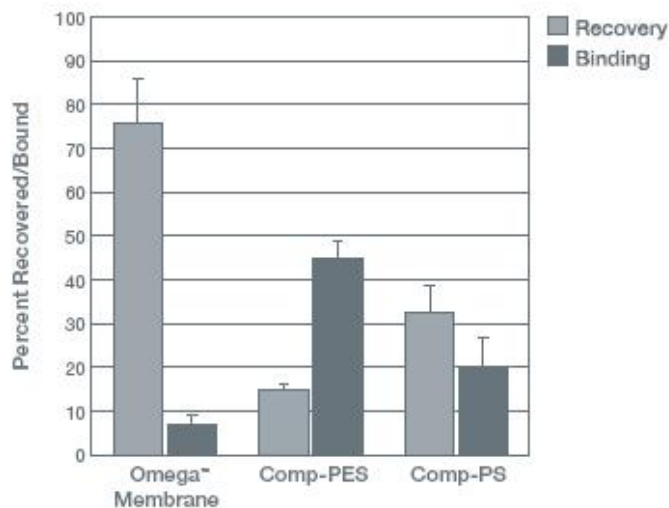
Ultrafiltration has been used successfully for years and is an excellent and gentle method to purify and concentrate protein samples. Ultrafiltration can be used to replace precipitation, evaporation, dialysis, lyophilization, and gel filtration to concentrate and desalt protein without significant protein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



Nanosep devices (Omega membrane) and competitive devices (Comp-PES, Comp-PS) were used to filter and recover radioactively-labeled BSA (^{125}I -BSA). A 1 μg sample of labeled BSA was diluted into 500 μL 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 μL 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).

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 $\times g$ and were concentrated to a volume of 10 to 60 μL .

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 μL 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 μL for a Nanosep device) is used to rinse the membrane surface for remaining protein samples.

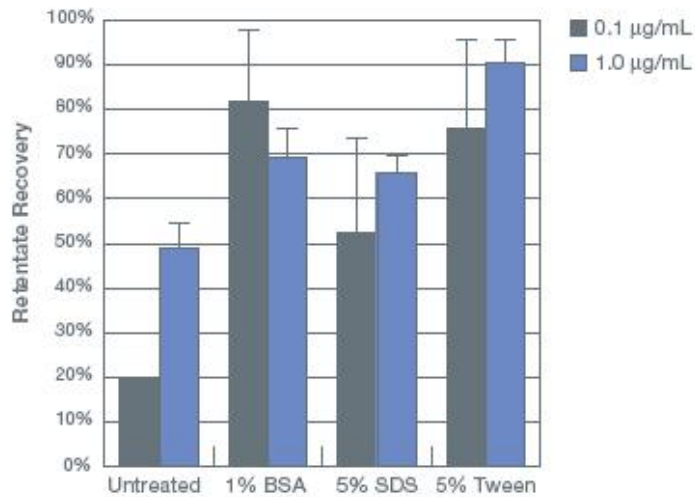
Improving the Recovery of Proteins from Dilute Solutions (Passivation)

Description

For very dilute protein solutions (< 10 $\mu\text{g}/\text{mL}$), 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).

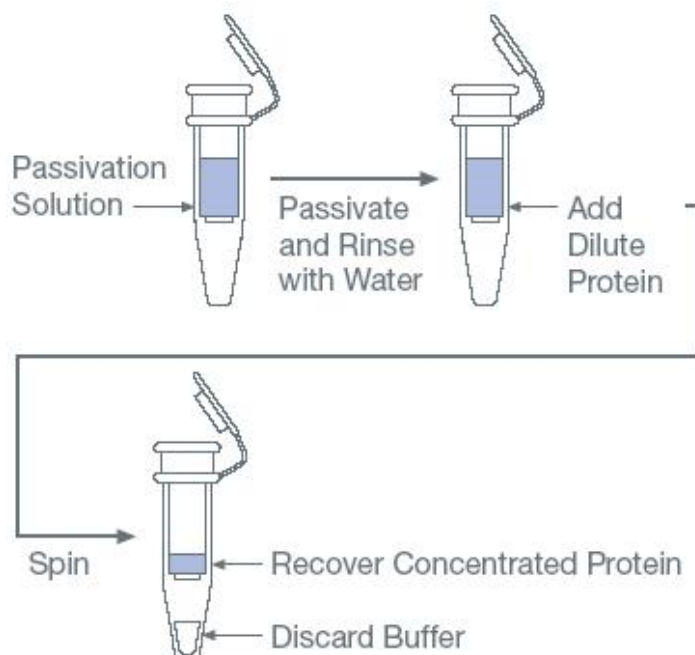
Figure 17
Use of Passivation to Increase Protein Recoveries



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
2. Discard the passivation solution by either pouring or pipetting it out of the device, and rinse the Nanosep device thoroughly with sterile distilled water.
3. 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.
4. 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).

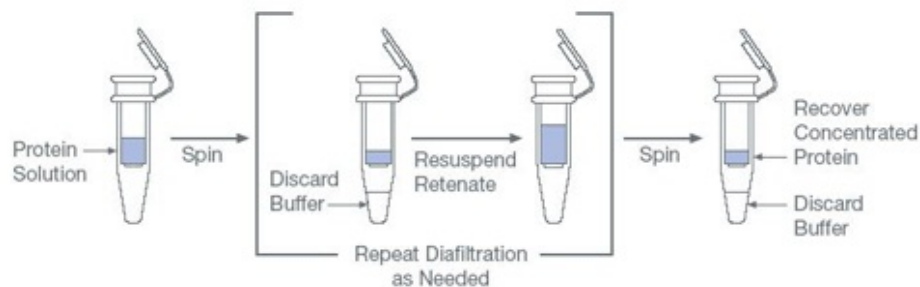
Table 5
Desalting Estimates

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 μ L to 25 μ 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).
2. To concentrate the sample without changing salt concentration: pipette 500 μ 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.
3. To desalt or exchange buffer, first concentrate the sample (step 2) then add new buffer or water to dilute the concentrated sample to 500 μ 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.
4. Recover the retained sample with a pipette tip. To maximize recovery, rinse the retentate cup twice with 10 to 20 μ L new buffer or water.



Gross Fractionation of Complex Protein Solutions

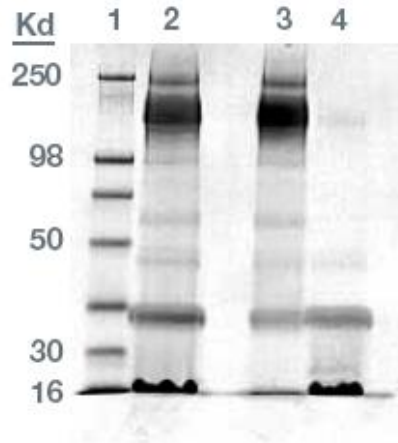
Description

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.

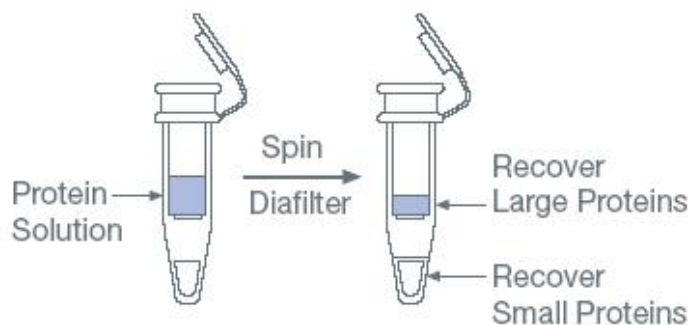
Figure 18
Gross Fractionation of Proteins



A 500 μL sample of a 5.0 mg/mL protein solution containing IgG (156 kD) and Cytochrome C (12.4 kD) was centrifuged at 1,000 \times g for 30 minutes in a Nanosep 100K device. The retentate was recovered in 500 μL ; 15 μL 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 μL of the protein mixture.
2. Centrifuge at low speed (500 to 1,000 \times 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 μL 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

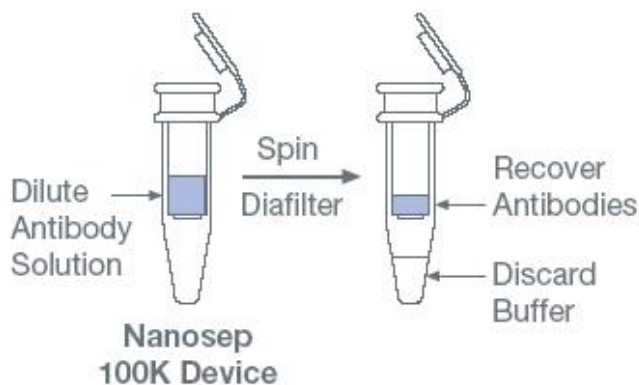
The concentration of monoclonal antibodies (mAbs) in culture supernatants is often too low to be useful for most in vitro and in vivo immunological techniques. Concentrated mAbs are obtained from ascites fluid induced in hybridoma cells. However, development of ascites from hybridoma cells is a complicated 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 \times 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.



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Miscellaneous Protocols

Preparation of Protein-free Samples for Analytical Applications

Description

Peptides and other low molecular weight bioactive materials can be analyzed and purified from complex biological sources using a variety of techniques such as HPLC, GC, and GC/MS. Ultrafiltration is an effective and fast way to remove proteins that may interfere with the analysis of other small molecules prior to these procedures (Table 6).

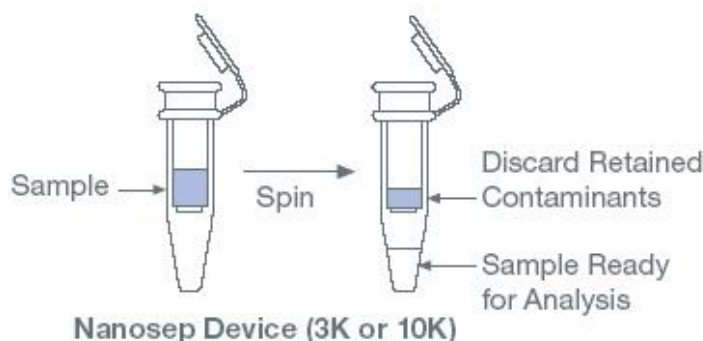
Table 6
Protein Removal for Analytical Applications

Nanosep® Device MWCO	Cyto C (12.5 kD)		BSA (67 kD)	
	3K	10K	3K	10K
% of Protein in Filtrate	<1%	6.5%	0	0

A 500 μL protein sample of 1.0 mg/mL in phosphate buffer saline was centrifuged at 14,000 $\times g$. A sample of filtrate (50 μL) was analyzed using a TOSOHAAS, TSK-GEL G3000WXL, 10 μm , 1000 \times 6.0 mm HPLC column and detected at 280 nm.

Step-by-Step Procedure

1. Select a Nanosep device with a 3K or 10K MWCO membrane, depending on the size of the protein to be removed.
2. Dilute the sample if possible to 500 μL . This step helps minimize membrane fouling; however, smaller volumes can be used.
3. Place the dilute sample into the upper sample reservoir.
4. Centrifuge the Nanosep device at 5,000 to 14,000 $\times g$ for up to 30 minutes.
5. Collect the protein-free filtrates from the bottom filtrate receiver. The sample is now ready for further analysis.
6. If needed, the concentrated/retained proteins can be recovered from the sample reservoir.



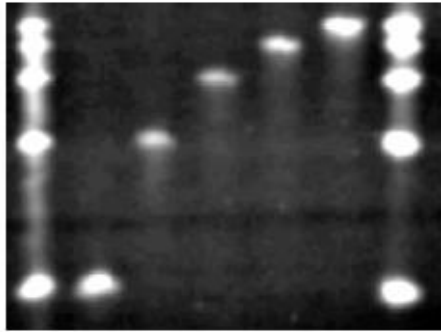
Purification of RNA Transcripts from Polyacrylamide Gels

Description

A number of applications require the purification of synthetic RNA transcripts from polyacrylamide gels

(PAGE). Often this is done to remove short or incomplete transcripts which may interfere with downstream applications. Once the synthetic mRNA band has been identified and excised from the gel, it is necessary to recover RNA from the gel matrix. The “crush and soak” method for mRNA recovery is often the method of choice because it is inexpensive, easy, and can be accomplished without extensive monitoring. Once the mRNA is released into solution, it can be desalted and concentrated in one easy step using ultrafiltration devices (Figure 19).

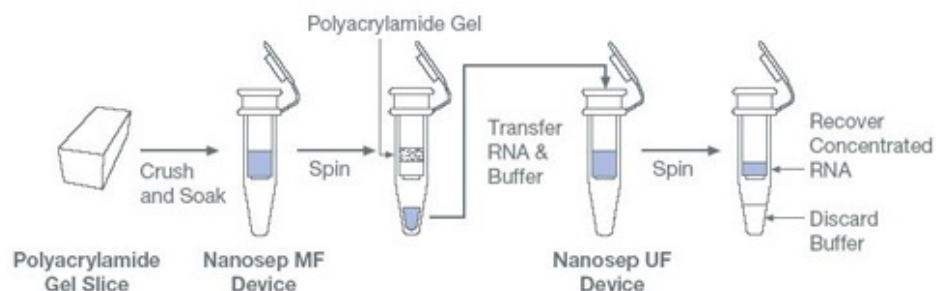
Figure 19
Recovery of RNA from Polyacrylamide Gels



RNA Century Markers prepared by in vitro transcription of Century Markers Templates (Ambion) were electrophoresed in 6% TBE (Tris-borate, EDTA)-urea gel. Gel slices containing RNA fragments from 100 to 500 bases long were excised, macerated with the Eppendorf fitting pestle and mixed with the extraction buffer. After 2 hours incubation at 37 °C, each slurry was transferred to a Nanosep MF 0.2 μm device and centrifuged at 14,000 x g. Each filtrate was transferred to a Nanosep 10K device and centrifuged at 5,000 x g for 15 minutes and diafiltered with TE. The desalted retentate was recovered in 20 μL TE and electrophoresed in a 6% TBE-urea gel alongside their respective RNA Markers. Equal band intensities approximate 100% recovery. Our recoveries ranged from 50 to 90%; no smearing due to RNase activity was detected.

Step-by-Step Procedure

1. Electrophorese the synthetic RNA transcripts in a 5% polyacrylamide/8 M urea PAGE gel.
2. Visualize RNA by staining the gel with ethidium bromide, or in the case of radioactive RNA, by brief exposure of the gel to X-ray film. (Do not overexpose the autoradiogram; a thinner band width allows a more accurate excision of the desired mRNA species.)
3. Place the excised band containing RNA in a standard 1.5 mL microcentrifuge tube.
4. Crush the excised band with microcentrifuge fitting pestle and add 500 μL gel extraction buffer (0.5 M Ammonium Acetate, 2 mM EDTA, 0.1% SDS).
5. Vortex and incubate at 37 °C from 2 hours to overnight depending on the size of the transcript. Small transcripts (< 200 nucleotides) will elute in approximately 2 hours; larger transcripts require longer incubation.
6. Transfer the slurry into a Nanosep MF device and spin at 14,000 x g for 10 minutes to remove the acrylamide and collect the mRNA in the filtrate.
7. RNA from the filtrate can be concentrated and desalted in a Nanosep device by centrifuging the filtrate at 5,000 x g in a device with the appropriate MWCO. For RNA molecules smaller than 50 bases, use a Nanosep 3K device, 50 to 500 bases use a Nanosep 10K device, 500 to 1000 bases use a Nanosep 30K device, and larger than 1000 bases use a Nanosep 100K device.
8. To remove salts (diafilter), add 500 μL of water or buffer to the upper sample receiver and centrifuge again. If necessary, repeat the dilution and concentration step.



Fast and Efficient Elution of Proteins from Polyacrylamide Gels

Description

Polyacrylamide Gel Electrophoresis (PAGE) is both an analytical and preparative tool widely used to separate proteins in an electric field according to their size, shape and charge. Recent advances in 2D-PAGE have revolutionized proteomics and its applications in biomedicine, biotechnology or pharmaceuticals. Complex protein samples are first separated by 2D-PAGE, stained, and then scanned, allowing for detailed measurements of expression levels and protein mapping. Further protein

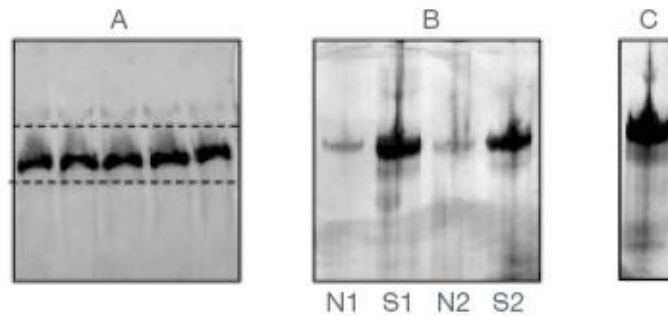
characterization often requires the extraction of specific proteins from the polyacrylamide gel matrix for further analyses such as mass spectrometry, HPLC, amino acid sequencing or composition analyses, and post transcriptional modification analyses (3,4).

Nanosep centrifugal devices provide a fast and inexpensive alternative to commonly used methods for the elution and concentration of proteins that have been separated on polyacrylamide gels. While Nanosep centrifugal devices allow for rapid and efficient elution of proteins from polyacrylamide gels, the amount of starting polyacrylamide gel material is the limiting factor for high protein recoveries. Reducing the amount of polyacrylamide not only increases the yields of eluted protein but also reduces centrifugation times significantly, a factor of consideration when eluting biologically active proteins (4).

For an in-depth scientific discussion on this protocol, see Pall Life Sciences technical report, "[Fast and Efficient Elution of Proteins from Polyacrylamide Gels Using Nanosep Centrifugal Units.](#)"

Figure 20

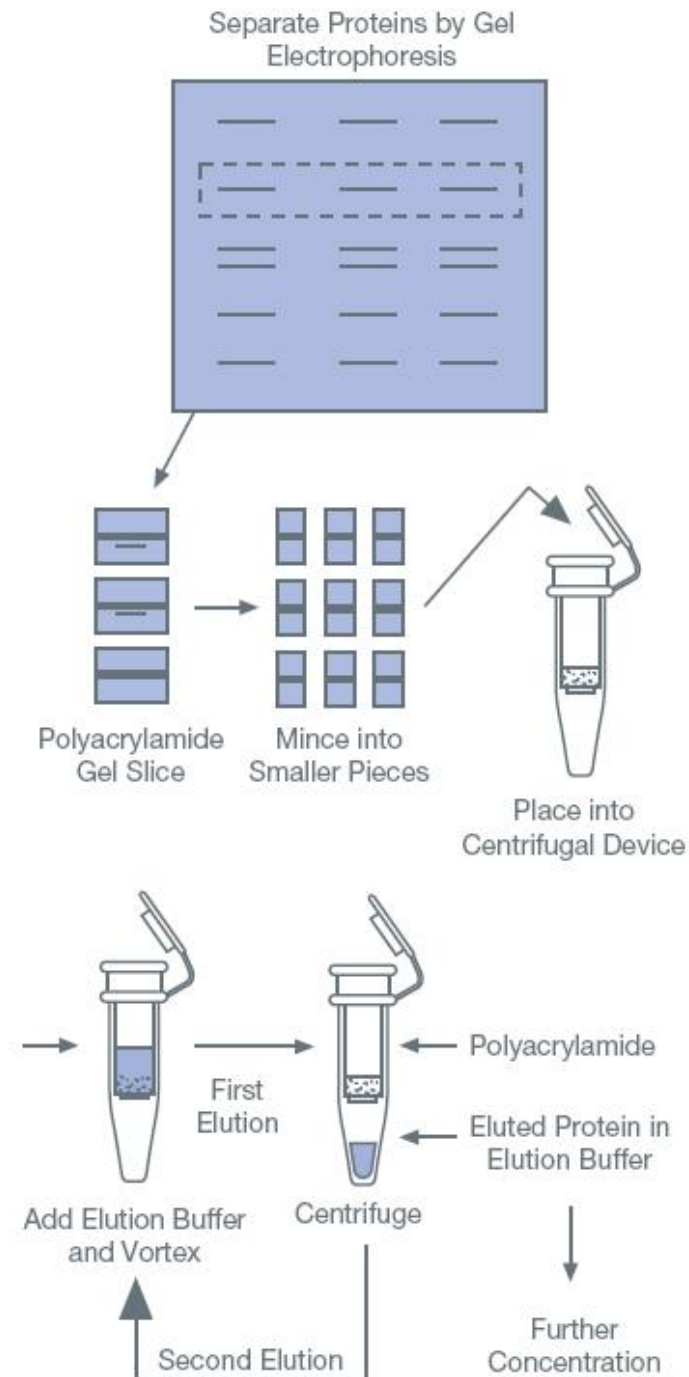
Elution of Bovine Serum Albumin (BSA) from Polyacrylamide in the Presence or Absence of SDS



BSA was subjected to SDS-PAGE and detected by Coomassie Blue R-250 staining (panel A). The protein in five lanes (30 μ g protein) was eluted using a 300K Nanosep Centrifugal Device in the presence or absence of SDS. Panel B shows the protein eluted in two consecutive runs (1 and 2), with (S) or without (N) SDS, after SDS-PAGE and detected by silver staining (not shown). A strip spanning the six lanes of the gel (8 μ g protein) was used in the ultrafiltration procedure in the presence of SDS. Panel C shows the protein eluted after SDS-PAGE and silver staining.

Step-by-Step Protocol

1. Identify the stained protein band or spot.
2. Excise stained plug or band with a clean razor blade and mince into smaller pieces to maximize the gel surface area during protein elution.
3. Place excised gel fragments into a Nanosep MF centrifugal device.
4. Add elution buffer (50 μ L or less) directly to the tube and mix with the gel slices by vortexing.
5. Centrifuge at 14,000 x g for 2 minutes; recover the filtrate containing the eluted protein.
6. Add an additional 20 μ L elution buffer, vortex and centrifuge again to maximize protein recovery.
7. Concentration of the protein can be done using a Nanosep device with the appropriate MWCO membrane.



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Appendices

Sanitizing Nanosep Devices

Nanosep centrifugal devices are sold non-sterile but can easily be sanitized prior to use with 70% ethanol by performing the following:

1. Fill device with 70% molecular biology grade ethanol. Close the cap and centrifuge at maximum g-force until all liquid passes through the membrane (10 to 30 minutes, depending on MWCO).
2. Discard filtrate. To remove residual ethanol, fill device with sterile water or the desired buffer and centrifuge again. Note: Use device within 20 minutes to prevent irreversible membrane damage caused by dehydration.

Nanosep Device Centrifugation Times

Filtration rates in centrifugal devices are affected by several operating parameters including membrane MWCO and material of construction, sample composition and viscosity, the centrifugal force used, and the operating temperature (Table 7).

Table 7

Approximate Spin Times in Nanosep Devices

Nanosep Device MWCO	Color Code	RCF	Spin Time at 25 °C
3K	gray	14,000 x g	20 minutes
10K	blue	14,000 x g	15 minutes
30K	red	14,000 x g	8 minutes
100K	clear	14,000 x g	5 minutes
300K	orange	14,000 x g	3 minutes

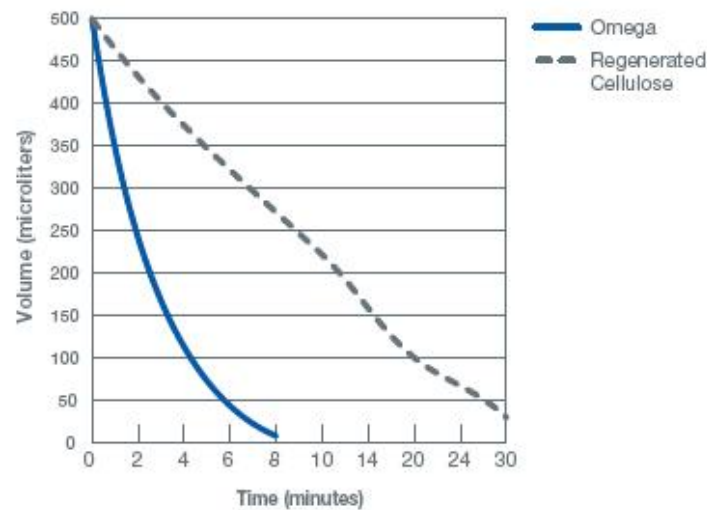
A 1.0 mg/mL 500 μ L sample containing five proteins of molecular weights ranging from 200 Kd to 6 Kd was concentrated down to 20 to 25 μ L. (B-amylase, 200 Kd; Alcohol dehydrogenase, 150 Kd; Bovine serum albumin, 66 Kd; Trypsin inhibitor, 20 Kd; Aprotinin, 6 Kd).

Effect of UF Membrane Material on Filtration Rates

Pall Omega membrane (modified polyethersulfone) has a very fast flow rate compared to UF membrane made of regenerated cellulose. Figure 18 compares the time required to concentrate 500 μ L of 0.1 mg/mL BSA sample in a Nanosep device containing the Omega 10K MWCO membrane with a device containing a 10K regenerated cellulose membrane. Both devices were spun at 5,000 x g.

Figure 21

Filtration Rates in UF Devices with Different Membranes

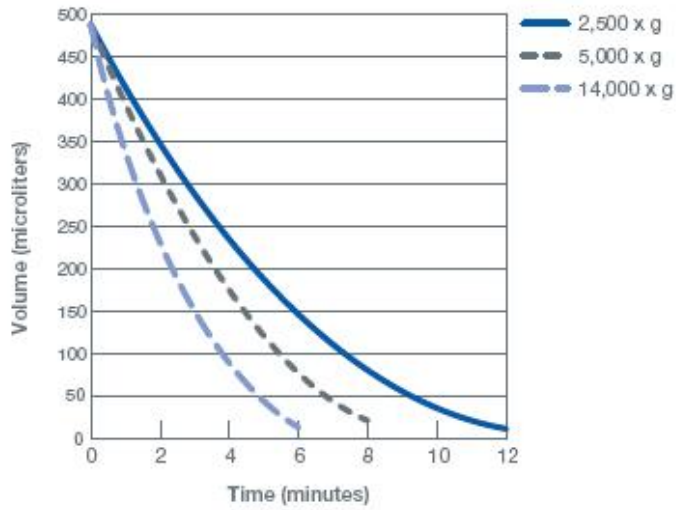


Effect of G-force on Filtration Rates

Centrifuge speed has a direct effect on the selectivity of membrane as well as the duration of the spin. A 500 μ L sample (0.1 mg/mL IgG) was concentrated in a Nanosep 100K device spun at different centrifugal forces (Figure 22). Higher g-forces result in faster filtration rates and a corresponding increase in molecule passage.

Figure 22

Effect of g-force on Filtration Rates

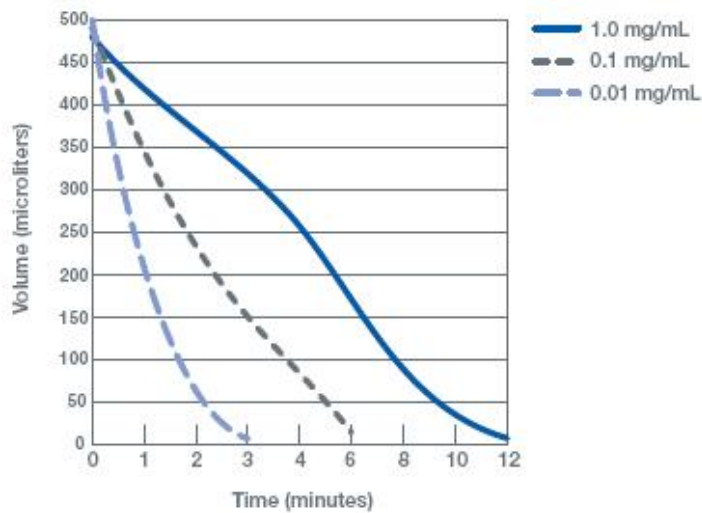


Effect of Sample Concentration on Filtration Rates

Sample concentration cannot only effect spin times but also may effect the performance of the nominally-rated MWCO. As protein concentration increases, there is a corresponding decrease in molecule passage (Figure 23).

Samples containing 500 μL IgG with initial concentrations of 0.01 mg/mL to 1 mg/mL were centrifuged at 14,000 x g in Nanosep 100K devices. Samples with higher starting concentrations require longer spin times to process.

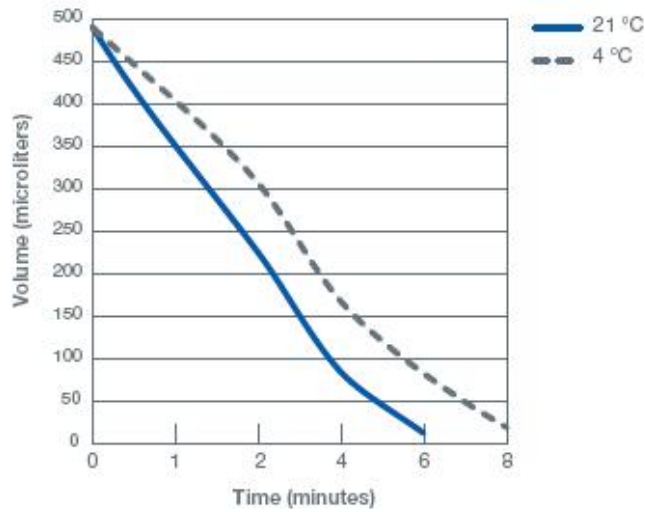
Figure 23
Effect of Sample Concentration on Filtration Rates



Effect of Temperature on Filtration Rates

As temperature decreases, there is a corresponding increase in centrifugation time (Figure 24). A 500 μL sample of 0.1 mg/mL IgG was centrifuged in a Nanosep 100K device at 21 °C or 4 °C.

Figure 24
Effect of Temperature on Filtration Rates



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References

1. Fang, Q., Seeley, K., and Short, T. 2000. Single-tube DNA Purification and Cloning Using Ultrafiltration Devices. Scientific and Technical Report, Pall Life Sciences.
2. Miller, J. S. 1988. Ultrafiltration for the Removal of Excess DNA Linkers Subsequent to Ligation. *BioTechniques*. 5:632-634.
3. Jenö, P. and M. Horst. 1996. Electroelution of Proteins from Polyacrylamide Gels, p. 207-214. In J. M. Walker (ed.), *The Protein Protocols Handbook*. Humana Press, Totowa, NJ.
4. Reguera, G. and Leschine, S. 2000. Fast and Efficient Elution of Proteins from Polyacrylamide Gels Using Nanosep Centrifugal Units. Scientific and Technical Report, Pall Life Sciences.

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Ordering Information

[Nanosep Centrifugal Devices with Omega™ Membrane](#)
[Nanosep MF Centrifugal Devices with GHP Membrane](#)
[Nanosep MF Centrifugal Devices with Bio-Inert® Membrane](#)

Scale Up

Many if not all procedures listed in this guide can scale up to larger-volume procedures. Pall Life Sciences offers centrifugal devices for processing volumes from < 100 µL to 60 mL. Order these precise, high performance products today for your critical applications. Devices are available in a range of sizes that accommodate the following sample volumes:

Sample Volume	Use
50 µL to 500 µL	Nanosep Device
500 µL to 3.5 mL	Microsep Device
3 mL to 15 mL	Macrosep Device
15 mL to 60 mL	Jumbosep Device

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