Guide to Gel Filtration or Size Exclusion Chromatography

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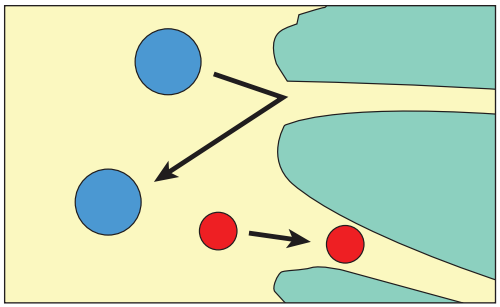
Gel Filtration

Gel Filtration also called size-exclusion chromatography can be used for protein DNA purification, buffer exchange, desalting, or for group separation in which the sample is separated in two major groups. Gel Filtration is an easy to use method for separation of molecules with different molecular sizes, using mild conditions. Gel Filtration uses the size of molecules in solution to determine separation. SpinColumns have short media packing so the samples are separated by size, the large molecules travel out of the column with the void volume the smaller molecules, salts, etc. remain in the column.

Gel Filtration columns are used not only to remove low molecular weight contaminants such as salt, but also for buffer exchange before and after different chromatography techniques and for the rapid removal of reagents to terminate a reaction. Examples of group separations include

- Removal of unincorporated nucleotides during DNA sequencing
- Removal of free low molecular weight labels
- Termination of reactions between macromolecules and low molecular weight reactants
- Removal of products, cofactors or inhibitors from enzymes
- Removal of unreacted radiolabels such as [α-32P] ATP from nucleic acid labeling reactions



Small molecules are retained\retarded large molecules pass through the column

In Gel Filtration, maximum resolution can be obtained with sample volumes of 0.5% to 2% of the total column volume; however, up to 5% may give acceptable separation. Even larger samples volumes can be appropriate if the resolution between target protein and the impurities to be removed is high. To increase capacity, the sample can be concentrated before Gel Filtration or larger columns can be used.

Sample components are eluted isocratically (single buffer, no gradient). Separation can be performed within a broad pH, ionic strength, and temperature range, and the medium accepts a variety of additives: co-factor, protein stabilizers, detergents, urea, and guanidine hydrochloride.

Remember it is the size in solution of the molecules which relates to the molecular weight, but can deviate.

SpinColumn Specifications

Description	Ultra-Micro	Micro	Macro	96-Well Micro	96-Well Macro
Bed Volume	37.96µl	66.42µl	191.45µl	66.42µl	191.45µl
Sample Volume	10-25µl	25-75µl	75-150µl	25-75µl	75-150µl
Sample Concentration	3-30µg	5-60µg	30-300µg	5-60µg	30-300µg

The choice of buffer will not affect resolution, but a low concentration of salt, between, 25 and 150 mM NaCl, should be used to reduce weak electrostatic interactions between proteins and the Gel Filtration media.

The selected buffer conditions should be one that does not cause inactivation or precipitation, but maintains the biomolecules stability and target proteins activity. Proteins are large molecules and cannot enter the pores of chromatography beads, but a protein that fits into a pore of the beads, will be retained/retarded. Salt or other low molecular weight substances that can enter the entire pore of the beads during Gel filtration Chromatography are retained the result separation of molecules by size. When a mixture of different sized molecules passes through a column, they will be separated by their size in solution.

For separation by Gel filtration a column is packed with a size exclusion media. The media are porous spheres which are inert. The media is first equilibrated with buffer which fills the pores and the space between the spheres. The buffer in the pore of the sphere of the packing material will be in equilibrium with the buffer between the sphere the mobile phase.

Samples are eluted with buffer washes of the same buffer. Gel filtration can be used to separate small molecules from larger ones and/or for buffer exchange. Gel filtration can also be used for buffer exchange, clean up of nucleotide labels, clean up of labeled proteins, separation of small molecules from large ones and dye removal. Harvard Apparatus offers six types of media, G10, G-25, G-50, G-100, P-2, P-6 and P-30 for use in size exclusion applications. Large molecules can be eluted during or soon after the void volume V0 seeing they pass through the column at the same rate as the buffer in a packed column, the void volume is the volume equivalent to approximately 30% of the total column volume.

Small molecules and salts will be retained/retarded by the porous spheres as they migrate through the column. The small molecules and salts move as a front and are not separated from each other.

Size Fractionation

Gel Filtration can be used for size fractionation of different sized molecules in a sample.

The separation of molecules in a sample will be by molecular weight distribution. When separating by size fractionation the sample result you will obtain will contain a few components, large or small molecules, if separating a complex mixture of many components gel filtration is not a good fit. And will result in poor resolution.

Size Fractionation is a good final step for purification.

Sample Volume

For group separation use volumes up to 30% of the total column volume. A sample volume of 0.5% to 4% of total volume is recommended. For most applications the sample volume should not exceed 2% to achieve maximum resolution. The ratio of sample volume to column influences:

- The higher the ratio the lower the resolution

Description	Ultra-Micro	Micro	Macro	96-Well Micro	96-Well Macro
Bed Volume	37.96µl	66.42µl	191.45µl	66.42µl	191.45µl
Sample Volume	10-25µl	25-75µl	75-150µl	25-75µl	75-150µl
Sample Concentration	3-30µg	5-60µg	30-300µg	5-60µg	30-300µg

Note: Sample dilution will occur due to diffusion which will occur as the sample passes through the column. To minimize sample dilution use a sample volume that gives the resolution required.

Samples and Buffer

Samples should be free of particulate matter, especially when working with bead size of 34µm or less.

For post extraction clean-up two approaches are available, centrifugation and filtration.

Filtration

The sample extract mixture is passed through a filter membrane to remove the solid sample form the solute. Fresh solvent washes the sample from the filter into the collection vessel. Two to three washes can be used to prevent sample dilution.

Centrifugation

The sample extract mixture is centrifuges and the extract is decanted and removed. The residual sample is washed 2 to 3 times

Sample Buffer Concentration

The pH, ionic strength and composition will not significantly affect resolution as long as they do not alter the size and stability of the protein or nucleic acid to be separated.

The sample buffer does not have to be the same buffer as the column. The sample is exchanged into the running buffer during separation. Prior to using all buffers should be filter through either a 0.45µm or a 0.22µm filter to eliminate debris.

Buffer Composition

The buffer composition will not directly influence the resolution unless it effects the shape or activity of the molecule. Extreme pH and ionic strength, denaturing agents and detergents can cause conformational changes, dissociation or association of protein complexes. To reduce dissociation avoid extreme pH changes and chaotropic agents and detergents, although detergents may help with sample recovery.

Sample Concentration and Viscosity

The viscosity of a sample can limit the sample concentration that can be used. The viscosity of the sample relative to the running buffer. High viscosity causes instability during the separation and an irregular flow pattern reducing resolution.

Buffer Selection

Select a buffer that supports protein stability and activity. The buffer should maintain the buffering capacity and constant pH, 25mM-150mM NaCl will avoid nonspecific, ionic interactions.

If using guanidine hydrochloride or urea to stabilize the protein, during extraction it should be included in the buffer.

Detergents are useful as stabilizing agents for proteins with low aqueous solubility and will not effect separation. If using detergents to stabilize a sample, they should be present in both the sample buffer and running buffer.

If a protein has been lyophilized use either ammonium acetate or ammonium bicarbonate.

Separation

Buffer: 0.05 M sodium phosphate, 0.15 M NaCl pH 7, or select sample buffer which the protein is soluble and stabile.

Use 0.15 M NaCl, or a buffer with equivalent ionic strength, to avoid pH-dependent ionic interactions with the matrix. At very low ionic strength, the presence of a small number of negatively charged groups on the medium may cause retardation of basic proteins.

1. The sample should be fully dissolved. Centrifuge or filter to remove particulate material
2. The temperature of the column and buffer should be the same to avoid introducing air into the column.
3. If working with a new sample try 0.55mM Sodium phosphate, 0.5mM NaCl, pH 7 or select the elution buffer.
4. If using high concentrations of detergents lower the centrifuge speed and increase the time. (viscosity of the buffer might change)

Harvard Apparatus Offers Six Types Of Media In Five Different Sizes

Media Selection			
Type	**Dia µm**	**Cut off Da**	**Application**
G-10	40-120	≤700	Desalting Peptides
G-25	40-120	≤1,500	Desalting Proteins & Nucleic Acids
G-50	50-150	100-5,000	Removal of Free labels from labeled macromolecules
G-100	40-150	1,000-10,000	Molecular weight determination
P-2	45-90	100-1,800	Rapid carbohydrate,& small peptide separations & desalting
P-6	45-90	1,000-6,000	Purification of proteins & polypeptides
P-30	45-90	2,500-40,000	Purification of proteins

SpinColumn Specifications					
Description	**Ultra-Micro**	**Micro**	**Macro**	**96-Well Micro**	**96-Well Macro**
Bed Volume	37.96µl	66.42µl	191.45µl	66.42µl	191.45µl
Sample Volume	10-25µl	25-75µl	75-150µl	25-75µl	75-150µl
Sample Concentration	3-30µg	5-60µg	30-300µg	5-60µg	30-300µg
Elution Volume	28.5µl	50µl	143µl	50µl	143µl

Gel Filtration SpinColumns G-10, G25, G50 and G-100

The Gel Filtration SpinColumns G-10, G25, G50 and G-100 are packed with Sephadex.

Sephadex is a highly cross linked porous agarose particles with is covalently bonded. The media has high physical and chemical stability, due to the highly cross-linked agarose matrix. The excellent properties are determined by the dextran chains. The stability of Superdex makes it suitable for use in SpinColumns where centrifugation at moderate speed spin protocols are required. Under normal chromatography conditions nonspecific interactions between proteins and Superdex are negligible when using buffers with ionic strengths in the range 0.15 M to 1.5 M.

Sephadex G Gel Filtration columns are stable in all commonly used aqueous buffers, pH 3–12, and additives such as detergents (1% SDS), denaturing agents (8 M urea or 6 M guanidine hydrochloride).

Gel filtration based on Sephadex enables group separation of biomolecules that are above the exclusion limit of the medium, from contaminants such as salts, dyes, and radioactive labels.

Sephadex is prepared by cross-linking dextran with epichlorohydrin. The different types of Sephadex vary in their degree of cross-linking and hence in their degree of swelling and selectivity for specific molecular sizes.

G-10 is well suited for the separation of biomolecules such as peptides (MW>700) from smaller molecules (MW <100).

G-50 is suitable for the separation of molecules MW >30 000 from molecules MW <1 500 such as labeled protein or DNA from unconjugated dyes. The medium is often used to remove small nucleotides from longer chain nucleic acids.

G-25 is recommended for the majority of group separations involving globular proteins. These media are excellent for removing salt and other small contaminants away from molecules that are greater than MW 5000. Using different particle sizes enables columns to be packed according to application requirements, see Table 5.1. The particle size determines the flow rates and the maximum sample volumes that can be applied. For example, smaller particles give higher column efficiency (narrow, symmetrical peaks), but may need to be run more slowly as they create higher operating pressures.

G-100 is recommended for molecular weight determination

Sephadex G-25 Application

Superfine For highest column efficiency (highest resolution), but operating pressures increase Small-scale separations 10µl to 150µl.

G-25 SpinColumns to remove salts and other low molecular weight compounds from proteins with MW > 5000 and Sephadex G-10 products for proteins with MW > 700.

Desalting (SEC) provides several advantages over dialysis for desalting.

Dialysis requires a longer period of time and a large volume of buffer. Additionally protein activity and/or stability can be compromised during dialysis if improperly handled.

During SEC sample volumes of up to 30% of the total volume of the column can be processed.

The speed and capacity of the separation allows sample volumes to be processed efficiently.

Sample capacity should not exceeded the recommended concentration and will not separation if with in the recommended range.

When SEC is the first chromatography step, the sample should first be clarified; centrifugation and/or filtration is recommended as a quick method for clarification.

Applications

Desalting columns are used not only to remove low molecular weight contaminants such as salt, but also for buffer exchange before and after different chromatography techniques and for the rapid removal of reagents to terminate a reaction. Examples of group separations include:

- Removal of phenol red from culture fluids prior to anion exchange chromatography or nucleic acid preparations
- Removal of unincorporated nucleotides during DNA sequencing
- Removal of free low molecular weight labels
- Termination of reactions between macromolecules and low molecular weight reactants
- Removal of products, cofactors or inhibitors from enzymes
- Removal of unreacted radiolabels such as [_-32P] ATP from nucleic acid labeling reactions

Note: Use 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate if volatile buffers are required, as with lypholized products.

P-2, P-6 and P-30 SpinColumns

P-2, P-6 and P-30 SpinColumns are porous polyacrylamide beads prepared by copolymerization of acrylamide and N,N'-methylene-bis-acrylamide. The SpinColumns are extremely hydrophilic and essentially free of charge, and provide efficient gel filtration of sensitive compounds. The composition and lack of soluble impurities eliminate sample contamination. The consistency of bead diameter gives high resolution separation by molecular weight.

P-2, P-6 and P-30 SpinColumns is compatible with dilute organic acids, 8 M urea, 6 M guanidine-HCl, chaotropic agents, reducing agents such as dithiothreitol and mercaptoethanol, and detergents such as SDS, CHAPS, and Triton® X-100.

P-2, P-6 and P-30 SpinColumns can use distilled water but, buffers of > 50 mM ionic strength are recommended for most protein separations.

Miscible organic solvents may be added to the eluants used with P-2, P-6 and P-30 SpinColumns.

Alcohol up to 20% will not substantially alter the exclusion properties of the gel, and will in some cases enhance separation of complex mixtures of slightly water soluble small molecules such as nucleotides, peptides, and tannins.

Harvard Apparatus Gel Filtration Products

Gel Filtration SpinColumns					
	Quantity of 24			Quantity of 1	
Description	Ultra-Micro	Micro	Macro	96-Well Micro	96-Well Macro
G-10	74-4420	74-4421	74-3821	74-5611	74-5651
G-25	74-4421	74-4504	74-3904	74-5612	74-5652
G-50	74-7222	74-4506	74-3906	74-5613	74-5653
G-100	74-7223	74-4507	74-3907	74-5614	74-5654
P-2	74-7224	74-4808	74-4308	74-5615	74-5655
P-6	74-7225	74-4809	74-4309	74-5616	74-5656
P-30	74-7199	74-4508	74-3908	74-5647	74-5665

	Quantity of 96		
Description	Ultra-Micro	Micro	Macro
G-10	74-7200	74-4504	74-3900
G-25	74-7201	74-4505	74-3901
G-50	74-7202	74-4506	74-3902
G-100	74-7203	74-4507	74-3903
P-2	74-7204	74-4808	74-4302
P-6	74-7205	74-4809	74-4303
P-30	74-7198	74-4499	74-3899

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