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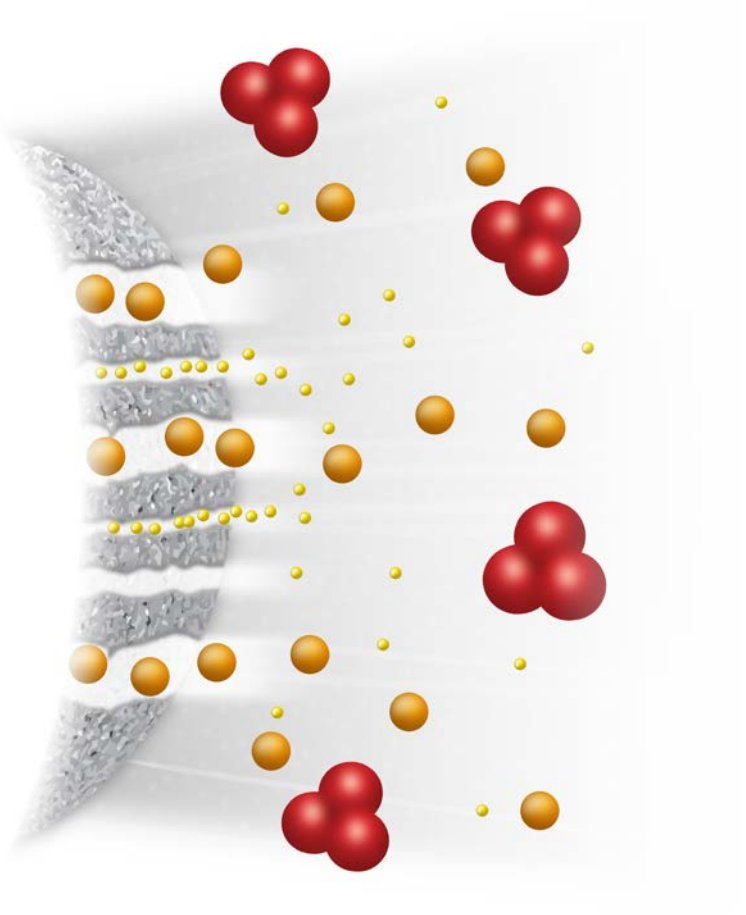
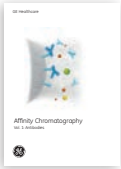


Size Exclusion Chromatography

Principles and Methods

Handbooks from GE Healthcare Life Sciences

For more information refer to gelifesciences.com/handbooks



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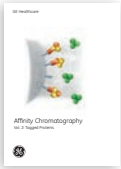

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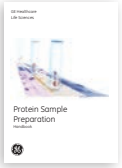

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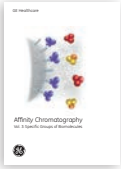

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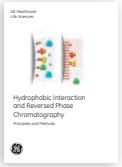

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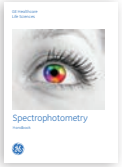

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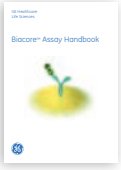

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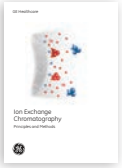

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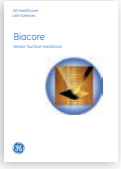

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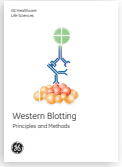

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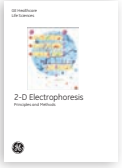

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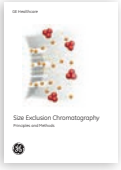

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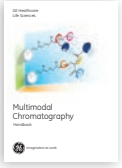

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Size exclusion chromatography

Principles and Methods

Contents

Symbols, acronyms and terminology used in this handbook

Symbols

This symbol indicates general advice on how to improve procedures or recommends measures to take in specific situations

This symbol indicates where special care should be taken

Highlights chemicals, buffers, and equipment

Outline of experimental protocol.

Common acronyms and abbreviations

^{3}H	tritium
^{32}P	phosphorous 32
A_{280}	UV absorbance at specified wavelength (in this example, 280 nm)
AC	affinity chromatography
AIEX	anion exchange chromatography
A_s	peak symmetry, expressed as asymmetry factor
ATP	adenosine triphosphate
AU	absorbance units
BSA	bovine serum albumin
CF	chromatofocusing
CIEX	cation exchange chromatography
CIP	cleaning-in-place
CIPP	capture, intermediate purification, polishing
CV	column volume(s)
DDM	n-dodecyl-β-maltoside
DM	n-decyl-β-maltoside and
DNA	deoxyribonucleic acid
DoE	design of experiments
DTE	dithioerythritol
DTT	dithiothreitol
dTTP	2´-deoxythymidine triphosphate
EDTA	Ethylenediaminetetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
GF	gel filtration (also referred to as SEC, size exclusion chromatography)
HCl	hydrochloric acid
HIC	hydrophobic interaction chromatography
HIV	human immnunodeficiency virus
HMW	high molecular weight
HPLC	high-performance liquid chromatography
i.d.	inner diameter
ID_{50}	inhibitory dose causing 50% inhibition
IEF	isoelectric focusing
IEX	ion exchange chromatography
IGF-1	insulin-like growth factor 1

IgG	immunoglobulin G
IgM	immunoglobulin M
IMAC	immobilized metal affinity chromatography
IU	inhibitor activity units
K_{av}	partition coefficient
K_d	distribution coefficient
K_o	specific permeability
LMW	low molecular weight
mAU	milli absorbance units
MPa	megaPascal
M_p	peak molecular weight
M_r	relative molecular mass
MS	mass spectrometry
NaCl	sodium chloride
NHS	N-hydroxysuccinimide
Ni	nickel
N/m, Nm^{-1}	column efficiency expressed as number of theoretical plates per meter
NM	n-nonyl-β-maltoside
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
pI	isolectric point
psi	pounds per square inch
PVDF	polyvinylidene fluoride
RI	refractive index
RNA	ribonucleic acid
RPC	reversed phase chromatography
R_s	resolution, the degree of separation between peaks
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography (also referred to as GF, gel filtration)
ssDNA, ssRNA	single-stranded DNA, RNA
SR	solvent resistant
TCM	traditional Chinese medicine
UDM	n-undecyl-β-maltoside
UV	ultraviolet
V_e	peak elution (retention) volume
V_0	void volume
V_i	volume of buffer inside the matrix
V_s	volume of stationary phase
V_{sep}	separation volume
V_t	total volume of the packed bed
v/v	volume/volume
W1/2	peak width at half peak height
w/v	weight/volume

Chromatography terminology

Adapter	Often used for the movable end pieces of columns; contains filter, flow distributor, and possibility to connect tubing.
Adsorption	Binding. The process of interaction between the solute (for example, a protein) and the stationary phase.
Affinity chromatography	A group of methods based on various types of specific affinities between target molecule(s), for example, a protein and a specific ligand coupled to a chromatography resin.
Asymmetry (asymmetry factor)	Factor describing the shape of a chromatographic peak.
Back pressure	The pressure drop across a column and/or a chromatography system.
Band broadening	The widening of a zone of solute (for example, a protein) when passing through a column or a chromatography system. Gives rise to dilution of the solute and reduces resolution. Also often called peak broadening or zone broadening.
Binding	Adsorption. The process of interaction between a solute (for example, a protein) and the stationary phase.
Binding buffer	Buffer/solution/eluent used for equilibration of the column before sample loading.
Binding capacity	The maximum amount of material that can be bound/mL of chromatography resin. See also Dynamic binding capacity.
Capacity factor	The degree of retention of a solute (for example, a protein) relative to an unretained peak.
Chromatofocusing	Method that separates proteins on the basis of pI.
Chromatogram	A graphical presentation of detector response(s) indicating the concentration of the solutes coming out of the column during the purification (volume or time).
Chromatography	From Greek chroma, color, and graphein, to write.
Chromatography resin	The stationary phase. The chromatography resin is composed of a porous matrix that is usually functionalized by coupling of ligands to it. The matrix is in the form of particles or, rarely, a single polymer block (monolith).
CIP (cleaning-in-place)	Common term for cleaning chromatography columns and/or systems with the purpose of removing unwanted/ nonspecifically bound material.
Column	Usually column hardware packed with chromatography resin.
Column equilibration	Passage of buffer/solution through the chromatography column to establish conditions suitable for binding of selected sample components. For example, to establish correct pH and ionic strength, and ensure that proper counter ions or counter ligands are present.
Column hardware	The column tube and adapters. All pieces of the column except the chromatography resin/the packed bed.
Column hardware pressure	The pressure inside the column. Column hardware pressure that is too high can break the column.
Column packing	Controlled filling of the column hardware with chromatography resin to obtain a packed bed.

Column volume	The geometrical volume of the column interior/the chromatography bed.
Counter ion	Ion of opposite charge that interacts with an ion exchange chromatography resin after the column equilibration. The counter ion is displaced by a protein that binds to the ion exchanger. If a high concentration of the counter ion is applied, it will compete with the bound protein and elute it from the chromatography column.
Counter ligand	Substances that interact with ligands of a chromatography resin and can be displaced by a solute (for example, protein) binding to the ligand.
Dead volume	The volume outside the packed chromatography bed. Can be column dead volume or chromatography system dead volume. The dead volume contributes to band broadening.
Degassing	Removal of dissolved air from buffers/solutions.
Desorption	Elution. Release or removal of bound substances from the chromatography resin.
Design of experiments (DoE)	DoE allows use of a minimum number of experiments, in which several experimental parameters can be varied simultaneously. Based on the obtained data, a mathematical model of the studied process (e.g., a protein purification protocol or a chromatography step) is created. The model can be used to understand the influence of the experimental parameters on the outcome and to find an optimum for the process.
Dynamic binding capacity	The binding capacity determined by applying the target using flow through a column, as opposed to equilibrium binding capacity determined by batch experiment.
Efficiency	Measured as number of theoretical plates. High efficiency means that sharp peaks will be obtained.
Effluent	The mobile phase leaving the column (= eluate).
Eluate	The mobile phase leaving the column (= effluent).
Eluent	The buffer/solution used during chromatography (= mobile phase).
Elution buffer	Buffer/solution used for elution (desorption) of bound solutes (for example, proteins) from a column.
Elution volume	The volume of buffer/solution (eluent) required to elute the solute for example, a protein (= retention volume).
Elution time	The time required for elution of a solute (protein) (= retention time).
Flow rate	Volumetric flow (mL/min) or linear flow rate (cm/h). Measurement of flow through a column and/or chromatography system.
Flowthrough	Material passing the column during sample loading (without being bound).
Frit	Type of deep filter often used at top and bottom of columns.
Gel filtration (GF)	Size-exclusion chromatography. Separates solutes (for example, proteins) according to size.
Gradient elution	Continuous increased or decreased concentration of a substance (in the eluent) that causes elution of bound solutes (for example, proteins).

Hydrophobic interaction chromatography (HIC)	Method based on the hydrophobic interaction between solutes (for example, proteins) and the chromatography resin in the presence of high salt concentration.
Hydroxyapatite chromatography	Mixed-mode ion exchange chromatography method.
Immobilized metal ion affinity chromatography (IMAC)	Method based on the affinity of proteins with His, Cys, or Trp amino residues on their surface and metal ions on the chromatography resin.
Ion exchange chromatography (IEX)	Method based on electrostatic interactions between solutes (for example, proteins) and chromatography medium.
Isocratic elution	Elution of the solutes without changing the composition of the buffer/solution (eluent).
Ligand	The specific molecular group that is coupled to the matrix to give some decided function to the chromatography resin.
Ligand density	Related to ligand concentration. The distribution of ligands on the surfaces (also surfaces inside pores) of the chromatography matrix.
Linear velocity	The flow rate normalized by the column cross section (cm/h).
Mass transfer	Movement of a solute (for example, a protein) in and out of the stationary phase. Important factor for column efficiency.
Matrix	The matrix is the nonfunctional base for the chromatography resin. The matrix has a porous structure that provides a large surface that can be modified with ligands that introduce possibilities for protein binding.
Mobile phase	The fluid (buffer/solution) carrying the solutes during chromatography (= eluent).
Peak broadening	Same as band broadening.
Peak capacity	The number of peaks that can be separated using a chromatography column.
Peak fronting	Broadening at the beginning of a peak.
Peak tailing	Broadening at the end of a peak due to additional delay of a fraction of the solute. Results in increased asymmetry factor.
Pore	Cavity in a chromatography matrix.
Pore volume	The total volume of the pores in a chromatography resin.
Pressure over the packed bed	The pressure drop across the packed bed upon passage of solution through the column. Caused by flow resistance in the packed bed.
Recovery	The relative amount of target protein that is retrieved after purification compared with amount loaded on the column.
Resolution	Measurement of the ability of a packed column to separate two solutes (peaks).
Retention volume	Same as elution volume.
Retention time	Same as elution time.
Reversed phase chromatography (RPC)	Method based on hydrophobic interactions between solutes (sample components) and ligands coupled to the chromatography resin. Organic modifiers (for example, acetonitrile) in the eluent are used for elution.
Sample	The material loaded on the chromatography column/resin, or to be analyzed.

Sample application	Applying/loading sample on the column.
Sample loading	Loading/applying sample on the column.
Sample volume	Usually the volume of the sample loaded on the chromatography column/resin.
Selectivity	Measure of the relative retention of two solutes in a column. Related to the distance between two peaks.
Solute	The dissolved substance (for example, a protein) in, for example, the mobile phase.
Stationary phase	Often called resin, chromatography particles, chromatography material, chromatography medium or media.
Step gradient elution	Stepwise increase in concentration of the substance that affects elution of bound solutes.
Void volume	The elution volume of solutes that do not enter the pores or interact with the chromatography resin, thus passing between the particles in the packed bed.
Wash	Wash step. Removal of unbound or weakly bound material from a column after the sample loading.
Wash buffer	Buffer/solution used for washing the column after sample loading.
Wash volume	Volume of buffer/solution used for the wash step.
Yield	Amount of target protein (or other solute) obtained after a purification step, or after the entire purification (multiple steps).
Zone broadening	Same as peak broadening.

Chapter 1
Introduction

This handbook describes the use of size exclusion chromatography (SEC) for the purification and separation of biomolecules, with focus on practical information for optimized results.

Since the introduction of the first SEC resin, Sephadex™ in 1959, SEC has played a key role in the purification of proteins and enzymes, polysaccharides, nucleic acids, and other biological macromolecules. This handbook focuses on the most up-to-date SEC resins and prepacked columns from GE Healthcare Life Sciences. The resins available, selection criteria, and examples of the most common applications are included, as well as the theoretical principles behind the technique.

Biomolecules are purified using chromatography techniques that separate them according to differences in their specific properties, as shown in Figure 1.1.

Property	Technique
Size	Size exclusion chromatography (SEC), also called gel filtration (GF)
Charge	Ion exchange chromatography (IEX)
Biorecognition (ligand specificity)	Affinity chromatography (AC)
Hydrophobicity	Hydrophobic interaction chromatography (HIC) Reversed phase chromatography (RPC)
Various (e.g., charge, hydrophobicity, and hydrogen bonding)	Multimodal chromatography (MMC)

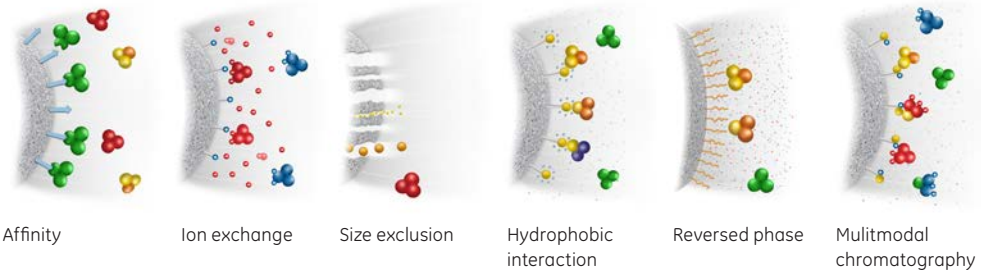


Fig 1.1. Schematic drawing of separation principles in chromatography purification. From left to right: AC, IEX, SEC, HIC, RPC, and MMC.

Versatility of size exclusion chromatography

SEC separates molecules by differences in size as they pass through a SEC resin packed in a column. Unlike techniques such as IEX or AC, molecules do not bind to the chromatography resin, which means that buffer composition does not directly affect resolution (the degree of separation between peaks). Consequently, a significant advantage of SEC is that conditions can be varied to suit the type of sample or the requirements for further purification, analysis, or storage without altering the separation.

SEC is a highly versatile separation technique. It is well suited for biomolecules sensitive to changes in pH, concentration of metal ions or cofactors, or harsh environmental conditions. Separations can be performed in the presence of essential ions, cofactors, detergents, urea, or guanidine hydrochloride at high or low ionic strength. Furthermore, SEC can be performed within a wide temperature range covering the requirements of most experiments. SEC is most commonly performed in the range +4°C to +30°C, but many SEC columns enable separations at even higher temperatures. Purified biomolecules may be collected in any chosen buffer.

Purification by size exclusion chromatography

To perform a separation, the resin is packed into a column to form a packed bed. SEC resins consist of a porous matrix of chemically and physically stable spherical particles with properties that minimize adsorption of biomolecules.

The packed bed is equilibrated with buffer, which fills the pores of the matrix and the space between the particles. The liquid inside the pores, or stationary phase, is in equilibrium with the liquid (buffer or mobile phase) outside the particles. Sample components are eluted isocratically, that is, the buffer composition remains constant throughout the separation. There is no need to use different buffers during the separation. However, a wash step using the running buffer is often included at the end of a separation to remove molecules that might have been retained on the column and to prepare the column for a new run.

SEC can be performed directly after IEX, HIC, or AC since the buffer composition will not generally affect the final separation.

Figure 1.2 illustrates the separation process of SEC.

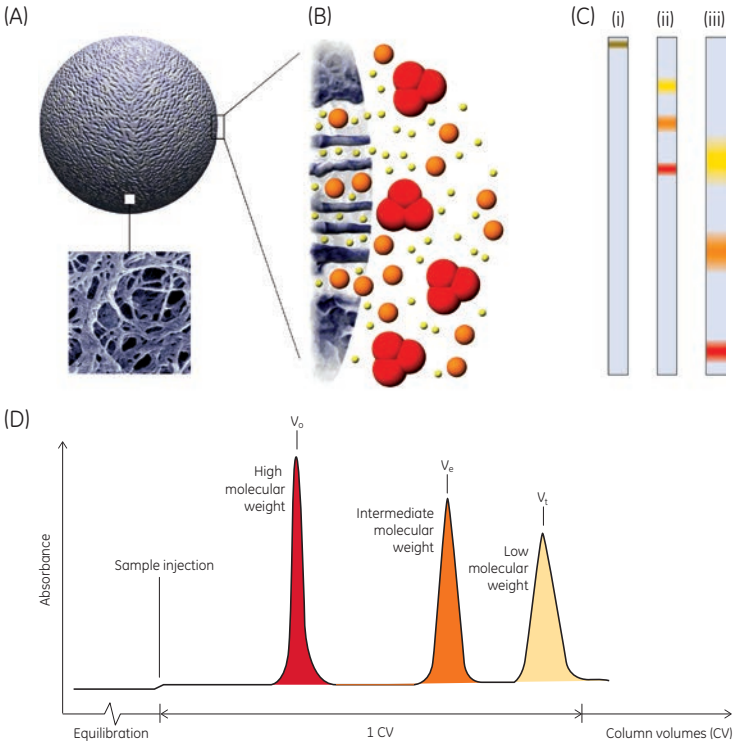


Fig 1.2. Process of SEC. (A) Schematic picture of a particle with an electron microscopic enlargement. (B) Schematic drawing of sample molecules diffusing into the pores of the particle. (C) Graphical description of separation: (i) sample is applied to the column; (ii) the smallest molecule (yellow) is more delayed than the largest molecule (red); (iii) the largest molecule is eluted first from the column. Band broadening causes significant dilution of the protein zones during chromatography. (D) Schematic chromatogram.

SEC can be categorized into three main application approaches:

1. Preparative size exclusion chromatography: a high-resolution size-based separation of biomolecules with fractionation. Preparative SEC is performed to isolate one or more components of a sample. Separated components can directly be transferred to a suitable buffer for assay or storage. Small sample volumes of 0.5% to 4% of the total column volume are applied at low flow rates using long columns, often 60 cm or longer. The particle size is commonly > 12 µm, providing low column back pressure. Resolution of preparative SEC varies from high to more moderate depending on the particle size. For optimal results, samples with few components such as those partly purified by other chromatography techniques are used. This makes preparative SEC well-suited for the final polishing step in a purification scheme; see handbook *Strategies for Protein Purification*. Figure 1.3 shows the chromatogram of a typical high-resolution separation. As the separation takes place in only 1 CV, it is essential to have a well packed column for good results in SEC. For convenience and optimal performance, prepacked SEC columns are recommended.

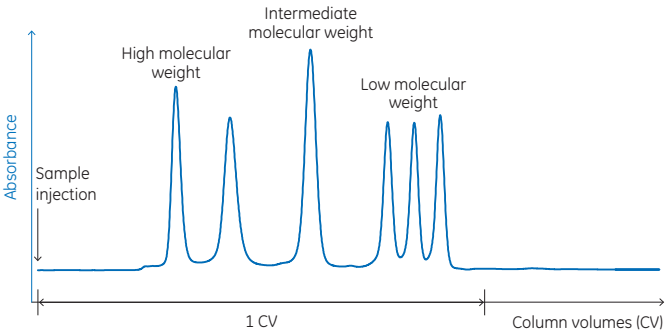


Fig 1.3. Typical high-resolution SEC separation.

2. Analytical size exclusion chromatography: a high-resolution size-based separation without fractionation (Fig 1.3). Often, the analysis is connected to highly selective detectors such as mass spectrometers and photodiode arrays (DAD), as well as multiangle light scattering (MALS) or fluorescence detectors.

Analytical SEC is performed to check the quality of the sample or to study the properties of a biomolecule. Small sample volumes, often 0.3% to 0.5% of the column volume are applied at low flow rates using long columns, typically 30 cm. The particle size is commonly 4 to 12 µm, providing high resolution. Prepacked SEC columns are an excellent choice for ensuring reliable results and for convenience.

Analytical SEC can also be used for rapid purity checks and screening. For this, shorter columns of typically 15 cm, which provide adequate resolution are used giving short cycle times together with small sample volume and low buffer consumption.

Note that shorter columns typically used for purity check/screening give lower resolution than longer columns.

3. Desalting and buffer exchange: a group separation where small molecules such as salt or free labels are separated from a group of larger molecules such as proteins. Samples can be prepared for storage or for other chromatography techniques and assays. Large sample volumes—up to 30% of the total column volume—can be applied at high flow rates using broad, short columns. The particle size is relatively large, providing low column back pressure and high flow rates.

Figure 1.4 shows the chromatogram of a typical group separation. Large molecules have no or limited access to the resin pores and are thus eluted in or just after the void volume, V_0. Small molecules such as salts have full access to the pores and move down the column more slowly. These molecules usually elute just before 1 CV of buffer has passed through the column.

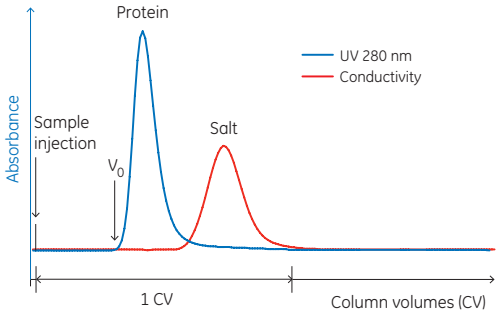


Fig 1.4. Typical group separation.

Information on available resins and prepacked columns as well as protocols are provided in Chapters 4 and 5.

Size exclusion chromatography in theory

Defining the process

Results from SEC are usually expressed as a chromatogram (elution profile) that shows the variation in concentration of sample components as they elute from the column in order of their molecular size.

Figure 1.5 shows a theoretical chromatogram of a high-resolution separation. Molecules that are larger than the largest pores in the matrix cannot enter the matrix. These molecules are eluted together in the void volume, V_0 as they pass directly through the column. For a well-packed column, the void volume is equivalent to approximately 30% of the total column volume. Molecules with partial access to the pores of the matrix are separated and elute from the column in order of decreasing size. Small molecules such as salts have full access to the pores and are not separated when they move down the column. These molecules usually elute at the total liquid volume (V_t), slightly before 1 CV (or V_c) of buffer has passed through the column.

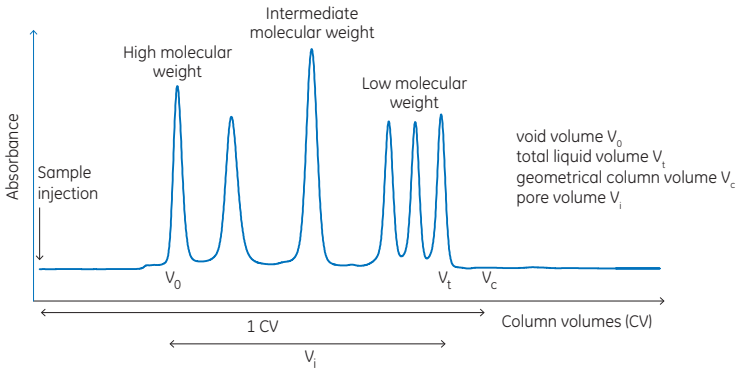


Fig 1.5. Theoretical chromatogram of a high-resolution separation.

From the chromatogram, the elution volume (V_e), often referred to as the retention volume (V_R), of a molecule is obtained. As shown in Figure 1.6, there are three different ways of measuring V_e depending on the volume of sample applied to the column.

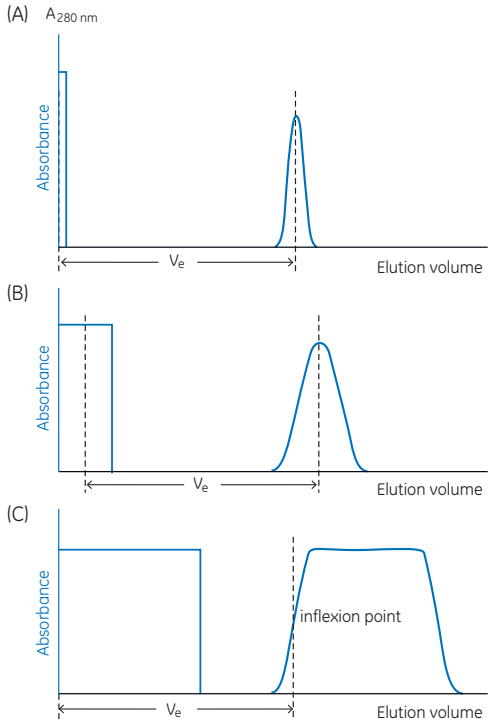


Fig 1.6. Measurement of elution volume, V_e. (A) Sample size negligible compared with volume of packed bed. (B) Sample size not negligible compared with volume of packed bed. (C) Sample giving elution curve with plateau.

V_e varies with the total volume of the packed bed (V_c) and the way in which the column has been packed. Consequently, the elution of a sample is best characterized by a distribution (or partition) coefficient (K_d). K_d is independent of column dimensions and thus allows comparison and prediction between columns with different sizes if the same resin is used. K_d describes how a molecule is distributed between the two phases:

$$K_d = \frac{\textit{Concentration of solute in stationary phase}}{\textit{Concentration of solute in mobile phase}}$$

The mobile phase of a SEC resin corresponds to the solution between the particles, that is, the void volume, V_0 (Fig 1.7).

The stationary phase corresponds to the pore volume, that is, the volume of solution inside the particles, V_i. Molecules with access to the entire pore volume elute at V_t. These molecules distribute freely between the mobile and stationary phases.

In other words, K_d represents the fraction of the resin's stationary phase that is available for diffusion of a given molecule:

$$K_d = \frac{V_e - V_0}{V_i} = \frac{V_e - V_0}{V_c - V_{matrix} - V_0} = \frac{V_e - V_0}{V_t - V_0}$$

Large molecules passing directly through the resin without entering the particles will have a K_d equal to 0 whereas small molecules with full access to the pores of the particle will have a K_d equal to 1.

In practice, the V_i is often difficult to determine. Instead, the average distribution constant, K_{av}, is often used. Here V_t is substituted by the more easily determined geometrical bed volume, V_c:

$$K_{av} = \frac{V_e - V_0}{V_c - V_0}$$

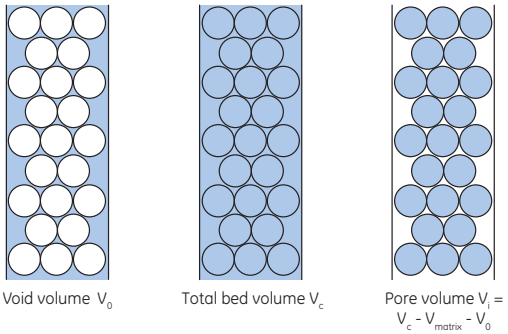
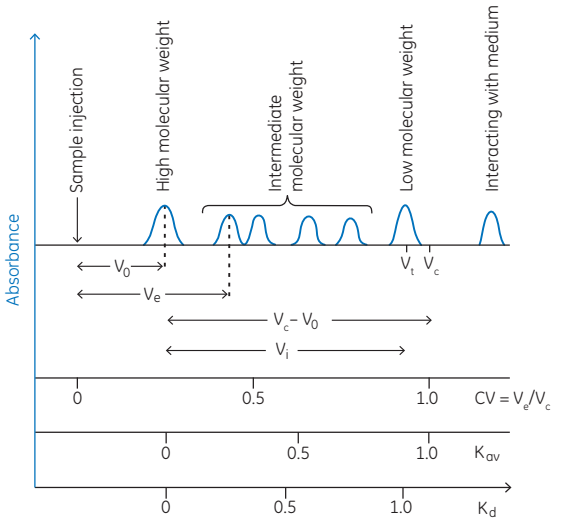


Fig 1.7. Diagrammatic representation of V_0, V_c and V_i. Note that the simplified expression $V_c - V_0$ will include the volume of the solid material which forms the matrix, V_{matrix}. From Fischer, L. *Laboratory Techniques in Biochemistry and Molecular Biology*. Vol. 1 part II. An Introduction to Gel Chromatography. North Holland Publishing Company, Amsterdam. Reproduced by kind permission of the author and publisher.

Since the simplified expression for the stationary phase ($V_c - V_0$) includes the volume of the solid matrix that is inaccessible to all solute molecules, K_{av} is not a true distribution coefficient. However, for a given resin there is a constant ratio between K_{av} and K_d, which is independent of the nature of the molecule or its concentration. K_{av} is easily determined and, like K_d, defines sample behavior independently of the column dimensions and packing. K_{av} is more affected by variations in system parameters such as flow and dead volumes than K_d. An accurate determination of the bed height is crucial for the determination of V_c. Other methods of normalizing data give values that vary depending upon how well the column is packed. The approximate relationships between some of these terms are shown in Figure 1.8.



$$K_{av} = \frac{V_e - V_0}{V_c - V_0}$$

$$K_d = \frac{V_e - V_0}{V_i} = \frac{V_e - V_0}{V_c - V_{matrix} - V_0} = \frac{V_e - V_0}{V_t - V_0}$$

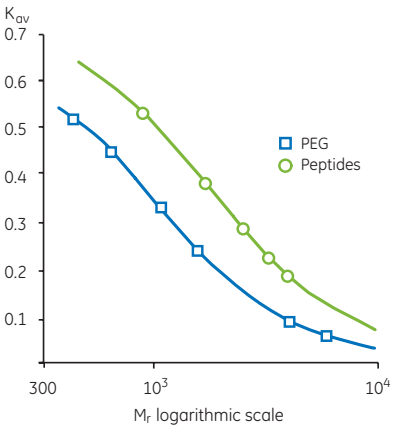
Fig 1.8. Relationship between several expressions used for normalizing elution behavior.

Selectivity curves and resin selection

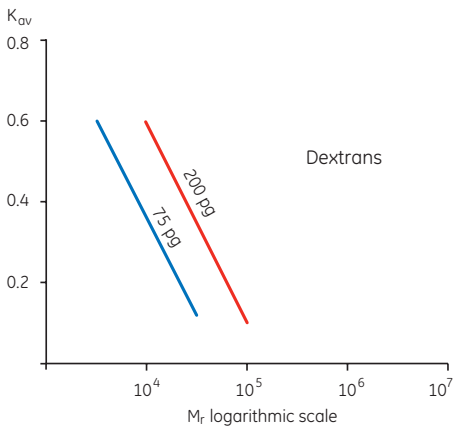
The distribution coefficients, K_d and K_{av} are related to the size of a molecule. Molecules of similar shape and density demonstrate a sigmoidal relationship between their K_d (or K_{av}) values and the logarithms of their relative molecular weights (M_r). Calibration curves constructed in this way are termed selectivity curves. The relationship between log M_r and K_d (or K_{av}) is virtually linear over a considerable range. The practical working range of K_d is approximately 0.1 to 0.9.

The selectivity of a SEC resin is an inherent property of the matrix. It depends solely on its pore size distribution and is visualized by the selectivity curve. Figure 1.9 shows selectivity curves for typical SEC resins. The slope of the selectivity curve is related to the shape of the analyzed molecules, that is, the selectivity curve will be steeper for rod-shaped molecules than for globular molecules of similar size (Fig 1.9).

Superdex™ 30 prep grade



Superdex 75 prep grade and Superdex 200 prep grade



Superdex 75 prep grade and Superdex 200 prep grade

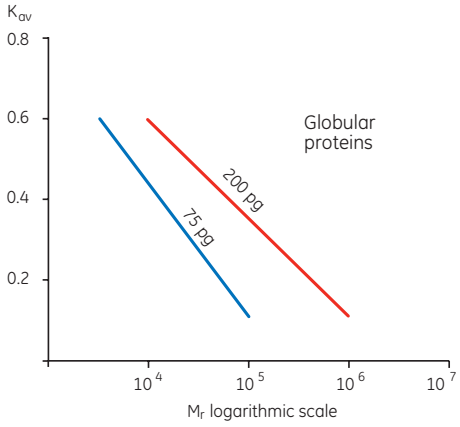


Fig 1.9. Selectivity curves for typical SEC resins.

The fractionation range of a resin defines the range of molecular weights that have partial access to the pores of the particle and can be separated. The exclusion limit of a SEC resin indicates the size of the molecules that are excluded from the pores and therefore elute in the void volume. The selectivity curve can be used to determine the fractionation range and estimate the exclusion limit of a SEC resin (Fig 1.10).

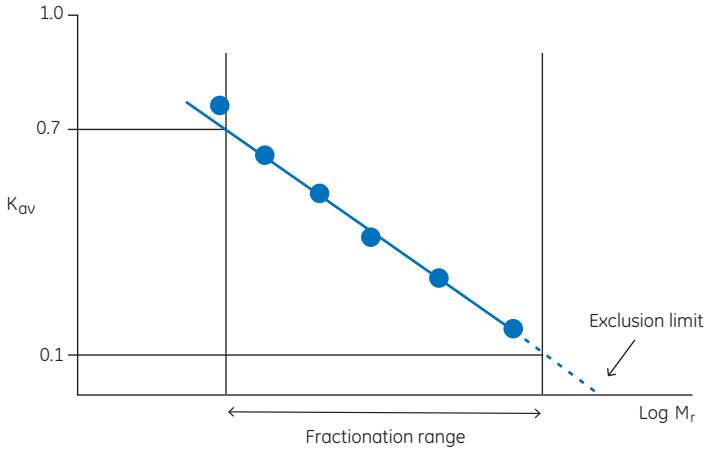


Fig 1.10. Defining fractionation range and exclusion limit from a selectivity curve.

A steeper selectivity curve means an improved separation (high selectivity) but a small separation range.

SEC resins should be selected so that the important components are found in the most linear part of the selectivity curve with minimum peak broadening or dilution and minimum time on the column. The lowest molecular weight substances should be eluted near V_t ($K_d = 1$).

Under ideal conditions, no molecules can be eluted with a K_d greater than 1 or less than 0.

The steeper the selectivity curve, the higher the resolution that can be achieved.

If K_d is greater than 1, nonspecific adsorption to the chromatographic resin might have occurred.

If K_d is less than 0, channeling in the chromatography bed might have occurred and the column must be repacked.

Deviations from ideal SEC: In ideal SEC, only steric effects contribute to the separation of biomolecules. An advantage of SEC is that the separation is generally insensitive to the eluent composition. However, deviation from the linear log M_r:K_d relationship might still occur. A nonideal behavior is caused by interactions between the resin and the biomolecules and can also influence the separation. Nonspecific interactions are generally only of concern when separating highly charged substances at an ionic strength that is too low; or when separating aromatic molecules on dense resins with a high matrix content (higher dry weight of resin). Hydrophobic interactions might also occur in some cases. Interactions can usually be avoided by changing the running buffer. Small biomolecules are more susceptible to interactions with SEC resins than larger.

Note that the molecular shape can also explain an unexpected elution position.

Resolution

Resolution is a function of the *selectivity* of the resin and the *efficiency* of that resin to produce sharp, narrow peaks with minimal peak broadening.

Resolution (R_s) can be expressed as follows:

$$R_s = \frac{V_{e2} - V_{e1}}{\frac{1}{2}(W_1 + W_2)}$$

where:

V_{e1} and V_{e2} are the elution volumes for two adjacent peaks measured at the center of the peak. W_1 and W_2 are the respective peak widths.

$(V_{e2} - V_{e1})$ represents the distance between the peaks and $(W_1 + W_2)/2$ the mean peak width of the two peaks as shown in Figure 1.11.

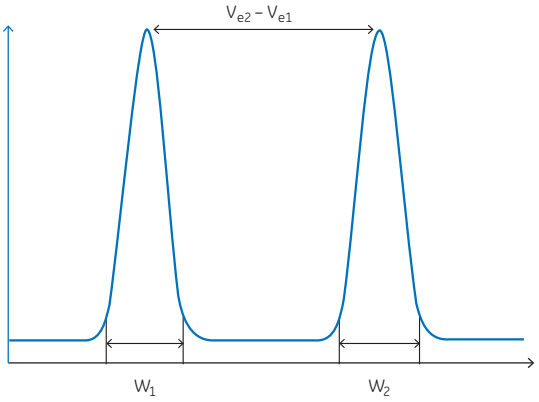


Fig 1.11. Parameters used to define resolution (R_s).

The final resolution, that is the degree of separation between peaks, depends both on the selectivity of the resin and the degree of peak broadening (Fig 1.12). The resin efficiency and other experimental factors contribute to peak broadening. Some factors such as particle size, particle size distribution, porosity, column dimensions, and column packing are related to the SEC column. Other factors include sample volume, flow rate, viscosity of the solution, and the equipment used. The success of SEC depends primarily on choosing conditions that give sufficient selectivity and counteract peak broadening effects during the separation.

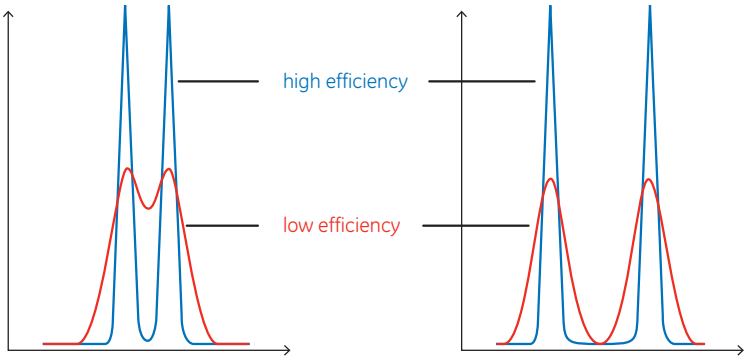


Fig 1.12. Resolution depends both on the selectivity and the efficiency of the resin.

The homogeneity of the packed bed and of the particles influences uniformity of flow through the column and hence affects the shape and width of peaks in the chromatogram. SEC resins with high uniformity and narrow particle size distribution result in the elution of molecules in sharp peaks.

SEC resins with smaller particle sizes facilitate diffusion of sample molecules in and out of the particles by reducing the time to achieve equilibrium between mobile and stationary phases. Peak width is reduced and resolution is thus improved.

Sample dilution is inevitable because diffusion occurs. To minimize sample dilution, a maximum sample volume is used within the limits set by the separation distance, that is, the resolution required between the peaks of interest. The sample can be regarded as a zone passing down the column. Figure 1.13 shows how, if no zone broadening occurs, the maximum sample volume could be as great as the separation volume (V_{Sep}):

$$V_{Sep} = V_{eB} - V_{eA}$$

where:

V_{eA} and V_{eB} are the elution volumes of molecule peaks A and B, respectively.

However, due to eddy diffusion, nonequilibrium between the stationary phase and the mobile phase, and longitudinal diffusion in the bed, the zones will always be broadened. Therefore, the sample volume must always be smaller than the separation volume.

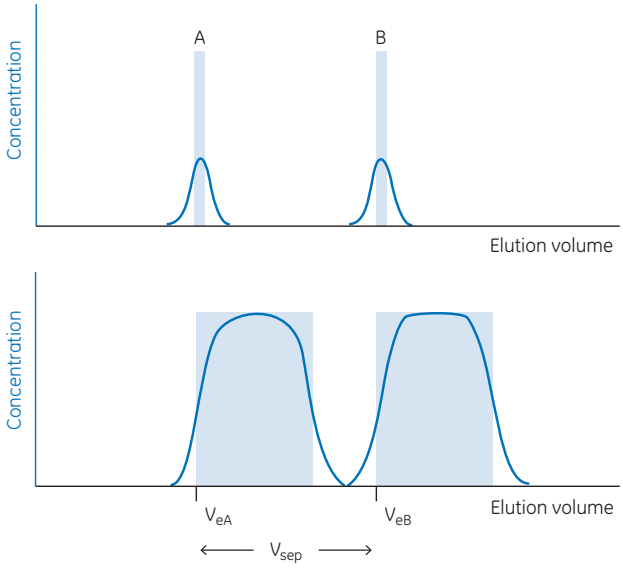


Fig 1.13. Elution curves for different sample sizes. The top diagram corresponds to the application of a small sample. The bottom diagram corresponds to the maximum sample volume to obtain complete separation in the conditions of the experiment. The shaded areas correspond to the elution profiles that would be obtained if there was no zone broadening.

Chapter 2
Size exclusion chromatography in practice

General advice

Columns and resin preparation

Efficient column packing is essential, particularly for high-resolution separations. Column bed heights of between 30 and 60 cm are recommended for preparative or analytical SEC separation. Rapid screening experiments may be performed on shorter columns (15 cm). For buffer exchange, a bed height of 10 cm is often sufficient. The required bed volume and hence the diameter of the column is determined by the sample volume.

A well-packed column should produce narrow symmetrical peaks with minimal peak broadening. This is especially important in SEC where the separation is limited by 1 CV. The uniformity of the packed bed and the particles influences the flow through the column and hence affects the shape and width of the peaks. High-performance SEC resins with high bed uniformity (smaller and more uniform particles) give decreased peak widths and improved resolution. Two parameters are generally used to characterize the column performance (see Appendix 2, *Determination of column efficiency and asymmetry factor* for further details):

- **Efficiency** (theoretical plate number per meter, N/m) describes the column ability to produce narrow peaks.
- **Peak asymmetry factor (A_s)** describes the symmetry of the peak.

Always perform a column efficiency test before first-time use. The values from the test should be used as the baseline for the column performance. Note that the system used, including the capillaries and other dead volumes can affect measured column efficiency. This means that the measured column efficiency can deviate from values given in the specification for the column. Refer to Appendix 1 and 2 for further information on column packing and column efficiency, respectively.

For optimal performance and reproducible results, use of prepacked columns is highly recommended.

Storage solutions and preservatives should be washed away thoroughly before using any SEC resin. Then equilibrate the column with 1 to 2 column volumes (CV) of buffer before starting a separation. Buffers and columns must have the same temperature before use.

Ensure sufficient buffer for the entire run. SEC columns that run completely dry must be repacked.

Scaling up

After establishing a separation on a small column, larger columns can be packed to process larger sample volumes in a single step. Note that small particles generate a high back pressure, which can restrict separation scale-up.

When comparing results between columns of different size, the flow velocity (cm/h) is a useful measurement. Results obtained at the same flow velocity on different sized columns will be comparable as far as the effects of flow velocity are concerned.

The volumetric flow rate (for example mL/min) is however often used in practice. To convert between volumetric flow rate and flow velocity, see Appendix 5.

General guidelines for scaling up are shown in Table 2.1.

Table 2.1. General guidelines for scaling up SEC separations

Maintain	Increase
Bed height	Column diameter
Flow velocity (cm/h)	Flow rate (mL/min)
Sample composition	Sample volume

To scale up a SEC separation, follow this advice:

1. Optimize the separation at small scale, see *Optimizing your separation* later in this chapter.
2. Maintain the sample-to-column volume ratio.
3. Maintain the bed height and increase the column volume by increasing the cross-sectional area of the column.
4. Run the separation at the same flow velocity (cm/h) as used on the smaller column (see Appendix 5).

Different factors related to the equipment can affect the performance after scale-up. Peak broadening can occur on a larger system if dead volumes are introduced or if the distribution system of the larger column has lower flow efficiency. This will cause extra dilution of the target molecule or even loss of resolution if the application is sensitive to variations in efficiency.

Sample and buffer preparation

Removal of particles in the sample, buffers, and cleaning solutions is extremely important for SEC. Clarifying a sample before applying it to a column will minimize the risk of blockage, reduce the need for stringent washing procedures and extend the lifetime of the column.

A running buffer of 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0 works well for many separations. Alternatively, select a suitable buffer for the next step (e.g., further purification, analysis, or storage). See advice on buffer composition later in this chapter.

Use high-quality water and chemicals. Solutions should be filtered through 0.45 µm or 0.22 µm filters before use. Degas buffers before any SEC separation since air bubbles can reduce performance. Buffers are automatically degassed if filtered under vacuum.

Buffers and columns must have the same temperature before use. Rapid changes in temperature, for example removing packed columns from a cold room and applying buffer at room temperature, can introduce air bubbles in the packed bed, resulting in poorer separation.

Samples must be clear and free from particulate matter, especially when working with particle sizes of 34 µm or smaller. For small sample volumes, a syringe-tip filter of cellulose acetate or polyvinylidene difluoride (PVDF) might be sufficient.

Filtration

Filtration removes particulate matter. Whatman™ syringe filters, which give the least amount of nonspecific binding of proteins, are composed of cellulose acetate (CA), regenerated cellulose (RC), or polyvinylidene difluoride (PVDF; Table 2.2).

Table 2.2. Whatman syringe filters for filtration of samples

Filter pore size (µm)	Up to sample volume (mL)	Whatman syringe filter[1]	Membrane
0.8	100	Puradisc FP 30	CA
0.45	1	Puradisc 4	PVDF
0.45	10	Puradisc 13	PVDF
0.45	100	Puradisc 25	PVDF
0.45	10	SPARTAN™ 13	RC
0.45	100	SPARTAN 30	RC
0.45	100	Puradisc FP 30	CA
0.2	1	Puradisc 4	PVDF
0.2	10	Puradisc 13	PVDF
0.2	100	Puradisc 25	PVDF
0.2	10	SPARTAN 13	RC
0.2	100	SPARTAN 30	RC
0.2	100	Puradisc FP 30	CA

[1] The number indicates the diameter (mm) of the syringe filter.

For sample preparation before chromatography, select a filter pore size in relation to the bead size of the chromatographic medium (Table 2.3).

Table 2.3. Selecting a sample filter based on the bead size of the chomatographic medium used

Nominal pore size of filter (µm)	Particle size of chromatographic medium (µm)
1.0	90 and upwards
0.45	30 or 34
0.22	3, 10, 15 or when extra clean samples or sterile filtration is required

Check the recovery of the target protein in a test run. Some proteins adsorb nonspecifically to filter surfaces.

Equipment selection

Accurate, reproducible control of the separation is not only essential for good results, but also for reliability in routine preparative work and repeated experiments. Use a liquid chromatography system (rather than a peristaltic pump or gravity flow) to maximize column performance. When working with small prepacked columns such as HiTrap™ Desalting, a syringe or standalone pump can also be used. For buffer exchange of small sample volumes, gravity columns and spin columns are also available. Appendix 3 provides a guide to the selection of suitable chromatography systems. Note that the chromatography system and the injection technique used can affect the resolution of the separation, see *System configuration* later in this chapter.

Setting column pressure limits

Pressure is generated by the flow through the chromatographic system. To protect the column hardware and the packed bed of the chromatographic resin, it is highly important to set maximum pressure limits. See Appendix 4 for further details on setting column pressure limits.

Maintenance of SEC columns

Routine cleaning of the SEC column should be performed to prolong column lifetime. Cleaning is performed to remove any precipitated proteins or other contaminants that build up on the column. The frequency of cleaning depends mainly on the sample, but once every 20 runs is a guideline. Insufficient cleaning might lead to discoloration, loss in resolution, and increase in back pressure. Cleaning procedures for each SEC resin are given in Chapter 4 and 5 and in the instructions for each product.

Remove the cleaning solution thoroughly and re-equilibrate the column with at least 1 to 2 CV of buffer before the next separation. Check that pH is neutral.

In case of increased back pressure, see also *Troubleshooting* section in this chapter.

Optimizing your separation

The success of SEC depends primarily on choosing conditions that give sufficient selectivity and counteract peak broadening effects during the separation. Prepacked columns are delivered with recommended running conditions that give satisfactory results in most situations but optimization might sometimes be necessary to reach the required resolution. Resolution is a function of the selectivity of the resin and the efficiency of that resin to produce narrow peaks (minimal peak broadening), as illustrated in Chapter 1.

Final resolution is influenced by many factors (Table 2.4). After selection of SEC resin, sample volume and column dimensions are the two most critical parameters that will affect the resolution of the separation. Chromatography system-related factors also affect resolution, particularly when working with small columns for high-resolution separations. Note that conditions leading to the highest resolution usually conflict with other experimental objectives, for example, separation time. Careful evaluation of overall requirements is therefore necessary.

Table 2.4. Factors that influence resolution

Resin-related factors	Selectivity Match between pore size and size of target molecule Particle size Particle uniformity
Column-related factors	Bed height Column packing quality
Chromatography system-related factors	Tubing dimensions (diameter and length) Volumes in system components
Experimental-related factors	Flow rate Sample volume Viscosity Buffer composition

If optimization is needed, follow these steps (given in order of priority):

1. Select a resin with a suitable fractionation range providing optimal resolution; see Figure 4.1.
2. Select a column with a bed height providing the required resolution. A bed height between 30 and 100 cm is recommended for preparative and analytical separation whereas 10 cm is often sufficient for buffer exchange and desalting.
3. Select a column size appropriate for the volume of sample that needs to be processed.
4. Select the highest flow rate that maintains resolution and minimizes separation time.

Resin selection

For highest resolution, select a SEC resin with suitable fractionation range and a small particle size. In cases where two resins have similar fractionation ranges, select the resin with the steepest selectivity curve for optimal resolution of all the sample components. For a specific component, select a resin where the log of molecular weight for the target component falls in the middle of the selectivity curve. Efficiency can be improved by using a resin with smaller particle size. However, using a smaller particle size will increase the back pressure. See Chapter 4 and 5 and *Ordering information* for available resins from GE.

For buffer exchange and desalting, select a SEC resin that elutes high molecular weight-molecules in the void volume to minimize peak broadening and dilution. The lowest molecular weight substances should appear within 1 CV of buffer.

An example of the effects of different selectivities between two similar SEC resins is shown in Figure 2.1. Superdex 200 Increase and Superose™ 6 Increase are both resins for small-scale preparative purification and analysis, with the same high-flow agarose base matrix (average particle size 8.6 µm). They differ in fractionation range, which results in very different chromatography profiles for the same sample mix. Superdex 200 Increase gives excellent resolution for biomolecules with molecular weight less than 440 000, while larger biomolecules elute together with aggregates in the void volume. Superose 6 Increase, on the other hand, has good separation between the largest biomolecules.

Columns:	Superdex 200 Increase 10/300 GL and Superose 6 Increase 10/300 GL
Sample:	1. IgM (M_r ~ 970 000)[1], 0.5 mg/mL
	2. Thyroglobulin (M_r 669 000), 1 mg/mL
	3. Ferritin (M_r 440 000), 0.1 mg/mL
	4. Bovine serum albumin (M_r 66 000), 1 mg/mL
	5. Myoglobin (M_r 17 000), 0.5 mg/mL
	6. Vitamin B_{12} (M_r 1355), 0.05 mg/mL
Sample volume:	100 µL
Flow rate:	0.5 mL/min
Buffer:	PBS (10 mM phosphate buffer, 140 mM sodium chloride, pH 7.4)
System:	ÄKTAmicro

[1] *Sample also contained aggregated forms of IgM.*

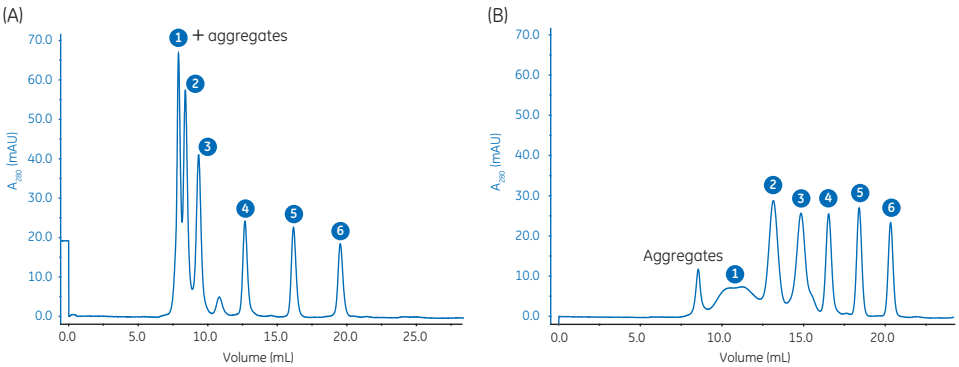


Fig 2.1. Chromatograms showing high-resolution SEC of six standard proteins on (A) Superdex 200 Increase 10/300 GL and (B) Superose 6 Increase 10/300 GL. The broad peak of IgM was confirmed by light scattering to include different isoforms and aggregates of IgM.

Sample volume and column dimensions

Sample volume is one of the most important parameters in SEC. Smaller sample volumes help to avoid overlap if closely spaced peaks are eluted. Figure 2.2 illustrates how sample volume can influence a high-resolution separation.

Column: Superdex 200 Increase 5/150 GL
Sample: 1. Thyroglobulin (M_r 669 000), 3 mg/mL
2. Aldolase (M_r 158 000), 3 mg/mL
3. Conalbumin (M_r 75 000), 3 mg/mL
4. Carbonic anhydrase (M_r 29 000), 3 mg/mL
5. Ribonuclease A (M_r 13 700), 3 mg/mL
Sample volumes: 50 and 4 µL
Buffer: PBS (10 mM phosphate buffer, 140 mM sodium chloride, pH 7.4)
Flow rate: 0.45 mL/min
System: ÄKTAmicro

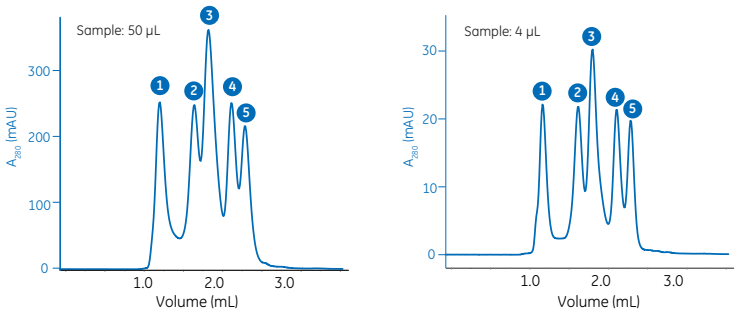


Fig 2.2. Comparison of protein separation on Superdex 200 Increase 5/150 GL using different sample volumes.

The sample volume can be expressed as a percentage of the total column volume (packed bed). This sample-to-column volume ratio influences resolution. As shown in Figure 2.3, higher ratios give lower resolution. Note that sample dilution is inevitable since diffusion occurs as the sample passes through the column. Small samples will be diluted more relative to the column volume.

Column: HiLoad™ 16/600 Superdex 200 prep grade
Sample: 1:1 solution of transferrin (M_r 81 000) and IgG (M_r 160 000)
Sample concentration: 8 mg/mL
Buffer: 50 mM sodium phosphate, 100 mM sodium chloride, pH 7.2
Flow rate (flow velocity): 1 mL/min (30 cm/h)

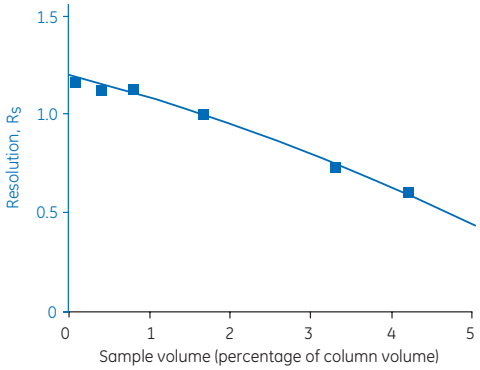


Fig 2.3. Influence of sample–to-column volume ratio on the resolution of transferrin and IgG on HiLoad 16/600 Superdex 200 prep grade.

For buffer exchange and desalting, use sample volumes of up to 30% of the total column volume (V_c).

For high-resolution separation, a sample volume from 0.3 to 4% of V_c is recommended, depending on the type of resin used. For most applications, the sample volume should not exceed 2% to achieve high resolution. Depending on the nature of the specific sample, it might be possible to load larger sample volumes, particularly if the peaks of interest are well separated. This can only be determined experimentally.

For analytical separations and separations of complex samples, start with a sample volume of 0.5% of the total column volume. Sample volumes of less than 0.3% do not normally improve resolution.

Concentrating samples can increase the capacity of a SEC separation. Avoid excessively high protein concentrations as viscosity effects can interfere with the separation. See section *Sample concentration and viscosity*, later in this chapter.

Normally, the column size is selected according to the sample volume to be processed. Larger sample volumes can require significantly larger columns; it might be beneficial to repeat the separation several times on a smaller column and pool the fractions of interest or to concentrate the sample prior to SEC.

The height of the packed bed affects both resolution and the separation time. Resolution in SEC increases with the square root of bed height. Doubling the bed height gives a 40% increase in resolution ($\sqrt{2} = 1.4$). For high resolution, long columns will give the best results.

If higher resolution is required, the effective bed height can be increased by using columns, containing the same resin, coupled in series. Alternatively, try a resin with the same or similar fractionation range, but with a smaller particle size.

For rapid purity check and screening, shorter columns with small bed volumes providing short cycle time, small sample volume, and low buffer consumption are suitable.

Elution and flow rates

The goal for most separations is to achieve the required resolution in the shortest possible time. Generally, a lower flow rate will allow time for molecules to diffuse in and out of the matrix and improve the resolution. The effect is most pronounced for large molecules whereas decreasing the flow rate can even have a negative impact on the resolution for very small molecules.

Figures 2.4 to 2.6 show the effect of flow rate on resolution. Each separation must be optimized to find the optimal flow rate. For most molecules, maximum resolution is obtained with a long column and a low flow rate. Maximum speed, however, is obtained with a short column and a high flow rate. The advantage of a higher flow rate, and consequently a faster separation, often outweighs the loss of resolution in, for example, screening experiments.

Column:	Superose 6 Increase 10/300 GL
Sample:	1. Thyroglobulin (M_r 669 000), 3 mg/mL
	2. Ferritin (M_r 440 000), 0.3 mg/mL
	3. Aldolase (M_r 158 000), 3 mg/mL
	4. Ovalbumin (M_r 44 000), 3 mg/mL
	5. Ribonuclease A (M_r 13 700), 3 mg/mL
	6. Aprotinin (M_r 6500), 1 mg/mL
Sample volume:	100 µL
Buffer:	PBS (10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4)
Flow rates:	1.5 and 0.25 mL/min, room temperature
System:	ÄKTAmicro

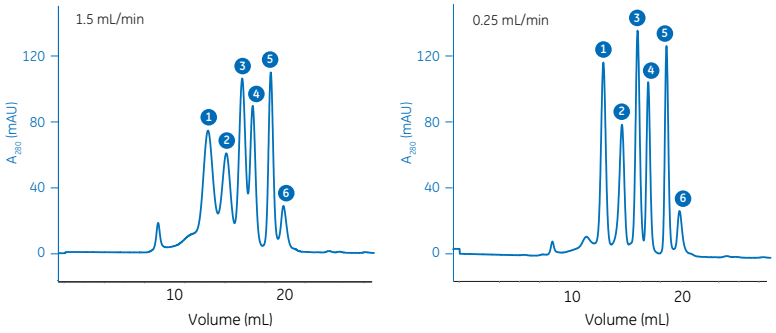


Fig 2.4. Comparison of protein separation on Superose 6 Increase 10/300 GL at different flow rates.

Column:	HiLoad 16/600 Superdex 30 prep grade
Sample:	Insulin-like growth factor 1 (IGF-1, M_r 7650) containing monomers and dimers
Sample volume:	1 mL (0.8% × V_c)
Sample concentration:	(A) 1.25 mg/mL
	(B) 5 mg/mL
Buffer:	50 mM sodium acetate, 100 mM sodium chloride, pH 5.0
Flow rates (flow velocities):	0.5 to 2.5 mL/min (15 to 80 cm/h)

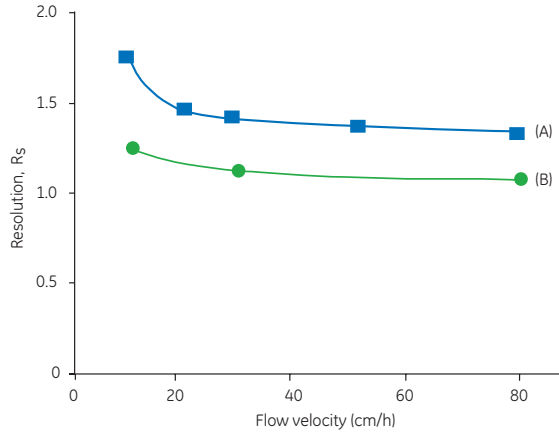


Fig 2.5. Resolution between (A) 1.25 mg/mL and (B) 5 mg/mL IGF-1 containing monomers and dimers at different flow velocities.

Columns:	HiPrep™ 16/60 Sephacryl™ S-100 HR HiPrep 16/60 Sephacryl S-200 HR HiPrep 16/60 Sephacryl S-300 HR
Sample:	IgG, ovalbumin, cytochrome C, 1:2:1
Sample volume:	2.4 mL (2% × V_c)
Sample load:	8 mg
Buffer:	50 mM sodium phosphate, 150 mM sodium chloride, 0.02% sodium azide, pH 7.0

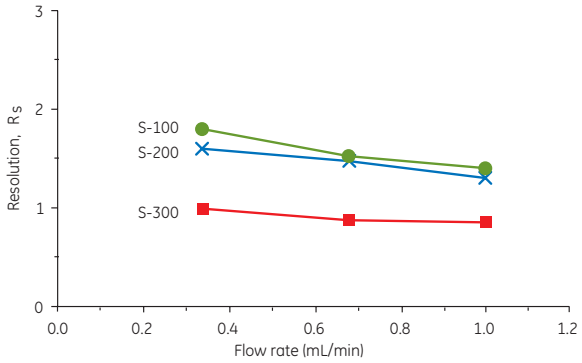


Fig 2.6. Influence on resolution between IgG, ovalbumin, and cytochrome C using different SEC resins, at different flow rates.

If peaks are well separated at a low flow rate, the separation time can be decreased by increasing the flow rate or by using a shorter column. Alternatively, increase the sample volume and benefit from a higher sample volume capacity without significant loss of resolution.

Sample concentration and viscosity

Sample mass and hence sample concentration has little effect on resolution in SEC (Fig 2.7 and 2.8). High resolution can be maintained despite high sample concentration. The viscosity of the sample might, however, limit the concentration that can be used.

Column:	Superose 6 Increase 10/300 GL
Sample:	Mix of three standard proteins: 1. Thyroglobulin (M_r 669 000) 2. Conalbumin (M_r 75 000) 3. Cytochrome C (M_r 12 400)
Sample volume:	100 µL
Buffer:	PBS (10 mM sodium phosphate, 140 mM sodium chloride, pH 7.4)
Flow rate:	0.5 mL/min

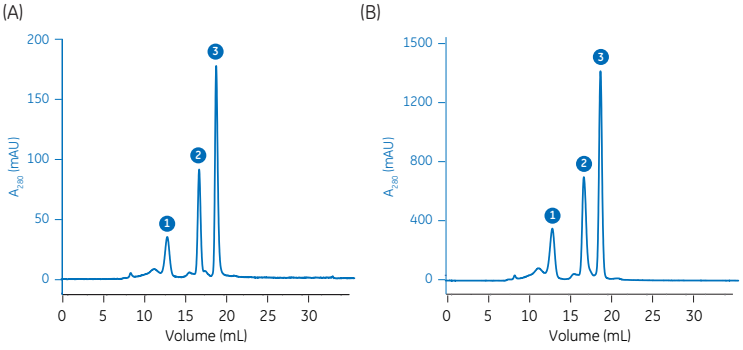


Fig 2.7. Comparison of separation of mixture with total protein concentration of (A) 10 and (B) 100 mg/mL on Superose 6 Increase 10/300 GL.

Column:	XK 16/70 (140 mL)
Resin:	Superdex 200 prep grade
Sample:	Solution of transferrin (M_r 81 000) and IgG (M_r 160 000) by equal weight
Sample volume:	1.1 mL (0.8% × V_c)
Buffer:	50 mM sodium phosphate, 100 mM sodium chloride, pH 7.2
Flow rate (flow velocity):	1 mL/min (30 cm/h)

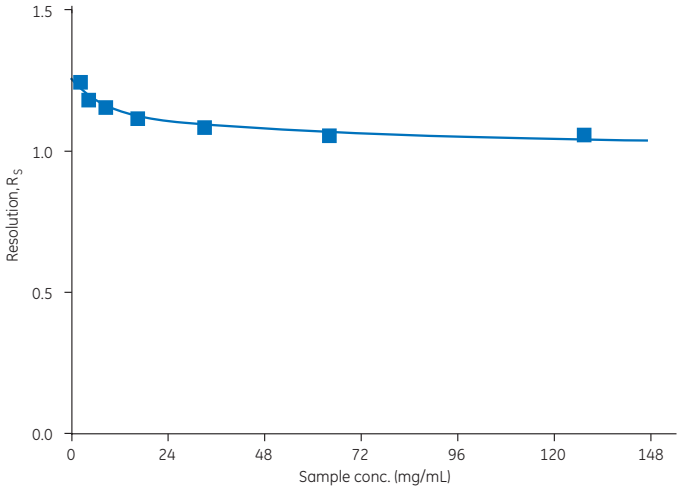


Fig 2.8. Influence of sample concentration on the resolution of transferrin and IgG on Superdex 200 prep grade.

A critical variable is the viscosity of the sample relative to the running buffer, as shown by the change in elution profiles of hemoglobin and sodium chloride at different sample viscosities in Figure 2.9.

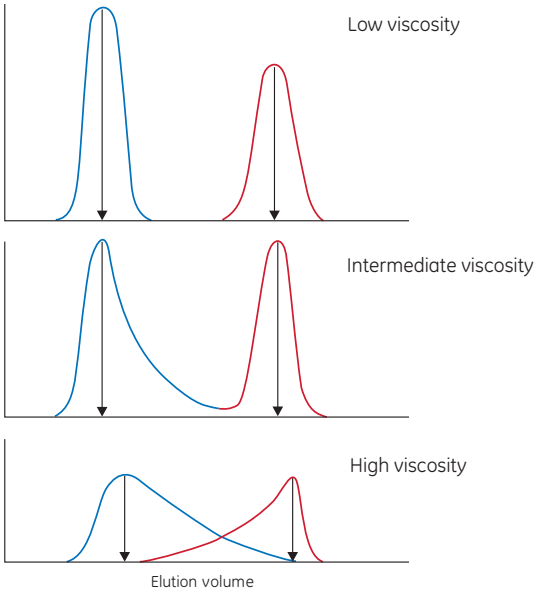


Fig 2.9. Deteriorating separation caused by increasing sample viscosity. Elution profiles obtained when hemoglobin (blue) and sodium chloride (red) were separated. Experimental conditions were identical except that the sample viscosities were altered by the addition of increasing amounts of dextran.

Dilute viscous samples, but not more than necessary to keep the sample volume low. For example, for proteins such as human serum albumin, the concentration should generally not exceed 70 mg/mL. Remember that viscosity varies with temperature.

Sample concentration does not generally influence the separation if the viscosity does not differ by more than a factor of 1.5 from that of the buffer used. If the viscosity of the sample is very high, this may be compensated for by increasing the viscosity of the eluent, for example by addition of sucrose or dextran.

High sample viscosity causes instability of the separation and an irregular flow pattern. This leads to very broad and skewed peaks and back pressure might increase.

The maximum flow rate for a column will be reduced using high-viscosity solutions or samples and the flow rate needs to be decreased accordingly. See Appendix 4 for further details.

Buffer composition

The pH, ionic strength, and composition of the buffer will not significantly affect resolution as long as these parameters do not alter the size or stability of the biomolecules to be separated and are within the stability range of the SEC resin. Extremes of pH and ionic strength and the presence of denaturing agents or detergents can cause conformational changes, dissociation, or association of protein complexes. The sample does not have to be dissolved in the running buffer since the buffer is exchanged during the separation, which is an added benefit of SEC.

Use a buffer concentration that maintains buffering capacity and constant pH. Sodium chloride (300 mM) may be included to suppress nonspecific ionic interactions, which might be seen as delays in peak elution or broad peaks. SEC of proteins at two different NaCl concentrations is shown in Figure 2.10. An increase in NaCl concentration had little effect on retention volume for the negatively charged α-acid glycoprotein (pI 2.7 to 3.2) and myoglobin (pI 6.8 to 7.2). However, for the positively charged cytochrome C (pI ~ 10), the behavior was different; at low NaCl concentration, the strongly charged protein interacted with the column (cation interaction), leading to a decrease in retention volume.

Note that interactions in some cases may be used to improve resolution.

Column:	Superose 6 Increase 10/300 GL
Sample:	Mix of three proteins 1. α-acid glycoprotein (M_r 41 to 43 000) 2. Myoglobin (M_r 16 700) 3. Cytochrome C (M_r 12 400)
Sample volume:	100 µL
Buffer:	(A) 10 mM sodium phosphate, 140 mM NaCl (B) 10 mM sodium phosphate, 300 mM NaCl
Flow rate:	0.5 mL/min

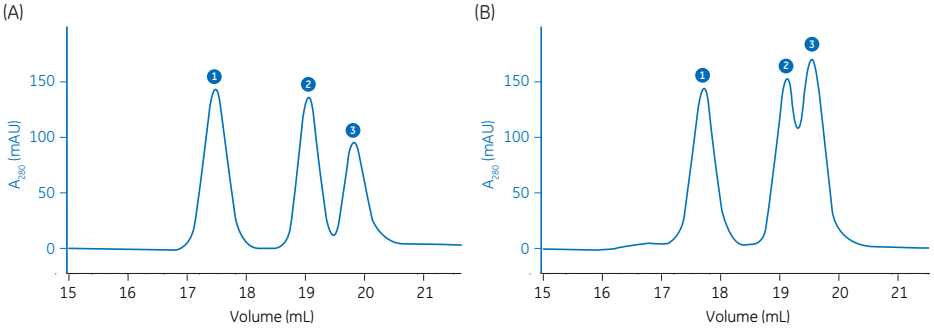


Fig 2.10. Separation of α-acid glycoprotein, myoglobin, and cytochrome C, using (A) 140 and (B) 300 mM NaCl in sample and running buffer.

Some proteins can precipitate in low ionic strength solutions.

Avoid using unnecessarily high salt concentrations as this might increase hydrophobic interaction.

Addition of organic solvent can be beneficial for hydrophobic substances (Fig 2.11).

Column:	Superdex 30 Increase 10/300 GL
Sample:	Somatostatin (M_r 1600)
Sample volume:	50 µL
Buffer:	50 mM sodium phosphate, 250 mM NaCl, pH 7.4 with addition of 0% to 30% acetonitrile
Flow rate:	0.8 mL/min

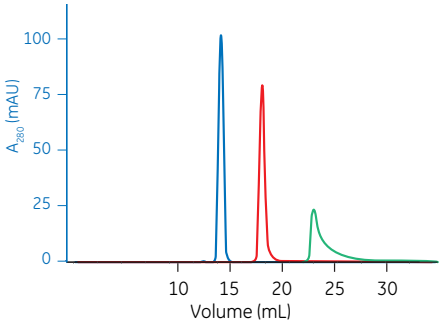


Fig 2.11. Elution profiles of somatostatin separated in 50 mM sodium phosphate, 250 mM NaCl, pH 7.4 with 30% (blue), 15% (red), and 0% (green) acetonitrile.

Note that peptides and other small biomolecules are more susceptible than larger biomolecules to interactions with SEC resins, as peptides display very little or no tertiary structure leaving amino acid side chains exposed. Detergents are useful as solubilizing agents for proteins with low aqueous solubility, such as membrane components, and will not affect the separation. Sometimes, detergents are necessary to maintain the solubility of the sample. Additives must be present all the time, both in the running buffer and the sample.

- Choose buffer conditions suitable for protein stability and activity. An increase of the sodium chloride concentration to 300 mM or including additives such as detergents or organic solvents can improve the result. See further information regarding denaturing (chaotropic) agents below.
- Note that some additives increase buffer viscosity. Use lower flow rates for high-viscosity solutions and/or low temperature (see Appendix 4).
- Volatile buffers such as ammonium acetate or ammonium bicarbonate should be used if the separated product is to be lyophilized.
- SEC can be used to exchange the detergent environment of a protein. For example, a protein solubilized in sodium dodecyl sulfate (SDS) could be transferred to a milder detergent such as Tween™ 20 without losing solubility.
- Avoid extreme changes in pH or other conditions that can cause inactivation or even precipitation. If the sample precipitates in the SEC column, the column will be blocked—possibly irreversibly—and the sample might be lost.

Denaturing (chaotropic) agents

Denaturing agents such as guanidine hydrochloride or urea may be used for initial solubilization of a sample as well as in SEC buffers to maintain solubility. However, since the proteins will denature, avoid chaotropics unless denaturation is specifically desired.

Urea and guanidine hydrochloride are very useful for molecular weight determination. The presence of these denaturing agents in the running buffer maintains proteins and polypeptides in an extended configuration. For accurate molecular weight determination, calibration standards must be run under the same conditions as the sample.

Note that selectivity curves are usually determined using globular proteins and do not reflect the behavior of denatured samples.

System configuration

Depending on the system setup, the contributions from internal system volumes to band broadening vary. For the highest resolution, it is important that the chromatography system used has the smallest possible internal system volumes. This is especially important when working with small columns for high-resolution separations. Internal system volumes may be decreased by, for example, using short, narrow-diameter capillaries (Fig 2.12).

Consider actions to reduce system volumes if sufficient resolution is not achieved. For optimal configuration of ÄKTA™ pure 25 for small-scale SEC, see *Optimal configuration of ÄKTA pure 25 for small-scale SEC*, 29181181.

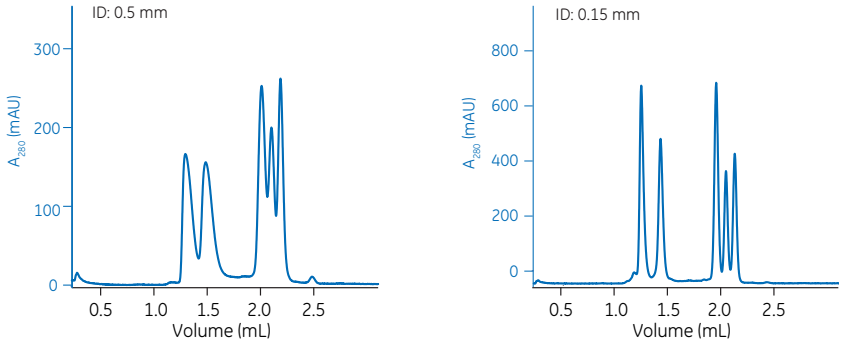


Fig 2.12. Comparison of peptide separation on Superdex 30 Increase 3.2/300 using different diameters of a 42 cm long capillary connected to the column.

Another way to minimize dead volumes in the system is to use components with small internal volumes. Figure 2.13 shows the effect of different internal volumes in column valves on ÄKTA pure 25. In Figure 2.13A, the resolution on Superdex 75 Increase 5/150 GL column (V_c 3 mL) is highly affected by a larger internal volume in the column valve compared with Fig 2.13B, where Superdex 75 Increase 10/300 GL column (V_c 24 mL) is less affected.

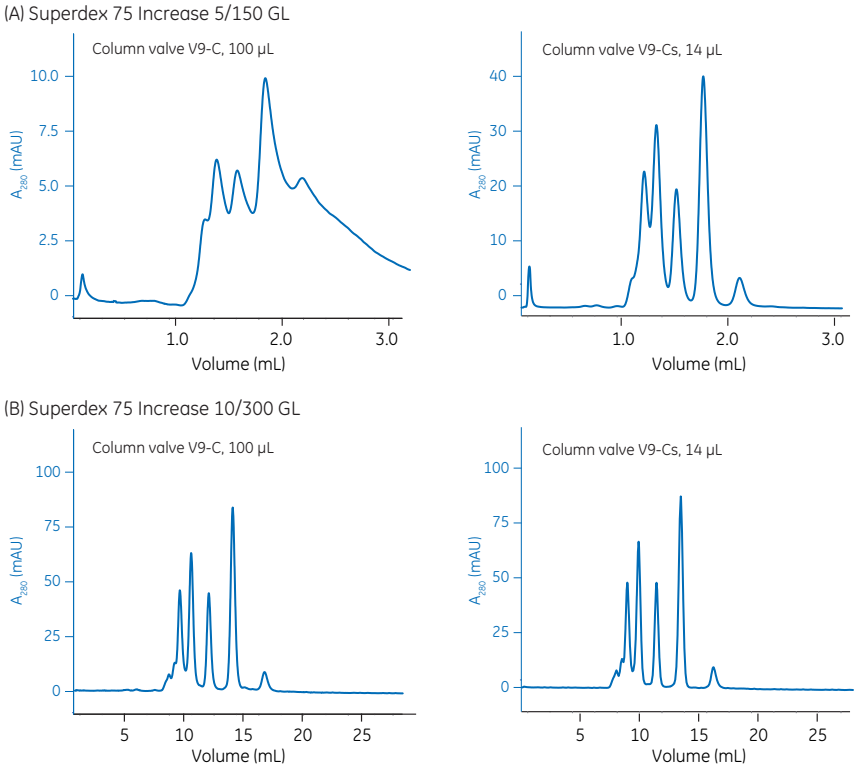


Fig 2.13. Comparison of protein separation on (A) Superdex 75 Increase 5/150 GL and (B) Superdex 75 Increase 10/300 GL. Purification was performed with ÄKTA pure 25 chromatography system using either column valve V9-C (internal volume 100 µL) or column valve V9-Cs (internal volume 14 µL). Note that column valve V9-C is not recommended for use with 5/150 GL column.

The injection technique might also affect the resolution and needs to be considered when working with high-resolution separations. Figure 2.14 shows an example of how resolution can be improved by decreasing the volumes used to empty the loop during sample injection. This is a common way of working when analytical studies are performed. For detailed information on injection techniques, see *ÄKTA Laboratory-scale Chromatography Systems Handbook*, 29010831.

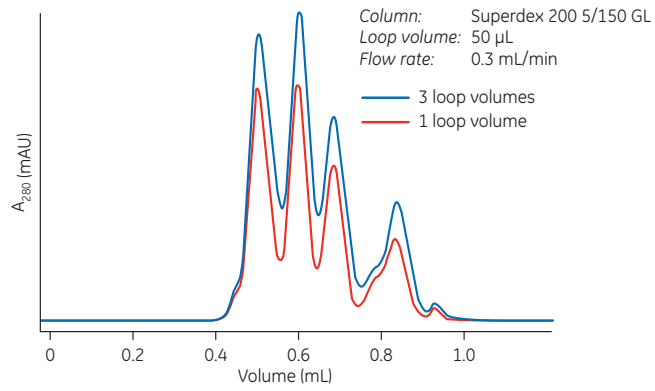
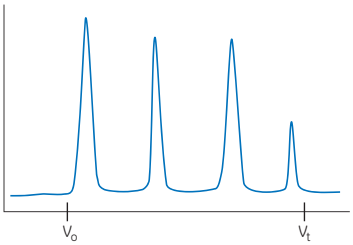


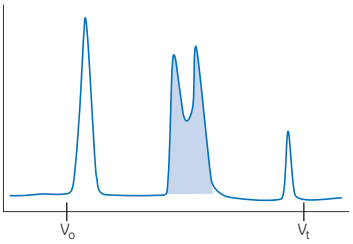
Fig 2.14. The chromatogram shows how the separation in SEC is affected by the different volumes used to empty the loop during sample injection.

Troubleshooting

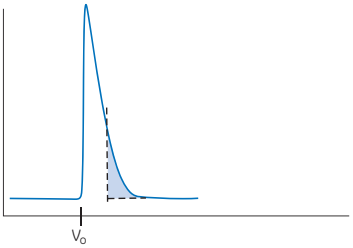
This section focuses on practical problems that can occur during SEC. Figure 2.15 indicates how the elution profile in a chromatogram can deviate from normal during a separation. Table 2.5 on the following pages contains further suggestions of possible causes and their solutions.



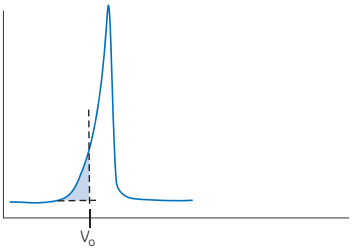
Satisfactory separation
Well-resolved, symmetrical peaks.



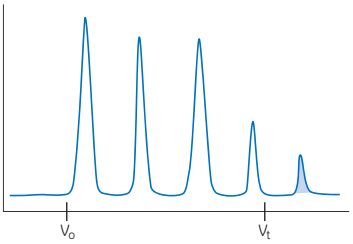
Poor resolution
Review factors affecting resolution, including the selectivity of the resin, particle size, sample-to-column volume ratio, flow rate, and the equipment used.



Asymmetric peaks: Tailing
Tailing peaks can be caused by an underpacked column, that is, a column packed at a very low pressure or flow rate. Ensure the resin is evenly packed. Tailing peaks can also be explained by uneven sample application or interactions with the column.



Asymmetric peaks: Fronting
Fronting peaks can be caused by an overpacked column, that is, a column packed at excessive pressure or flow rate. Peaks appearing before the void volume indicate channeling in the packed bed.



Late elution
Peaks eluting after 1 CV of buffer can be caused by nonspecific interactions with the resin. Include a wash step or extend the elution to ensure elution of late eluting molecules. Changing the buffer composition might also be beneficial.

Fig 2.15. Normal chromatograms and chromatograms deviating from normal behavior.

Table 2.5. Troubleshooting guide for SEC

Situation	Possible cause	Solution
Peak of interest is poorly resolved from other major peaks.	Sample volume is too high.	Decrease sample volume and apply sample carefully.
	Sample is too viscous.	Dilute with buffer, but check maximum sample volume. Maintain protein concentration below 70 mg/mL.
	Sample contains particles.	Re-equilibrate column, filter sample, and repeat.
	Column is poorly packed.	Check column efficiency (see Appendix 2). Repack if needed[1]. Use prepacked columns.
	Column is dirty.	Clean and re-equilibrate.
	Incorrect resin.	Check selectivity curve. Check for adsorption effects. Consider effects of denaturing agents or detergents if present.
	Large dead volumes.	Minimize dead volumes in tubing and connections.
	Column is too short.	Increase bed height.
	Flow rate is too high.	Check recommended flow rates. Note that the maximum flow rate for a packed column will vary with running conditions.
Molecule does not elute as expected.	Ionic interactions between molecule and matrix.	Maintain ionic strength of buffers above 50 mM (preferably include up to 300 mM sodium chloride).
	Hydrophobic interactions between molecule and matrix.	Reduce salt concentration to minimize hydrophobic interaction. Add suitable detergent or organic solvent, e.g., 5% isopropanol. Changing pH might also affect hydrophobic interactions.
	Sample has not been filtered properly.	Clean the column, filter the sample.
	Sample has changed during storage.	Prepare fresh samples.
	Column is not equilibrated sufficiently.	Repeat or prolong the equilibration step.
	Proteins or lipids have precipitated on the column.	Clean the column or use a new column.
	Column is overloaded with sample.	Decrease the sample load.
	Precipitation of protein in the column filter and/or at the top of the bed.	Clean the column, exchange or clean the filter, or use a new column.
	Microbial growth has occurred in the column.	Microbial growth rarely occurs in columns during use. To prevent infection of packed columns, store in 20% ethanol when possible.
Molecule elutes later than expected or even after running a total column volume.	Hydrophobic and/or ionic interactions between molecule and matrix.	Reduce salt concentration to minimize hydrophobic interaction. Increase pH. Add suitable detergent or organic solvent, e.g., 5% isopropanol.
		Increase salt concentration (up to 300 mM) to minimize ionic interaction.
Peaks elute late and are very broad.	Column is dirty.	Clean and re-equilibrate.
Molecule elutes earlier than expected (before the void volume).	Channeling in the column.	Repack column[1]. Using a thinner slurry of resin might prevent channeling during repacking.
Fronting or very rounded peaks in chromatogram.	Column overloaded.	Decrease sample load and repeat.
Tailing peaks.	Column is packed too loosely.	Check column efficiency (see Appendix 2). Repack using a higher flow rate. Use prepacked columns[1].
Fronting peaks.	Column is packed too tightly.	Check column efficiency (see Appendix 2). Repack using a lower flow rate. Use prepacked columns[1].

[1] *Not all prepacked columns may be repacked.*

Situation	Possible cause	Solution
Low recovery of activity but normal recovery of molecule.	Molecule is unstable or inactive in the buffer.	Determine the pH and salt stability of the molecule.
	Enzyme separated from cofactor or similar.	Test by pooling aliquots from the reactions and repeat the assay.
Lower yield than expected.	Protein has been degraded by proteases.	Add protease inhibitors to the sample and buffers to prevent proteolytic digestion. Run sample through a resin such as Benzamidine 4 Fast Flow (high sub) to remove trypsin-like serine proteases.
	Adsorption to filter during sample preparation.	Use another type of filter.
	Sample precipitates.	Can be caused by removal of salts or unsuitable buffer conditions. Change the buffer conditions.
	Hydrophobic molecules.	Decrease the ionic strength. Use denaturing agents, or detergents. Addition of organic solvent or changing pH might be beneficial.
	Nonspecific ionic adsorption.	Increase salt concentration in the buffer, up to 300 mM sodium chloride.
More target substance is recovered than expected.	Molecule is coeluting with other substances.	Optimize conditions to improve resolution. Check buffer conditions used for assay before and after the run. Check selection of resin.
Obtained column efficiency is not the same as in the column specification.	Result for column efficiency is dependent on the system and cannot be expected to be the same.	Use the obtained column efficiency for later comparisons in order to detect any changes in column performance. Consider system dead volumes when determining efficiency (see Appendix 2).
Results are not reproducible.	The new column is not saturated by molecule in the first two to three runs.	Perform test runs until the column is saturated with molecule and stability is obtained.
	Column is not sufficiently equilibrated.	Allow more time for equilibration.
Reduced or poor flow through the column.	Presence of lipoproteins or protein aggregates.	Remove lipoproteins and aggregates during sample preparation.
	Protein precipitation in the column caused by removal of stabilizing agents during separation.	Modify the eluent to maintain stability.
	Blocked column filter.	If possible, replace the filter or use a new column. Always filter samples and buffer before use.
	Blocked end-piece, adapter, or tubing.	If possible, remove and clean or use a new column.
	Precipitated proteins.	Clean the column using recommended methods or use a new column.
	Bed compressed.	Clean or repack the column[1]. Alternatively, use a new column[1].
	Microbial growth.	Microbial growth rarely occurs in columns during use. To prevent infection of packed columns, store in 20% ethanol when possible. Always filter samples and buffers. Clean the column.
	Fines (Sephadex).	Decant fines before column packing. Avoid using magnetic stirrers that can break the particles.
Back pressure increases during a run or during successive runs.	Turbid sample.	Improve sample preparation. Improve sample solubility by the addition of ethylene glycol, detergents, or organic solvents.
	Precipitation of protein in the column filter and/or at the top of the bed.	Clean using recommended methods. If possible, exchange or clean filter or use a new column. Include any additives that were used for initial sample solubilization in the running buffer.
	Clogged on-line filters.	Exchange on-line filters.

[1] *Not all prepacked columns may be repacked.*

Situation	Possible cause	Solution
Air bubbles in the bed.	Column packed or stored at cool temperature and then warmed up.	Note that small amounts of air will normally not affect the performance of the column. Remove air bubbles by passing degassed buffer in an up-flow direction at low flow rate. Note that reverse flow should not be used for Sephacryl columns. Repack column if needed[1] (see Appendix 1).
	Buffers not properly degassed.	Degas buffers thoroughly. Take special care if buffers are used after storage in a fridge or cold-room.
Gap between resin bed and adapter.	Back pressure increase or bed insufficiently packed.	Clean the column if dirty. Adjust the adapter to the resin bed. Make sure that the pressure limits are set appropriately. Perform a column performance test.
	Flow rate too high.	Do not exceed maximum flow rate for resin or packed column. Note that the maximum flow rate for a packed column will vary with running conditions.
Cracks in the bed.	Large air leak in column.	Check all connections for leaks. Repack the column[1].
Distorted bands as sample runs into the bed.	Air trapped at top of column or in inlet adapter.	If possible, re-install the adapter taking care to avoid air bubbles. Perform a column performance control.
	Particles in buffer or sample.	Filter or centrifuge the sample. Cover bottles to avoid particles in the buffer.
	Blocked or damaged net/filter in upper adapter.	If possible, dismantle the adapter, clean or replace the net.
		Keep particles out of samples and eluents.
Distorted bands as sample passes down the bed.	Column poorly packed.	Suspension too thick or too thin. Bed packed at a temperature different from run.
		Bed insufficiently packed (packing pressure too low, equilibration time too short). Column packed at excessively high pressure.
Resin/particles appear in eluent.	Bed support end piece is loose or broken.	Replace or tighten.
	Filter pores are too large.	Use a filter with smaller pores appropriate for the size of the resin particles.
	Column operated at excessively high pressure.	Do not exceed recommended operating pressure for medium or column.

[1] *Not all prepacked columns may be repacked.*

Reference

1. Janson, J.-C. ed. In *Protein Purification: Principles, High Resolution Methods, and Applications,* pp 59, equation 3.16 third edition, Wiley Online Library (2011). doi: 10.1002/9780470939932

Chapter 3
Considerations for analytical SEC

Introduction

SEC is widely used in several different analytical applications from basic research to quality control of biotherapeutics. It is a very effective method for protein analysis and it allows true size profiling of protein samples due to the mild separation conditions that can be used to obtain high-resolution separations. A protein can occur in different "size forms" (monomeric, aggregated, degraded, complexed, Fig 3.1) and those different forms exhibit different functions. As many protein "size forms" are held together by noncovalent means, they are often weak and could be easily broken if using nonnative (i.e., denaturing) conditions.

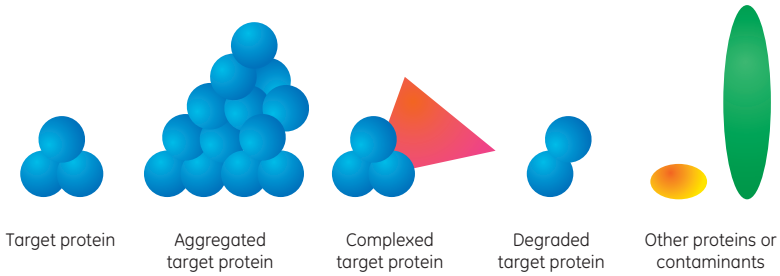


Fig 3.1. Schematic illustration of different "size forms" of a protein.

The use of SEC makes it possible to size profiling the sample, for example when you need to:

- Monitor protein preparation quality
- Evaluate protein stability
- Study complex formation
- Evaluate tendency to aggregate and quantity of aggregates
- Evaluate tendency to degrade and quantity of degraded forms
- Identify protein interaction partners and interaction conditions

Factors to consider when performing analytical SEC

As for all types of SEC, the goal for analytical SEC separations is to achieve the required resolution in the shortest possible time. The requirements on resolution are however often considerably higher in analytical SEC compared to preparative SEC.

In addition, the need for a robust and consistent method is of utmost importance, especially for methods used for quality control (QC). Careful evaluation of critical method parameters such as running conditions and column lot-to-lot variation and their influence on accuracy and precision need to be identified.

To confirm that an analytical SEC method continuously delivers reliable results, a system suitability test can be performed. Typically, a system suitability test involves numerical acceptance limits for predefined chromatographic parameters such as theoretical plates, retention time, and resolution.

As described in Chapter 2, resolution is influenced by many factors (see Table 2.2). After the selection of SEC resin, column dimensions, sample volume, and flow rate are the most important factors for the separation. When performing analytical SEC, factors such as those marked in blue in Table 3.1 need to be considered and are further discussed in this chapter. Note that these factors do not necessarily influence the resolution the most.

Table 3.1. Factors affecting analytical SEC[1]

Resin properties	Chemical composition Particle size and distribution Pore size and selectivity Fractionation range
Chromatography running conditions	Sample volume and injection technique Flow rate Buffer composition
Chromatography system-related factors	Tubing diameter Volume of the flow path

[1] *General information about the factors described in this table is provided in Chapter 2.*

Resin properties

Chemical composition

The most commonly used SEC resins for analytical purposes are based on either silica or agarose. Agarose is obtained from natural sources and contains a very low amount of ionic and hydrophobic groups that could interact with the compound of interest. In contrast, silica-based resins have silanol groups that require coating before use in SEC.

Properties of silica-based and agarose-based resins are listed in Table 3.2.

Table 3.2. Properties of silica-based and agarose-based resins[1]

Property	Silica particles	Agarose particles
Chemical composition	SiO_2	Polysaccharide (D-galactose-3,6-Anhydro-L-galactose)
Surface coating	Silanols are blocked to minimize adsorption	Not required
pH stability	Typically, pH 2 to 8 (operational)[2]	Typically, pH 3 to 11, operational[2] pH 1 to 12, cleaning-in-place (CIP)[3]
Mechanical stability	Rigid, high pressure stability	Rigidity achieved by chemical cross-linking
Porosity	Can be controlled to achieve the desired separation range	Can be controlled to achieve the desired separation range

[1] *This information is taken from product information on vendor's web pages.*
[2] *pH range where resin can be operated without significant change in function.*
[3] *pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.*

Silica-based resins are stable below a pH of about 7 but do not tolerate pH above 8. Efficient cleaning prolongs the column lifetime and minimizes the risk of carry-over between runs allowing the same SEC column to be used for different samples. For analytical SEC columns, it may be sufficient to clean the column by regular injections of a small volume of NaOH.

Particle size and size distribution

Particle size is important for the resolution in SEC. Small particles and narrow size distribution usually give the highest resolution. Particle size ranges used for analytical SEC currently available on the market including benefits and challenges are listed in Table 3.3.

The size range of 4 to 12 µm in diameter is traditionally the standard for analytical SEC resins. However, there is a trend towards even smaller particle sizes (< 2 µm) used with ultra-high-performance liquid chromatography (UHPLC) systems for even faster separations in high-throughput mode. Columns packed with particles < 4 µm typically provide rapid runs and high resolution. However, the high pressure obtained under UHPLC conditions generates high temperatures and shear stress, which might affect sensitive proteins.

Table 3.3. Particle size ranges currently available for analytical SEC

4 to 12 µm particles (standard for analytical SEC)	**< 4 µm particles (includes particles for UHPLC)**
Benefits	*Benefits*
• Usually provides adequate resolution and run times	• Rapid runs
• Standard HPLC equipment can be used	• High resolution
• Milder conditions can be used than with UHPLC	*Challenges*
	• Very high back pressure; demands specific equipment
	• Loss of resolution due to LC system dead volumes
	• Heat generation and shear stress at high flow rates could affect proteins

Column-related factors

A well-packed column is essential for high-resolution SEC separations. Use of prepacked columns is highly recommended, especially for routine use of analytical SEC where lot-to-lot consistency is highly important. Smaller particles are more difficult to pack with good results and are predominantly supplied prepacked.

As described in Chapter 2, the height of the packed bed affects both resolution and separation time. The efficiency in SEC increases with the bed height. As high resolution is often important for analytical SEC, bed heights of 30 cm are typically used. Longer columns however also mean longer run times. There is a tradeoff between resolution and runtime that needs to be defined by the purpose of the analysis. Suitable columns for rapid purity check and screening need to provide short cycle times and are invariably shorter—typically around 15 cm in length. When using columns packed with very small particle size (< 4 µm), the resolution increases significantly allowing usage of even shorter columns, high flow velocities, and thus reduction in separation time.

Running conditions

Running conditions to be considered are sample volume and injection method, buffer composition, and flow rate. Ensure that the sample is stable and soluble at the concentration used. When developing an analytical SEC method for routine use, for example in a QC lab, it is highly recommended to use an experimental design approach, for example, a design of experiments (DoE). DoE allows several parameters to be studied simultaneously in a minimum number of experiments. Critical method parameters can be identified and their influence on accuracy and precision estimated.

In analytical SEC, the sample volume should be approximately 0.3% of the bed volume to achieve optimal results. It is also relevant to consider how the sample loop (capillary) is filled and emptied, a factor that is often overlooked. For detailed information on injection techniques, see *ÄKTA Laboratory-scale Chromatography Systems Handbook,* 29010831.

Chromatography system configuration

To maximize the performance of a high-resolution SEC column, it is crucial to optimize the configuration of the liquid chromatography (LC) system. For maximum resolution, small internal (dead) volumes are required. Use short, narrow capillaries and bypass unnecessary system components.

It is important to understand how the configuration of the chromatography system contributes to band broadening. If system volumes in the chromatography system are too large, optimization of other parameters affecting resolution will not have the intended effect.

Modifications of the system configuration might affect results. Modifications made after setting up a high-performance SEC method must be evaluated carefully.

Agarose-based resins for analytical SEC

Superdex Increase and Superose Increase resins and prepacked columns have high reproducibility, buffer stability, and lifetime stability including stability in high pH. Agarose-based resins have low nonspecific interactions, which is also important for performance. The narrow specification for particle size range and selectivity of these resins provide low batch-to-batch variation and thus high consistency and reproducibility. See Chapter 4 for further information about Superdex Increase and Superose Increase columns.

It is important to clean the column and check the performance at regular intervals (see Appendix 2). A column performance test should also be carried out before first-time use of a column to create a baseline for the column performance. It is highly recommended to set up criteria for when a column should be replaced. Suitable criteria may include changes in plate number, resolution, retention time, and peak asymmetry. Before use, column conditioning may be performed by repeated injections of a suitable sample.

High reproducibility

Results from batch-to-batch reproducibility of different resin lots of Superdex 200 Increase are shown in Figure 3.2. Six different resin lots were compared for resolution and retention volume and show minor differences in relative standard deviation (RSD); < 6% for resolution and < 10% for retention volume.

Columns:	Superdex 200 Increase 10/300 GL
Sample:	mAb5 containing monomer, dimer, and Fab fragments
Sample volume:	50 µL (0.2% V_c)
Buffer:	20 mM NaH_2PO_4, 300 mM NaCl, pH 7.4
Flow rate:	0.75 mL/min

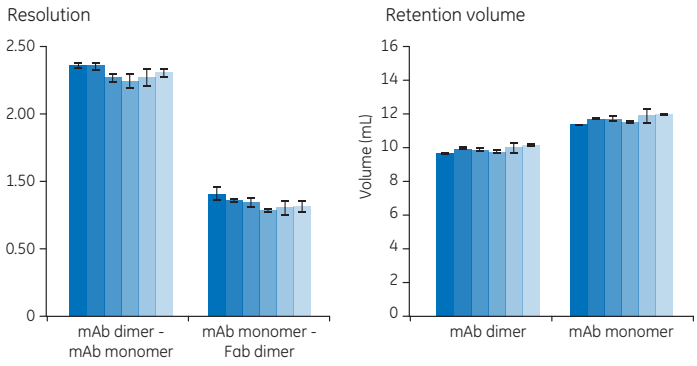


Fig 3.2. Comparison of six different Superdex 200 Increase batches of resin on resolution and retention volume in the purification of mAb, mAb aggregates, and fragments.

Lifetime stability

Reproducible results are essential in all research. The long working life and high reproducibility of Superdex Increase and Superose Increase prepacked columns are the result of optimized design, stable properties of the resin, and controlled production procedures. The high tolerance of Superdex Increase and Superose Increase columns for NaOH enables efficient cleaning, which promotes a long column lifetime. In Figure 3.3, a total of 350 injections of a sample mix consisting of low concentrations of proteins and peptides was performed on Superdex 30 Increase 10/300 GL column. Peak areas and resolution were essentially unchanged during the study.

Column:	Superdex 30 Increase 10/300 GL
Sample:	1. Cytochrome C (M_r 12 400), 0.16 mg/mL 2. Aprotinin (M_r 6500), 0.16 mg/mL 3. [Ile^7]-Angiotensin III (M_r 897), 0.08 mg/mL 4. Triglycine (M_r 189), 0.16 mg/mL 5. Glycine (M_r 75), 5.6 mg/mL
Sample volume:	50 µL
Buffer:	20 mM phosphate buffer, 280 mM NaCl, pH 7.4
Flow rate:	0.8 mL/min
System:	HPLC

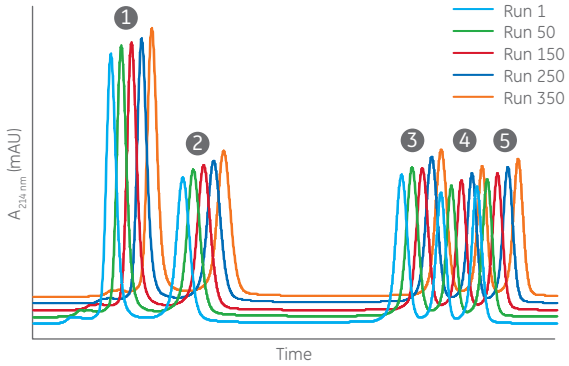


Fig 3.3. Repeated injections of a sample mix consisting of proteins and peptides on Superdex 30 Increase. Results from run 1, 50, 150, 250, and 350 are shown. Peaks of the proteins and peptides are labeled 1 to 5.

Applications

Superdex Increase and Superose Increase columns from GE have been used in several published studies for analytical SEC applications including: molecular mass determination, analysis of oligomeric state, SEC-multiangle light scattering (MALS) experiments, and complex formation studies. The columns have also been used to prepare samples for protein structure determination using cryo-EM or X-ray crystallization.

A selection of applications where Superdex and Superose Increase columns have been used are highlighted in the following examples.

Screening of mAb aggregates

Screening for mAb aggregation is important in the production of mAb. When performing aggregate analysis on mAbs there is often no issues when the aggregate level is above 1%. At lower levels, however, problems can arise during integration of the aggregate peak. Figure 3.4 shows that it is possible to determine ≥ 0.1% aggregate with 4 min run time on Superdex 200 Increase 5/150 GL. Thus, this column is a good screening tool to evaluate conditions for mAbs.

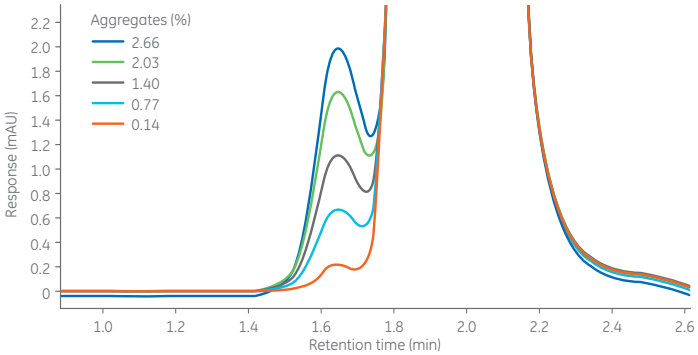


Fig 3.4. Screening of mAb aggregates on Superdex 200 Increase 5/150 GL at run times down to 4 min.

Studying aggregation and degradation over time

In structural and functional protein studies, it is essential that the prepared protein does not aggregate, oligomerize, or degrade. With analytical SEC, tendency to aggregate and other size changes can be studied under different conditions, such as in storage stability analyses of biopharmaceuticals.

In the examples in Figures 3.5 and 3.6, Superdex 75 Increase 5/150 GL was used to monitor small changes in size homogeneity of two proteins stored under different conditions and over different time periods (weeks). The HPLC system used was equipped with an autosampler to allow analysis of many samples during long, unattended, overnight SEC runs.

Column: Superdex 75 Increase 5/150 GL
Sample volume: 10 µL
Flow rate: 0.5 mL/min
System: HPLC

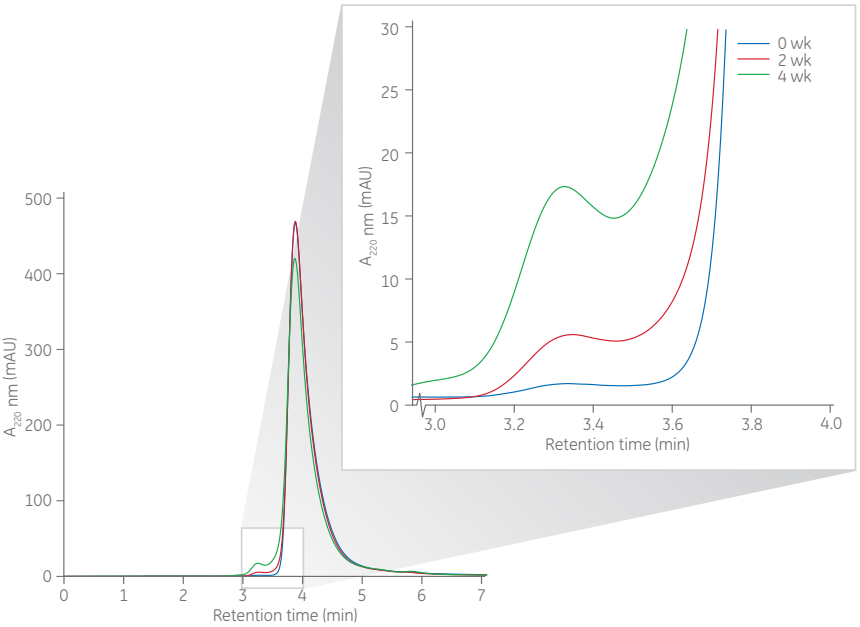


Fig 3.5. Monitoring of size-homogeneity changes of proteins during storage (0, 2, and 4 wk) using Superdex 75 Increase 5/150 GL for rapid analyses of higher molecular weight variants. The area under the curves for the much larger main peaks decreased over time and corresponded to the area increase seen for the small fronting peaks.

Column: Superdex 75 Increase 5/150 GL
Sample volume: 10 µL
Flow rate: 0.5 mL/min
System: HPLC

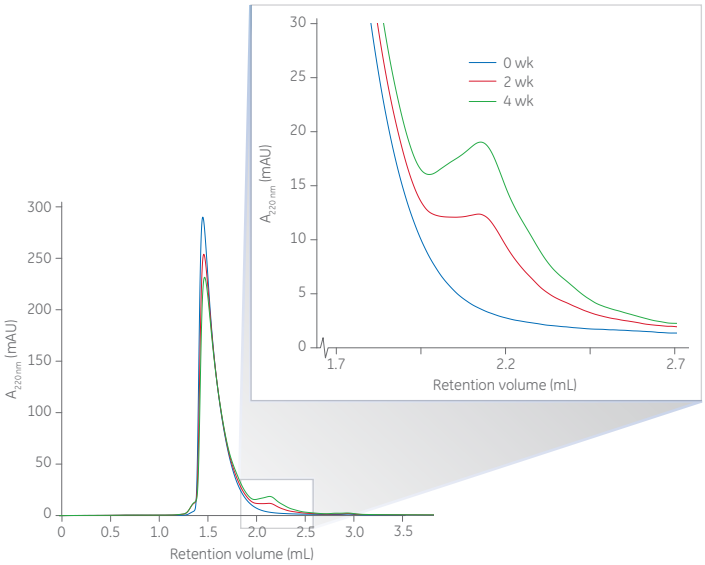


Fig 3.6. Monitoring of size-homogeneity changes of proteins during storage (0, 2, and 4 wk) using Superdex 75 Increase 5/150 GL for rapid analyses of degraded products. The area under the curves for the much larger main peaks decreased over time and corresponded to the area increase seen for the small tailing peaks.

Studying the initiation of α-synuclein aggregation by an aldehyde

Lewy bodies are abnormalities found inside nerve cells in patients with Parkinson's disease and related neurodegenerative disorders. Lewy bodies are composed of mainly aggregated forms of the protein α-synuclein. Aldehydes formed during oxidative stress are believed to be involved in the formation of Lewy bodies. Superose 6 Increase 3.2/300 was used for analysis of oligomerized α-synuclein for further *in vitro* and *in vivo* studies (Fig 3.7).

Column: Superose 6 Increase 3.2/300
Sample volume: 10 µL
Flow rate: 0.05 mL/min
System: HPLC

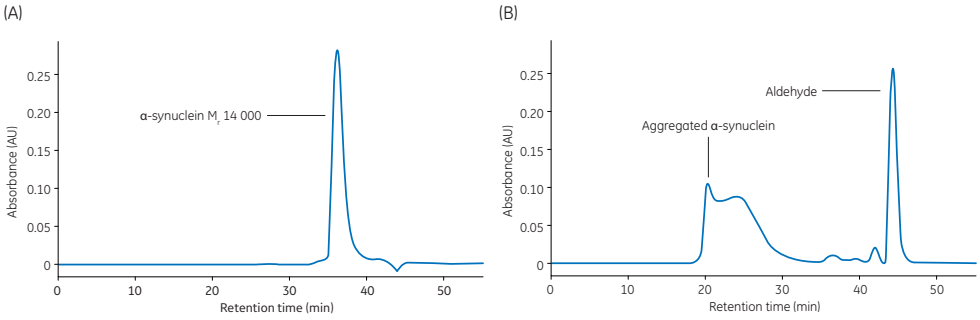


Fig 3.7. Superose 6 Increase 3.2/300 was used for characterizations during the preparation of oligomerized α-synuclein for further *in vitro* and *in vivo* studies. In chromatogram (A) a monomeric form of α-synuclein is seen. In chromatogram (B), no monomers of the protein remain after incubation with aldehydes; only oligomerized α-synuclein is present. Data courtesy of Dr. Joakim Bergstrom, Rudbeck Laboratory, Uppsala University.

Purity of a peptide: different lots of [Ile^7]-Angiotensin III

Peptides are attracting increasing interest as therapeutics since they are considered potent and specific, but also relatively safe and easy to manufacture. Characterization of different types of impurities is important not only for release of a drug, but also for initial functionality studies in early drug discovery phases. Analytical SEC offers quantitative assessment of aggregates, multimers, and other size homogeneities of the active peptide under native conditions.

Figure 3.8 illustrates the separation of a peptide from two different lots using Superdex 30 Increase 10/300 GL. The amount of main impurity (peak 1) was 1.2% of the total peak area for lot A and 0.1% for lot B. Total impurity was 3.2% and 0.7% for lots A and B, respectively. Additional mass spectrometry analysis showed that the main impurity (peak 1) contained peptide with a mass suggesting acetylation of an amino acid and that impurity in peak 2 contained peptide with a mass suggesting a missing amino acid in the sequence.

Column:	Superdex 30 Increase 10/300 GL
Sample:	Different lots of [Ile^7]-Angiotensin III (M_r 897)
Sample volume:	100 µL
Buffer:	20 mM phosphate buffer, 280 mM NaCl, pH 7.4
Flow rate:	0.5 mL/min
System:	HPLC

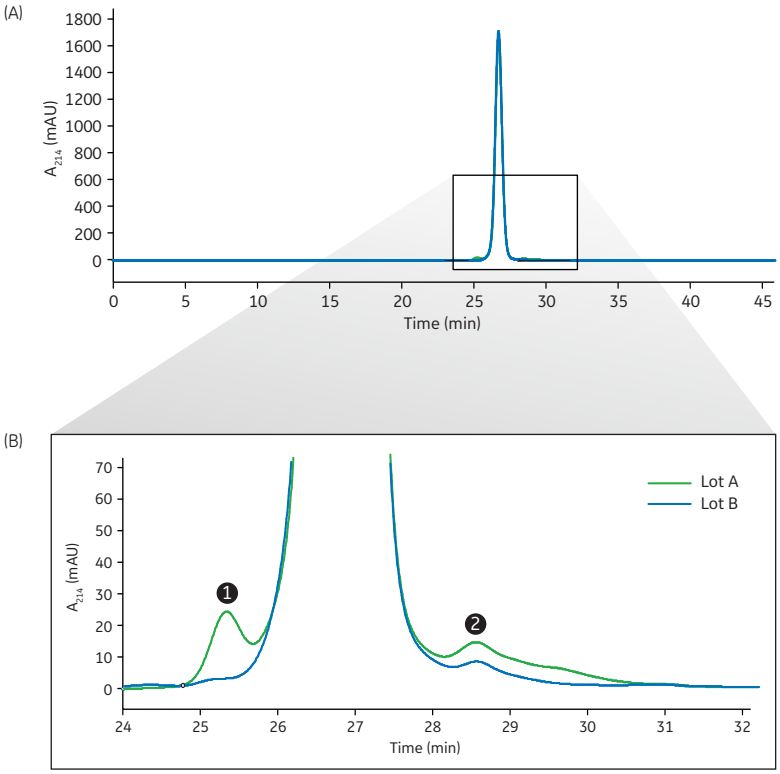


Fig 3.8. (A) Separation of two different lots of [Ile^7]-Angiotensin III on Superdex 30 Increase. (B) Magnified chromatogram showing impurity profile.

Chapter 4
Superdex, Superose, and Sephacryl resins: high-resolution SEC

SEC resins from GE cover a broad molecular weight range offering separation of a very wide range of biomolecules.

When choosing a resin, consider three main factors:

1. The aim of the experiment (analytical SEC, preparative SEC or buffer exchange).
2. The molecular weights of the target proteins and contaminants to be separated.
3. The final scale of the purification.

An overview of the SEC resins available from GE is shown in Figure 4.1. Almost all resins are available in prepacked columns. If you are not very experienced in column packing, prepacked columns are recommended. Further information about buffer exchange and Sephadex resins is found in Chapter 5.

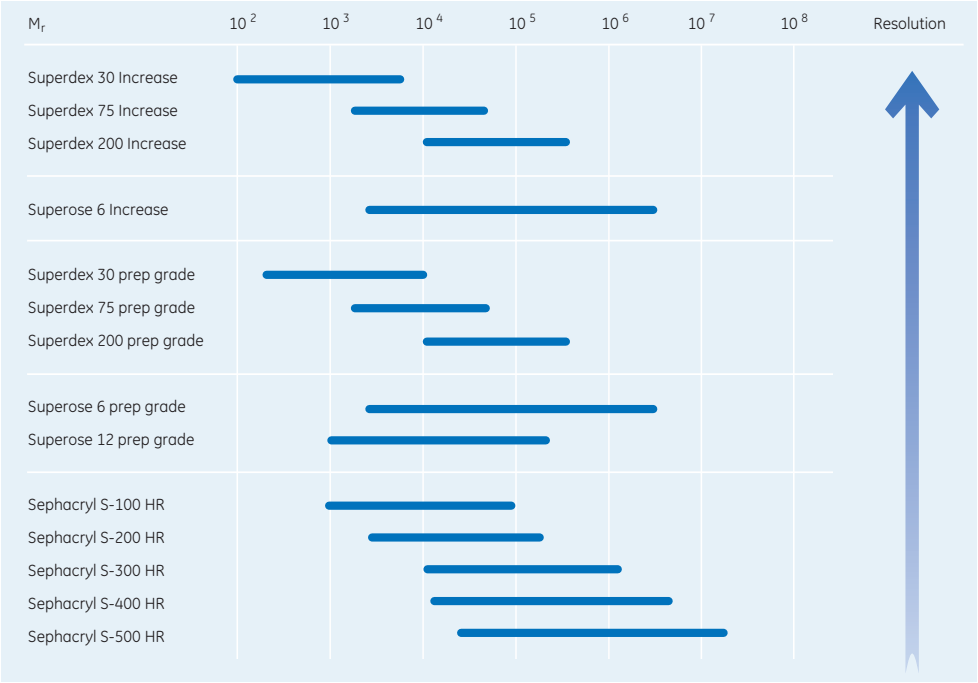


Fig 4.1. SEC resin fractionation ranges (globular proteins). Sephacryl S-500 HR fractionation range is for dextrans.

Superdex Increase and Superose Increase are designed for high resolution, short run times, and high recovery.

Superdex prep grade, Superose prep grade, and Sephacryl are suitable for fast, high-recovery separations at laboratory and industrial scale.

Superdex, Superose, and Sephacryl are available with a wide variety of fractionation ranges. In cases when two resins have similar fractionation ranges, select the resin with the steepest selectivity curve for optimal resolution of all the sample components (see Chapter 1 for definition of selectivity curve and later in this section for further information about each resin). For a specific component of interest, select the resin where the log of molecular weight for the target component falls in the middle of the selectivity curve.

Superdex resins

Superdex resins consist of a composite base matrix of dextran and agarose (Fig 4.2). This matrix combines the excellent SEC properties of dextran with the physical and chemical stability of cross-linked agarose, resulting in a resin with outstanding selectivity and high resolution. In addition, low nonspecific interactions permit high recovery of biological material. Together, these properties make Superdex an excellent choice for all applications from laboratory to process scale.

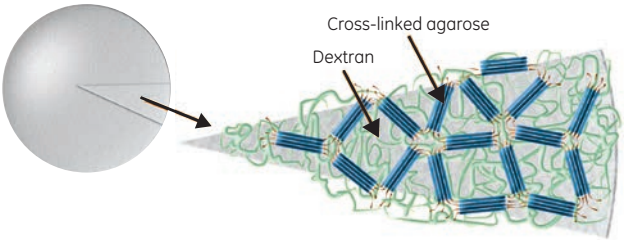


Fig 4.2. A schematic section of a Superdex particle. In Superdex, the dextran chains are covalently linked to a highly cross-linked agarose matrix.

Two types of Superdex resins are available: Superdex Increase, which replaces the previously available Superdex resin, and Superdex prep grade.

Figure 4.3 shows the fractionation ranges for all Superdex resins available. The fractionation ranges of the discontinued Superdex resins were equivalent to those of the Superdex Increase resins.

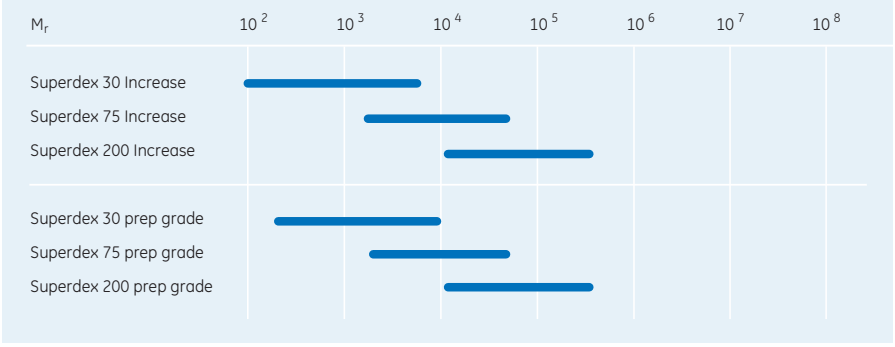


Fig 4.3. Fractionation ranges (globular proteins) for Superdex resins.

The high rigidity of Superdex resins allows even viscous eluents, such as 8 M urea, to be run at relatively high flow rates. Superdex is chemically stable in commonly used aqueous buffers and additives such as detergents, denaturing agents (8 M urea or 6 M guanidine hydrochloride), and 30% acetonitrile.

Superdex can be cleaned using up to 1 M NaOH. Cleaning with NaOH is highly efficient, prolongs column life, and minimizes the risk for carry-over between runs.

A packed column should be used at the temperature range 4°C to 40°C. Further characteristics of Superdex are found in Table 4.1 and in the instructions for each product.

Table 4.1. Superdex resin characteristics

Product	Efficiency[1]	Operational[2]	Cleaning-in-place (CIP)[3]	Particle size, d_{50v} (µm)[4]
Superdex 30 Increase	> 43 000	3 to 12	1 to 14	~ 9
Superdex 75 Increase	> 43 000	3 to 12	1 to 14	~ 9
Superdex 200 Increase	> 48 000	3 to 12	1 to 14	~ 8.6
Superdex 30 prep grade	≥ 13 000	3 to 12	1 to 14	~ 34
Superdex 75 prep grade	≥ 13 000	3 to 12	1 to 14	~ 34
Superdex 200 prep grade	≥ 13 000	3 to 12	1 to 14	~ 34

[1] *Theoretical plates per meter (prepacked columns only).*
[2] *pH range where resin can be operated without significant change in function.*
[3] *pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.*
[4] *Median particle size of the cumulative volume distribution.*

Superdex

The legacy Superdex resin from GE has a particle size of ~ 13 µm and three different fractionation ranges: Superdex Peptide (M_r 100 to 7000), Superdex 75 (M_r 3000 to 70 000), and Superdex 200 (M_r 10 000 to 600 000). These products have been replaced by Superdex Increase.

Superdex Increase

The next generation Superdex resin, Superdex Increase, offers improved performance providing high resolution and shorter run times. This is achieved by using a high-flow agarose base matrix with higher pressure-flow tolerance, smaller particle size (~ 9 µm), and narrower particle size distribution. The design enables high-resolution separations in short run times, resulting in excellent analytical results and high final purity.

Superdex Increase resins cover a molecular weight range from peptides to larger biomolecules (M_r 100 to 6×10^5, for globular proteins). Three resins with different fractionation ranges are available; Superdex 30 Increase, Superdex 75 Increase, and Superdex 200 Increase. Superdex 30 Increase has a fractionation range from M_r 100 to 7000 allowing separation of peptides and other small biomolecules. Superdex 75 Increase has a fractionation range that allows separation of proteins ranging from M_r 3000 to 70 000. Superdex 200 Increase has a fractionation range that allows separation of proteins from M_r 10 000 to 600 000 with an optimized resolution for the antibody molecular weight range of M_r 100 000 to 300 000 (see Fig 4.4 and 4.5).

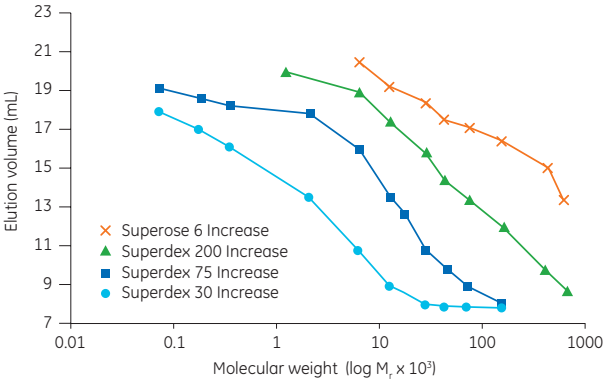
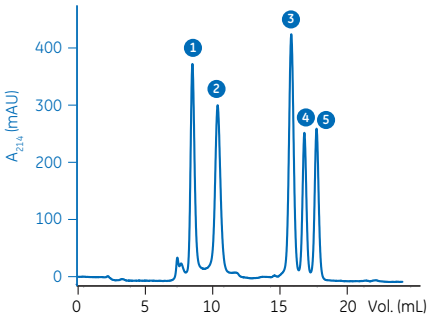


Fig 4.4. Fractionation range (globular proteins and peptides) for Superdex 30 Increase 10/300 GL, Superdex 75 Increase 10/300 GL, Superdex 200 Increase 10/300 GL, and Superose 6 Increase 10/300 GL. Note that the whole fractionation range of Superose 6 Increase is not covered.

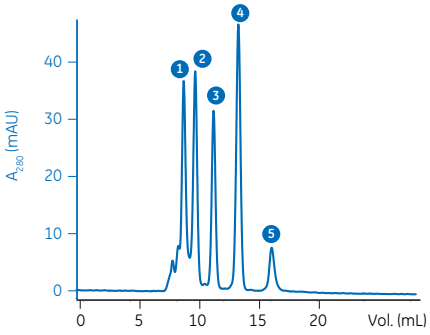
(A)

Column:	Superdex 30 Increase 10/300 GL
Sample:	1. Cytochrome C (M$_r$ 12 400) 0.2 mg/mL 2. Aprotinin (M$_r$ 6500) 0.2 mg/mL 3. Vitamin B$_{12}$ (M$_r$ 1355) 0.07 mg/mL 4. Triglycine (M$_r$ 189) 0.2 mg/mL 5. Glycine (M$_r$ 75) 7 mg/mL
Sample volume:	100 µL
Eluent:	2× PBS (20 mM phosphate buffer, 280 mM NaCl, 6 mM KCl, pH 7.4)
Flow rate:	0.8 mL/min, room temperature
System:	ÄKTA pure 25



(B)

Column:	Superdex 75 Increase 10/300 GL
Sample:	1. Conalbumin (M$_r$ 75 000) 1.5 mg/mL 2. Ovalbumin (M$_r$ 44 000) 4 mg/mL 3. Carbonic anhydrase (M$_r$ 29 000) 1.5 mg/mL 4. Ribonuclease A (M$_r$ 13 700) 3 mg/mL 5. Aprotinin (M$_r$ 6500) 1 mg/mL
Sample volume:	100 µL
Eluent:	PBS (10 mM phosphate buffer, 140 mM NaCl, 3 mM KCl, pH 7.4)
Flow rate:	0.8 mL/min, room temperature
System:	ÄKTA pure 25



(B)

Column:	Superdex 200 Increase 10/300 GL
Sample:	1. Thyroglobulin (M$_r$ 669 000) 3 mg/mL 2. Ferritin (M$_r$ 440 000) 0.3 mg/mL 3. Aldolase (M$_r$ 158 000) 3 mg/mL 4. Conalbumin (M$_r$ 75 000) 3 mg/mL 5. Ovalbumin (M$_r$ 44 000) 3 mg/mL 6. Carbonic anhydrase (M$_r$ 29 000) 3 mg/mL 7. Ribonuclease A (M$_r$ 13 700) 3 mg/mL
Sample volume:	100 µL
Eluent:	10 mM phosphate buffer, 140 mM NaCl, pH 7.4
Flow rate:	0.75 mL/min, room temperature

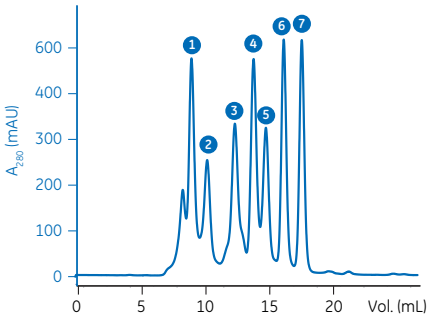


Fig 4.5. Typical chromatograms showing high-resolution separations of biomolecules on (A) Superdex 30 Increase 10/300 GL, (B) Superdex 75 Increase 10/300 GL, and (C) Superdex 200 Increase 10/300 GL.

Superdex prep grade

Superdex prep grade has a particle size of ~ 34 µm and is excellent for preparative purification and scale-up purposes. The high stability makes Superdex prep grade suitable for use in larger scale where high flow rates and fast, effective cleaning-in-place (CIP) protocols are required. Since these resins can withstand high flow rates during equilibration and cleaning, the overall cycle time can be kept short.

Superdex prep grade is available as Superdex 30 prep grade, Superdex 75 prep grade, and Superdex 200 prep grade for different fractionation ranges (Fig 4.3). Selectivity curves for the Superdex prep grade resins are shown in Figure 4.6.

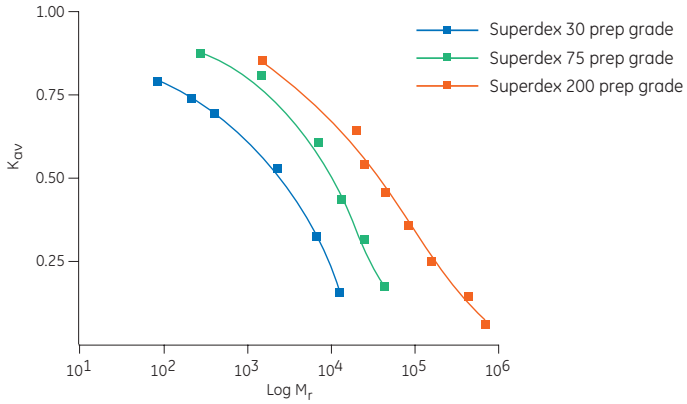


Fig 4.6. Selectivity curves for the Superdex prep grade resins.

A comparison of the selectivity of Superdex 200 prep grade and Superdex 75 prep grade using standard proteins is shown in Figure 4.7.

Columns:	(A) HiLoad 16/600 Superdex 75 prep grade (B) HiLoad 16/600 Superdex 200 prep grade
Sample:	1. Ferritin (M_r 440 000), 0.24 mg/mL 2. IgG (M_r 158 000), 0.2 mg/mL 3. Albumin (M_r 67 000), 5 mg/mL 4. Ovalbumin (M_r 43 000), 4 mg/mL 5. Myoglobin (M_r 17 000), 1.5 mg/mL
Sample load:	500 µL
Buffer:	50 mM phosphate buffer, 150 mM sodium chloride, 0.01% sodium azide, pH 7.0
Flow rate (flow velocity):	1.5 mL/min (45 cm/h)

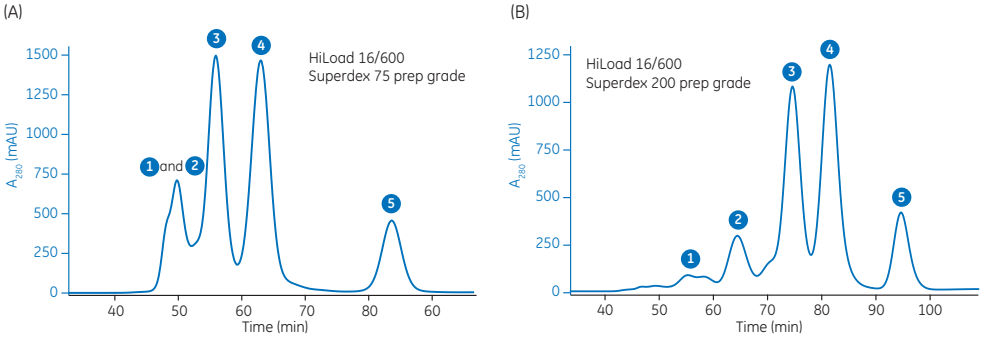


Fig 4.7. Comparison of the selectivity of Superdex 75 prep grade and Superdex 200 prep grade for standard proteins. Superdex 75 prep grade (A) gives excellent resolution of the three proteins in the molecular weight range of 17 000 to 67 000 while the two largest proteins (ferritin and IgG, peaks 1 and 2) elute together in the void volume (V_0). These peaks are completely resolved using Superdex 200 prep grade (B). Ferritin peak (peak 1) contains aggregates, which accounts for the observed double peak.

Product selection

Superdex is available in two different particle sizes and for three different fractionation ranges (see Table 4.2).

Superdex 200 Increase or Superdex 200 prep grade resins are especially suitable for the separation of antibody monomers from dimers and from contaminants of lower molecular weight, for example albumin and transferrin.

Superdex 30 Increase or Superdex 30 prep grade are suitable for separations of peptides, oligonucleotides and small proteins below M_r 10 000.

Superdex Increase resins are available in prepacked high-performance glass columns in three different sizes, offering versatility for different applications. Superdex Increase 10/300 GL is designed for high-resolution small-scale preparative purification and characterization. Superdex Increase 5/150 GL is the first choice for rapid screening, purity checks and for applications where buffer consumption needs to be low. Superdex Increase prepacked in 3.2/300 columns is an excellent choice when working with very small sample volumes where high sensitivity is crucial, and for high resolution microscale separations. Superdex prep grade is available as bulk resin and packed in two different column formats suitable for high-resolution preparative SEC; HiLoad 16/600 and HiLoad 26/600.

Table 4.2. Separation options with Superdex resins

Product	Fractionation range, M_r (globular proteins)	Sample volume capacity	Typical pressure drop over the packed bed (MPa)*	Recommended operating flow rate†
Superdex 30 Increase 10/300 GL	1×10^2 to 7×10^3	25 to 500 µL	3.0‡	0.8 mL/min
Superdex 30 Increase 3.2/300	1×10^2 to 7×10^3	4 to 50 µL	2.0‡	0.075 mL/min
Superdex 75 Increase 10/300 GL	3×10^3 to 7×10^4	25 to 500 µL	3.0‡	0.8 mL/min
Superdex 75 Increase 5/150 GL	3×10^3 to 7×10^4	4 to 50 µL	3.0‡	0.45 mL/min
Superdex 75 Increase 3.2/300	3×10^3 to 7×10^4	4 to 50 µL	2.0‡	0.75 mL/min
Superdex 200 Increase 10/300 GL	1×10^4 to 6×10^5	25 to 500 µL	3.0‡	0.75 mL/min
Superdex 200 Increase 5/150 GL	1×10^4 to 6×10^5	4 to 50 µL	3.0‡	0.45 mL/min
Superdex 200 Increase 3.2/300	1×10^4 to 6×10^5	4 to 50 µL	2.0‡	0.075 mL/min
HiLoad 16/600 Superdex 30 pg	$< 1 \times 10^4$	≤ 5 mL	0.3¶	1.0 mL/min
HiLoad 26/600 Superdex 30 pg	$< 1 \times 10^4$	≤ 13 mL	0.3¶	2.6 mL/min
HiLoad 16/600 Superdex 75 pg	3×10^3 to 7×10^4	≤ 5 mL	0.3¶	1.0 mL/min
HiLoad 26/600 Superdex 75 pg	3×10^3 to 7×10^4	≤ 13 mL	0.3¶	2.6 mL/min
HiLoad 16/600 Superdex 200 pg	1×10^4 to 6×10^5	≤ 5 mL	0.3¶	1.0 mL/min
HiLoad 26/600 Superdex 200 pg	1×10^4 to 6×10^5	≤ 13 mL	0.3¶	2.6 mL/min
Superdex 30 prep grade (bulk resin)§	$< 1 \times 10^4$	0.5% to 4% of total column volume	Column-dependent	10 to 50 cm/h
Superdex 75 prep grade (bulk resin)§	3×10^3 to 7×10^4	0.5% to 4% of total column volume	Column-dependent	10 to 50 cm/h
Superdex 200 prep grade (bulk resin)§	3×10^3 to 6×10^5	0.5% to 4% of total column volume	Column-dependent	10 to 50 cm/h

* *See Appendix 4 to convert between different pressure units.*

† *Do not exceed maximum flow rate limits. Note that the maximum flow rate will depend on the temperature and the viscosity of the solution. For further information, see Appendix 4.*

‡ *The actual value is individual for each column and needs to be determined (see Appendix 4 Column pressure limits and maximum flow rate).*

§ *Recommended operating flow velocity measured in a 20 cm diameter column with 83 cm bed height at room temperature using buffers with the same viscosity as water.*

¶ *For HiLoad columns, the values given correspond to max. pressure on the column*

Use columns prepacked with Superdex 30 Increase, Superdex 75 Increase, and Superdex 200 Increase for small-scale preparative purification and analytical runs with sample volumes up to 0.5 mL.

Use columns prepacked with Superdex 30 prep grade, Superdex 75 prep grade, or Superdex 200 prep grade resins for preparative purification with larger sample volumes, up to 13 mL.

Performing a separation

Buffer: 10 to 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0 to 7.4, or select the buffer in which the sample should be stored or solubilized for the next step.

Under normal conditions, nonspecific interactions are low when using buffers with ionic strengths in the range 0.15 M to 1.5 M. Some hydrophobic interactions have been noted, particularly for compounds such as smaller hydrophobic and/or aromatic peptides, membrane proteins, and/or lipoproteins, which can elute later than predicted. However, in some applications, these interactions can be an advantage for increasing the resolution of the separation.

The sample should be fully dissolved. Centrifuge or filter to remove particulate matter. Always use degassed buffers and maintain a constant temperature during the run to avoid introducing air into the column.

Ensure that the maximum flow rate and pressure limits of the column are not exceeded and that an appropriate pressure limit is set on the chromatography system. This is particularly important when working at low temperatures, such as in a cold room, or when the column is used with 20% ethanol or other viscous solutions. Use lower flow rates for high viscosity solutions and at low temperature (see Appendix 4).

Purification protocol

1. Remove the storage solution by applying 2 CV of distilled water at room temperature. Use a low flow rate to avoid gap formation (see Table 4.3 and Appendix 4).
2. For first-time use: Perform a column performance test (see Appendix 2) and determine the limit for maximum pressure over the packed bed if recommended (see Table 4.2).
3. Equilibrate with 2 CV of buffer at recommended flow rate (see Table 4.2).
4. Apply a sample volume equivalent to between 0.3% and 4% of the bed volume. For most applications, the sample volume should not exceed 2% to achieve high resolution. Smaller sample volumes generally lead to improved resolution.
5. Elute with 1 CV of buffer.
6. Before applying a new sample, re-equilibrate column with buffer until the baseline of the monitored signal is stable. Proteins can be monitored at 280 nm.

Note: To detect any column changes, the column performance should be checked at regular intervals (see Appendix 2).

Table 4.3. Recommended flow rates (room temperature) for removal of storage solution with 20% ethanol , and during cleaning-in-place using Superdex Increase or HiLoad Superdex prep grade prepacked columns

	Recommended flow rates (mL/min)				
	3.2/300	**5/150 GL**	**10/300 GL**	**HiLoad 16/600**	**HiLoad 26/600**
First-time use or after long-term storage (all resins except Superdex 200 Increase)	0.04	0.25	0.5	1 (30 cm/h)	2.6 (30 cm/h)
First-time use or after long-term storage (Superdex 200 Increase)	0.075	0.3	0.75	N/A	N/A
Cleaning-in-place, CIP	0.02	0.13	0.5	0.8 (25 cm/h)	2.2 (25 cm/h)

For further information about performing a separation and for optimization, see Chapter 2 *Size exclusion chromatography in practice* and the product instructions.

Cleaning

Routine cleaning of the SEC column should be done to prolong column lifetime. The frequency of cleaning depends mainly on the sample, but once every 20 runs is a guideline.

1. Wash with 1 CV of 0.5 M sodium hydroxide, alternatively 0.5 M acetic acid. Use a low flow rate during the entire CIP procedure (see Table 4.3).
2. Immediately wash with 1 CV of distilled water followed by at least 2 CV of buffer or until the baseline of the monitored signal and the pH of the eluent are stable. Further equilibration might be necessary if the buffer contains detergent.

Note: For other cleaning solutions and for extreme cases of contamination, check the instructions supplied with each product.

In special cases, it might be necessary to change the top filter or to remove and discard the top 2 to 3 mm of the resin. These operations must be done with extreme care to avoid serious loss of resolution. Note that this is not possible with the 3.2/300 column as it should not be opened.

Superdex prep grade may be autoclaved at 121°C for 20 min (1 cycle) without significantly affecting its chromatographic properties. The resin should be removed from the column prior to autoclaving.

Storage

Store bulk resin at 4°C to 30°C in 200 mM sodium acetate, 20% ethanol (Superdex 30 prep grade and Superdex 75 prep grade), or 20% ethanol (all other resins). Do not freeze.

For long-term storage of columns, wash with 2 CV of distilled water followed by 2 CV of 20% ethanol (or 200 mM sodium acetate, 20% ethanol for Superdex 30 prep grade and Superdex 75 prep grade) at low flow rate. Connect a storage/shipping device to prevent air from entering the column during storage. Store the column at 4°C to 30°C. Avoid changes in temperature, which can cause formation of air bubbles in the packed bed.

Applications

Figures 4.8 to 4.10 show applications on columns packed with Superdex Increase and Superdex prep grade resins.

Analysis of size distribution of a protein hydrolysate

Analysis of the size distribution is important for the characterization of a protein hydrolysate. The Superdex Increase resins offer the possibility to separate proteins and peptides according to size under native conditions and are hence well suited for this purpose. Figure 4.8 shows separations of hydrolyzed proteins from a lentil extract on Superdex 30 Increase, Superdex 75 Increase, and Superdex 200 Increase columns. The hydrolysate was most efficiently separated on Superdex 30 Increase with fraction range for the smallest molecules (M_r 100 to 7000). Superdex 75 Increase and Superdex 200 Increase columns are more suitable for separation of larger molecules such as the proteins in the lentil extract.

Columns:	Superdex 30 Increase 10/300 GL Superdex 75 Increase 10/300 GL Superdex 200 Increase 10/300 GL
Sample:	Tryptic hydrolysate from lentils
Sample volume:	50 µL
Buffer:	20 mM phosphate buffer, 280 mM NaCl, pH 7.4
Flow rate:	0.75 mL/min
System:	HPLC

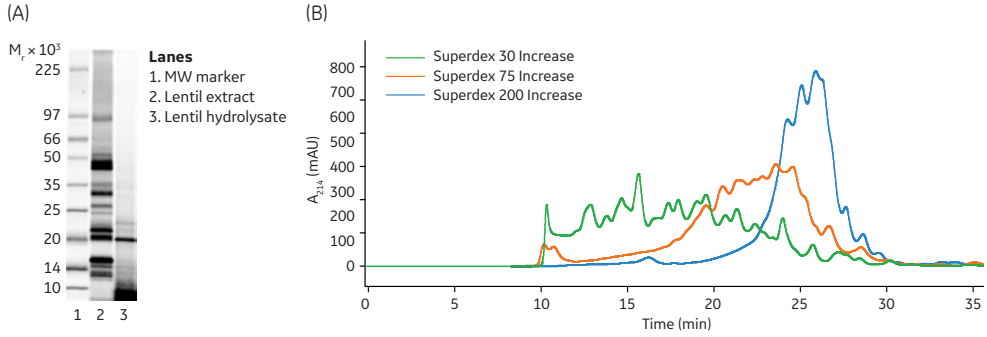


Fig 4.8. (A) SDS-PAGE analysis of lentil extract (lane 2) and lentil hydrolysate (lane 3). (B) Separation of a lentil hydrolysate sample on Superdex 30 Increase, Superdex 75 Increase, and Superdex 200 Increase columns

Rapid screening of detergents for crystallization

For evaluation of detergents for further crystallization of a membrane protein, a rapid screening of maltoside detergents of variable acyl chain length—n-Dodecyl-β-maltoside (DDM), n-Undecyl-β-maltoside (UDM), n-Decyl-β-maltoside (DM), and n-Nonyl-β-maltoside (NM)—was performed on Superdex 200 Increase 5/150 GL. Purified recombinant membrane protein CE07 was run on Superdex 200 Increase 5/150 GL, equilibrated in each respective detergent. The run time was approximately 6 min each and the results are shown in Figure 4.9.

Column: Superdex 200 Increase 5/150 GL
Sample: Recombinant integral membrane protein CE07
Sample volume: 25 µL
Buffer: 20 mM HEPES, 150 mM sodium chloride, 5% glycerol, 0.5 mM TCEP + maltoside detergent, pH 7.5
Detergent: 0.03% DDM, 0.2% UDM, 0.01% DM, or 0.3% NM
Flow rate: 0.5 mL/min
System: ÄKTAmicro

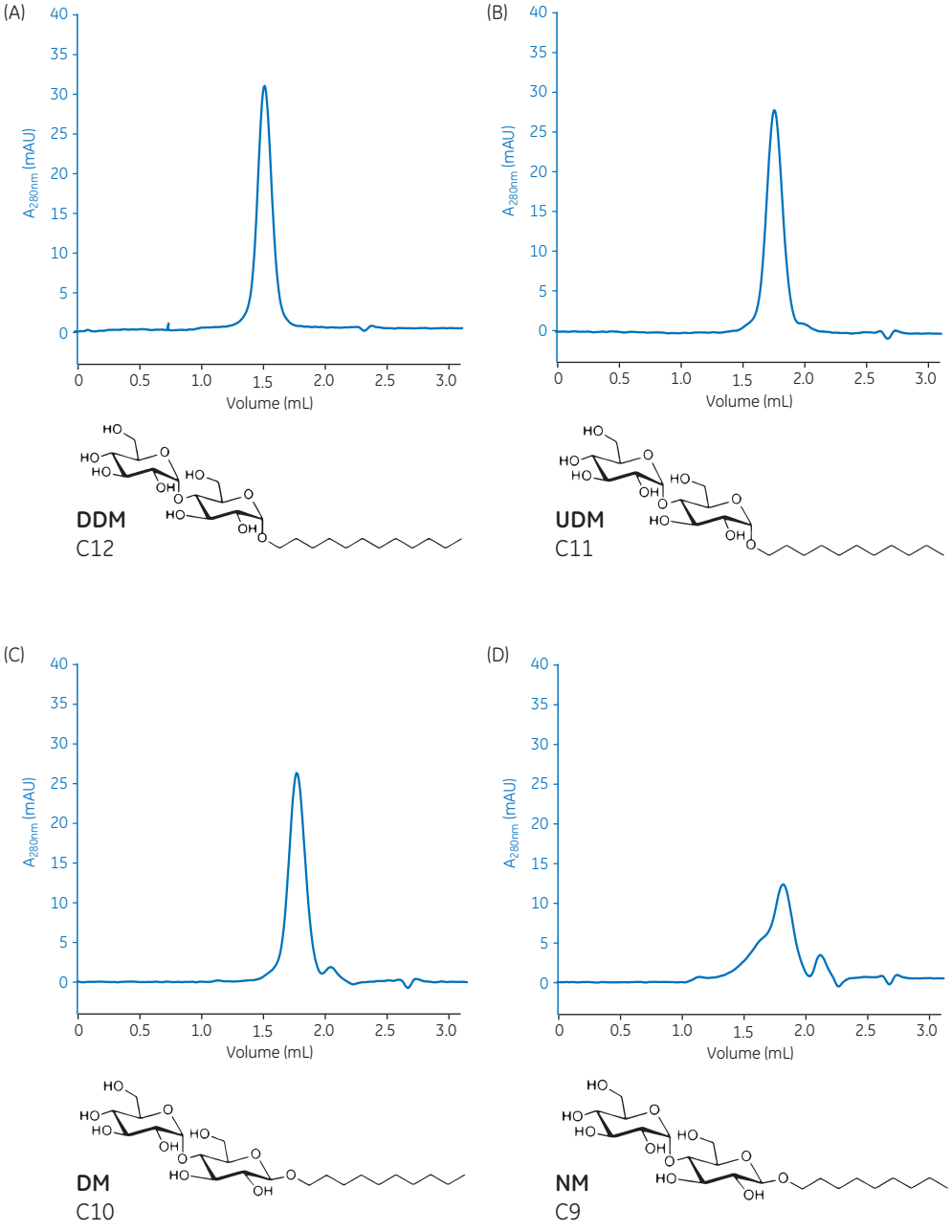


Fig 4.9. Rapid screening of detergents using Superdex 200 Increase 5/150 GL. Purified recombinant membrane protein CE07 was run in a column equilibrated with (A) DDM, (B) UDM, (C) DM, and (D) NM. The study was perfomed in collaboration with Dr. Per Moberg at the Karolinska Institute, Stockholm, Sweden.

Preparative purification of IGF-1

Figure 4.10 shows the separation of IGF-1 (M_r 7600) from its ZZ fusion protein partner (M_r 14 500) and uncleaved material on HiLoad 16/600 Superdex 75 pg. This column is an excellent choice since the resin separates proteins and peptides in the molecular weight range M_r 3000 to 70 000 and performs best between M_r 8000 and 50 000.

Column:	HiLoad 16/600 Superdex 75 pg
Sample:	IGF-1, ZZ fusion protein, and uncleaved material
Buffer:	0.15 ammonium acetate, pH 6.0
Flow rate (flow velocity):	0.75 mL/min (22.5 cm/h)

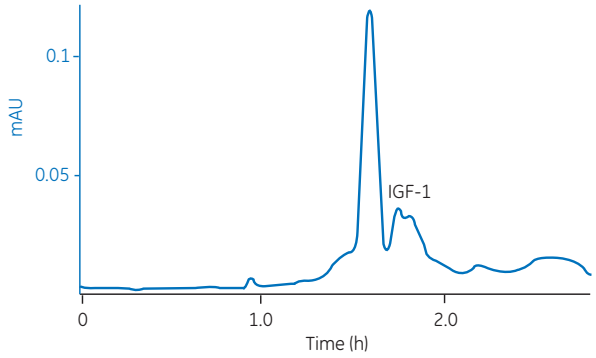


Fig 4.10. Separation of recombinant IGF-1 (M_r 7600) from its ZZ fusion protein partner (M_r 14 500) and uncleaved material.

Superose resins

Superose resins are based on cross-linked porous agarose particles. The resins have broad fractionation ranges and high physical and chemical stability. Under normal chromatographic conditions, nonspecific interactions between proteins and Superose are low.

Two types of Superose resins are available: Superose Increase (particle size ~ 8.6 µm) and Superose prep grade (particle size ~30 µm), see Table 4.4. Superose Increase replaces the legacy Superose resin previously available from GE.

Figure 4.11 shows the fractionation ranges for all Superose resins available.

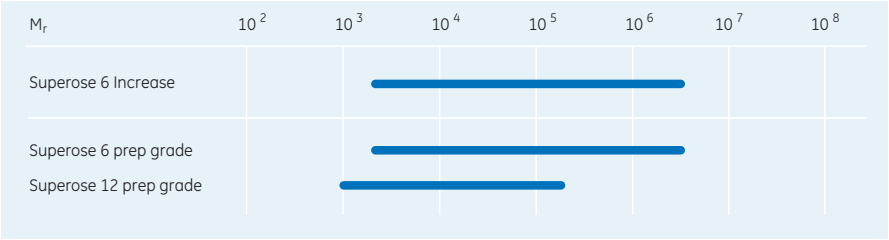


Fig 4.11 Fractionation ranges of Superose resins.

The high rigidity of Superose allows even viscous eluents, such as 8 M urea, to be run at relatively high flow rates. Superose is chemically stable in commonly used aqueous buffers and additives such as detergents, denaturing agents (8 M urea or 6 M guanidine hydrochloride), and 30% acetonitrile. Superose is agarose based and may be cleaned using up to 1 M sodium hydroxide. Cleaning with sodium hydroxide is highly efficient, prolongs column life and minimizes the risk for carry-over between runs. A packed column should be used at the temperature range 4°C to 40°C. Further characteristics of the resins are found in Table 4.4 and in the instructions for each product.

Table 4.4. Superose resin characteristics

Product	Efficiency[1]	pH stability		Particle size, d_{50v} (µm)[4]
		Operational[2]	Cleaning-in-place (CIP)[3]	
Superose 6 Increase	> 48 000	3 to 12	1 to 14	~ 8.6
Superose 6 prep grade	> 10 000	3 to 12	1 to 14	~ 30
Superose 12 prep grade	> 10 000	3 to 12	1 to 14	~ 30

[1] *Theoretical plates per meter (prepacked columns only).*
[2] *pH range where resin can be operated without significant change in function.*
[3] *pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.*
[4] *Median particle size of the cumulative volume distribution.*

Superose

Superose 6 has a fractionation range between 5000 and 5 000 000 (globular proteins) and a particle size of ~ 13 µm. This product has been replaced by Superose 6 Increase, which has the same fractionation range (see Fig 4.11).

Superose 12 has a fractionation range between 1000 and 300 000 (globular proteins) and a particle size of ~ 11 µm. Superose 12 will not be replaced by a Superose Increase resin with the same fractionation range. The most suitable replacement resin will thus depend on the size of the molecules to be separated.

To select a replacement resin for Superose 12, see Table 4.6 and Figure 4.16.

Superose Increase

Superose 6 Increase is the next generation Superose resin with improved performance providing higher resolution and increased flow rates. This is achieved by using a high-flow agarose base matrix, which has higher pressure-flow tolerance, smaller particle size (~ 8.6 µm), and narrower particle size distribution. The design enables high-resolution separations in short run times, resulting in excellent analytical results and high final purity.

Superose 6 Increase has a broad fractionation range for molecular weights between 5000 and 5 000 000 (globular proteins), and is especially valuable for its ability to separate large biomolecules, membrane proteins, and protein complexes. The selectivity curve for Superose 6 Increase is shown in Figure 4.12.

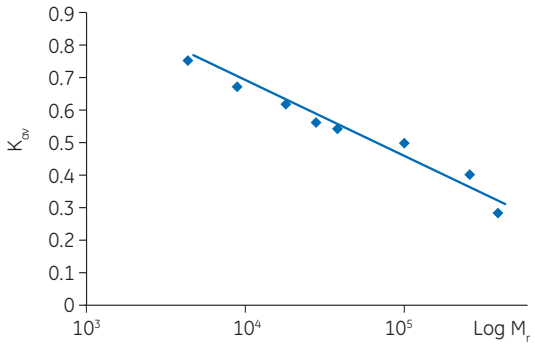


Fig 4.12. Superose 6 Increase selectivity curve for globular proteins.

Superose 6 Increase has higher resolution capabilities compared with the legacy Superose 6 resin. The improved resolution on Superose 6 Increase can be attributed to the reduced particle size of Superose 6 Increase resin (~ 8.6 µm) compared with the larger particles of Superose 6 (~ 13 µm) as well as the narrower particle size distribution. The higher rigidity of the chromatography resin and thereby enhanced flow properties enable faster runs on Superose 6 Increase.

A typical chromatogram showing high-resolution separation of a protein mix on Superose 6 Increase 10/300 GL is shown in Figure 4.13.

Column:	Superose 6 Increase 10/300 GL
Sample:	1. Thyroglobulin (M_r 669 000) 3 mg/mL
	2. Ferritin (M_r 440 000) 0.3 mg/mL
	3. Aldolase (M_r 158 000) 3 mg/mL
	4. Ovalbumin (M_r 44 000) 3 mg/mL
	5. Ribonuclease A (M_r 13 700) 3 mg/mL
	6. Aprotinin (M_r 6500) 1 mg/mL
Sample volume:	100 µL
Eluent:	10 mM phosphate buffer, 140 mM NaCl, pH 7.4
Flow rate:	0.5 mL/min, room temperature

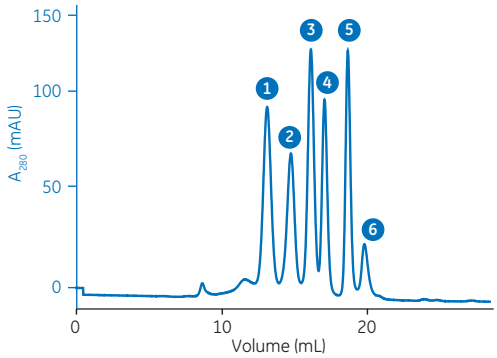


Fig 4.13. Chromatogram from a function test of Superose 6 Increase 10/300 GL showing high-resolution separation of a protein mix.

The excellent reproducibility of Superose 6 Increase over the duration of 200 injections of IgG_{2b} and 200 cleaning-in-place (CIP) cycles can be seen in Figure 4.14.

Column: Superose 6 Increase 10/300 GL
Sample: 1. Thyroglobulin (M_r 669 000), 2 mg/mL
2. Aldolase (M_r 158 000), 2 mg/mL
3. Ovalbumin (M_r 44 000), 2 mg/mL
4. Ribonuclease A (M_r 13 700), 0.6 mg/mL
Sample volume: 50 µL
Buffer: PBS (10 mM phosphate buffer, 140 mM sodium chloride, pH 7.4)
Flow rate: 0.5 mL/min
System: ÄKTAexplorer 10

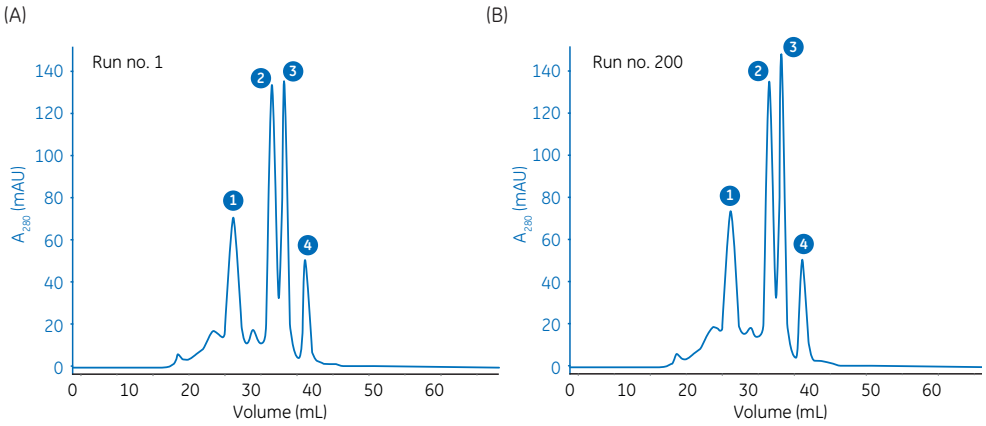


Fig 4.14. SEC of standard proteins on Superose 6 Increase 10/300 GL (A) before and (B) after 200 injections of IgG_{2b} including 20 CIP cycles with 0.5 M NaOH.

Superose prep grade

Superose 6 prep grade and Superose 12 prep grade have a particle size of ~ 30 µm. The resins have fractionation ranges of M_r 5000 to 5 000 000 and 1000 to 300 000 (globular proteins), respectively. Superose prep grade resins are designed for preparative purposes mainly at laboratory scale. The selectivity curves for Superose prep grade resins are shown in Figure 4.15.

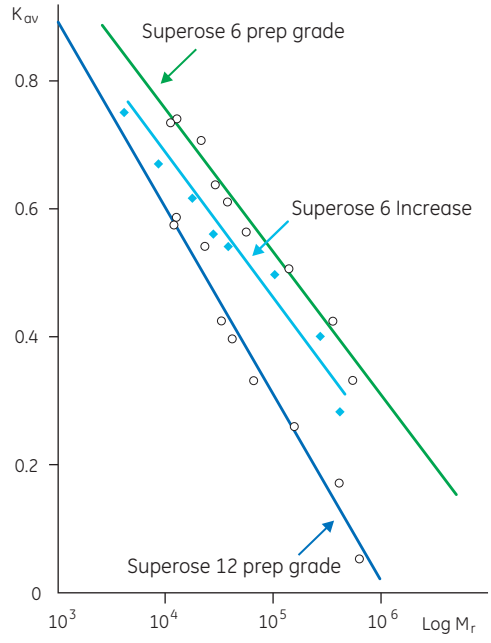


Fig 4.15. Selectivity curves (globular proteins) for Superose 6 prep grade, Superose 12 prep grade, and Superose 6 Increase.

Product selection

Superose resins are available in two different particle sizes and two different fractionation ranges (Table 4.4 and 4.5).

Superose 6 Increase is available prepacked in high-performance glass columns in three different sizes for separation of proteins between M_r 5000 and 5 000 000. Superose 6 Increase 10/300 GL is designed for high-resolution small-scale preparative purification, and protein characterization. Superose 6 Increase 5/150 GL is the first choice for rapid screening, purity checks, and for applications where buffer consumption needs to be low. Superose 6 Increase 3.2/300 is an excellent choice when working with very small sample volumes and for high resolution microscale separations.

Superose prep grade is only available as bulk resin. See Appendix 1 for column packing recommendations.

Table 4.5. Separation options with Superose resins

Product	Fractionation range, M_r (globular proteins)	Sample volume capacity	Typical pressure drop over the packed bed (MPa)*	Recommended operating flow rate/flow velocity†
Superose 6 Increase 10/300 GL	5×10^3 to 5×10^6	25 to 500 µL	3.0‡	0.5 mL/min
Superose 6 Increase 5/150 GL	5×10^3 to 5×10^6	4 to 50 µL	3.0‡	0.3 mL/min
Superose 6 Increase 3.2/300	5×10^3 to 5×10^6	4 to 50 µL	2.0‡	0.04 mL/min
Superose 6 prep grade (bulk resin)	5×10^3 to 5×10^6	0.5% to 4% of total column volume	Column-dependent	up to 40 cm/h
Superose 12 prep grade (bulk resin)	1×10^3 to 3×10^6	0.5% to 4% of total column volume	Column-dependent	up to 40 cm/h

* *See Appendix 4 to convert between different pressure units.*

† *Do not exceed maximum flow rate limits. Note that the maximum flow rate will depend on the temperature and the viscosity of the solution. For further information, see Appendix 4.*

‡ *The actual value is individual for each column and needs to be determined (see Appendix 4 Column pressure limits and maximum flow rate).*

Table 4.6. Guide to selecting a replacement resin for Superose 12

Peak	Protein	Mol. weight (M_r)	Superose 12	Superose 6 Increase	Superdex 200 Increase	Superdex 75 Increase	Superdex 30 Increase
		> 600 000		●			
1	Ferritin	440 000		●	●		
2	Conalbumin	75 000	●	●	●	●	
3	Ovalbumin	44 300	●	●	●	●	
4	Carbonic anhydrase	29 000	●	●	●	●	
5	Ribonuclease A	13 700	●	●	●	●	
6	Aprotinin	6500	●	●		●	●
7	Vitamin B_{12}	1200	●				●
8	Cytidine	240					●

● = *Suitable column for separation of the indicated protein*

Columns: 10/300 GL
Sample load: 100 µL
Buffer: PBS (10 mM phosphate buffer, 140 mM sodium chloride, pH 7.4)
Flow rate: 0.8 mL/min

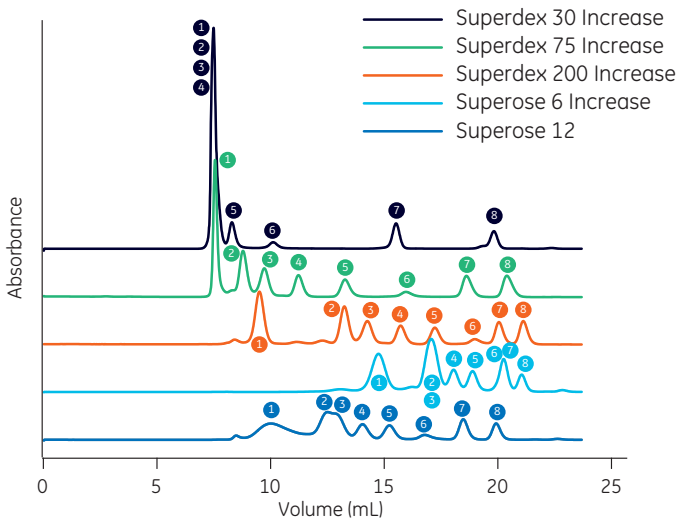


Fig 4.16. Chromatograms showing separation of eight molecules on Superdex 30 Increase, Superdex 75 Increase, Superdex 200 Increase, Superose 6 Increase and Superose 12. Peak numbers: 1. ferritin; 2. conalbumin; 3. ovalbumin; 4. carbonic anhydrase; 5. ribonuclease A; 6. aprotinin; 7. vitamin B_{12}; 8. cytidine.

For guidance on selecting a suitable replacement resin for Superose 12, see Table 4.6 and Figure 4.16.

Use prepacked Superose 6 Increase columns for small-scale preparative purification and analytical runs with sample volumes up to 0.5 mL.

Performing a separation

Buffer: 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0, or select the buffer in which the sample should be stored or solubilized for the next step.

Under normal chromatography conditions, nonspecific interactions are low when using buffers with ionic strengths in the range 0.15 M to 1.5 M. Some hydrophobic interactions have been noted, particularly for compounds such as smaller hydrophobic and/or aromatic peptides, membrane proteins and/or lipoproteins which can elute later than predicted. However, in some applications, these interactions can be an advantage for increasing the resolution of the separation.

The sample should be fully dissolved. Centrifuge or filter to remove particulate material. Always use degassed buffers and maintain a constant temperature during the run to avoid introducing air into the column.

Ensure that the maximum flow rate and pressure limits of the column are not exceeded, and that an appropriate pressure limit is set on the chromatography system. This is particularly important when working at low temperatures, such as in a cold room, or when the column is used with 20% ethanol or other viscous solutions. Use lower flow rates for high viscosity solutions and at low temperature (see Appendix 4).

Purification protocol

1. Remove the storage solution by applying 2 CV of distilled water at room temperature. Use a low flow rate to avoid gap formation (see Table 4.7 and Appendix 4).
2. For first-time use: Perform a column performance test (see Appendix 2) and determine the maximum pressure over the packed bed limit if recommended (see Table 4.6).
3. Equilibrate with 2 CV of buffer at recommended flow rate (see Table 4.6).
4. Apply a sample volume equivalent to between 0.3% and 4% of the bed volume. For most applications, the sample volume should not exceed 2% to achieve high resolution. Smaller sample volumes generally lead to improved resolution.
5. Elute with 1 CV of buffer.
6. Before applying a new sample, re-equilibrate the column with buffer until the baseline of the monitored signal is stable. Proteins can be monitored at 280 nm.

Note: To detect any column changes, the column performance should be checked at regular intervals (see Appendix 2)

Table 4.7. Recommended flow rates at different stages using columns containing Superose 6 Increase resin

	Flow rates (mL/min) at room temperature		
	3.2/300	**5/150 GL**	**10/300 GL**
First-time use or after long-term storage	0.04	0.25	0.5
Cleaning-in-place, CIP	0.02	0.1	0.5

For further information about performing a separation and for optimization, see Chapter 2 *Size exclusion chromatography in practice* and the product instructions.

Cleaning

Routine cleaning of the SEC column should be performed to prolong column lifetime. The frequency of cleaning depends mainly on the sample, but once every 20 runs is a guideline.

1. Wash with 1 CV of 0.5 M sodium hydroxide, alternatively 0.5 M acetic acid. Use a low flow rate during the entire CIP procedure (see Table 4.7).
2. Immediately wash with 1 CV of distilled water followed by at least 2 CV of buffer or until the baseline of the monitored signal and the pH of the eluent are stable. Further equilibration might be necessary if the buffer contains detergent.

Note: For other cleaning solutions and for extreme cases of contamination check the instructions supplied with each product.

In special cases, it might be necessary to change the top filter or to remove and discard the top 2 to 3 mm of the resin. These operations must be done with extreme care to avoid serious loss of resolution. Note that this is not possible with the 3.2/300 column as it should not be opened.

Superose prep grade may be autoclaved repeatedly at 121°C, pH 7.0 for 20 min without significantly affecting its chromatographic properties. The resin should be removed from the column prior to autoclaving.

Storage

Store bulk resin at 4°C to 30°C in 20% ethanol. Do not freeze.

For long-term storage of columns, wash with 2 CV of distilled water followed by 2 CV of 20% ethanol at low flow rate. Connect a storage/shipping device to prevent air from entering the column during storage. Store the column at 4°C to 30°C. Avoid changes in temperature, which can cause air bubbles in the packing.

Application

ATP synthase complex from *E. coli* membrane was captured with Ni Sepharose™ 6 Fast Flow and further purified on Superose 6 Increase 5/150 GL. As seen in Figure 4.17, the resolution between aggregates (Peak 1) and monomeric ATP synthase protein complex was good (Peak 2), as well as separation from degradation products (Peaks 3 and 4). In addition to small consumption of sample and buffer, the separation was achieved in a short run time, yet with sufficient resolution. The purified material was further used for structural and molecular mechanism studies.

Column:	Superose 6 Increase 5/150 GL
Sample:	ATP synthase complex from *E. coli* membrane
Sample volume:	25 µL
Buffer:	20 mM Tris-HCl, 150 mM sodium chloride, 10% glycerol, 250 µM magnesium chloride, 0.05% dodecyl maltoside, pH 8.0
Flow rate:	0.15 mL/min
System:	ÄKTAmicro in cold room

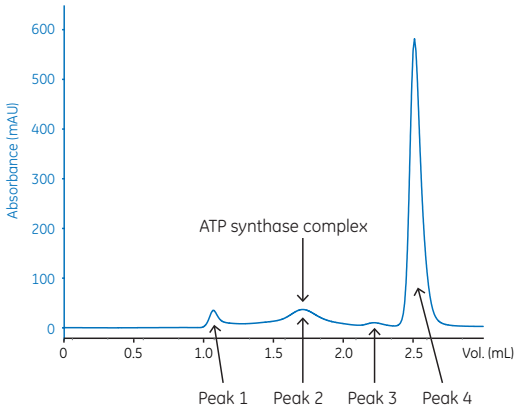


Fig 4.17. SEC using Superose 6 Increase 5/150 GL of ATP synthase complex from *E. coli* membrane. Peak 1: Aggregates; Peak 2: Monomeric ATP synthetase complex; Peak 3 and 4: Degradation products.

Sephacryl resins

Sephacryl High Resolution (HR) resins provide a useful alternative for applications that require a broad fractionation range. The particle size, ~ 50 µm, together with the rigidity of the matrix and high chemical stability make Sephacryl HR well suited for industrial use.

Sephacryl HR covers a wide molecular weight range from peptides to very large biomolecules (M_r 1×10^3 to 2×10^7). Figure 4.18 shows the fractionation ranges for the five Sephacryl HR resins available.

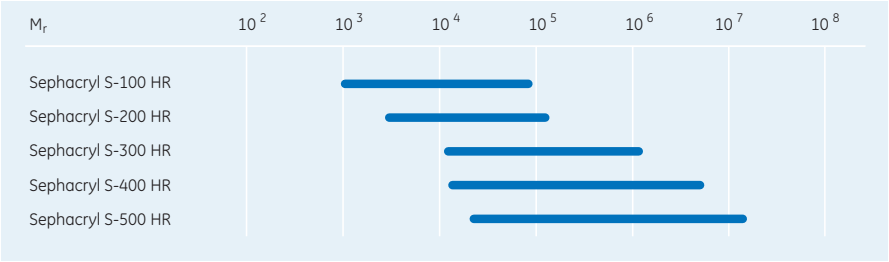


Fig 4.18. Fractionation ranges for Sephacryl HR. The fractionation range for Sephacryl S-500 HR is for dextrans; all other fractionation ranges are given for globular proteins.

Sephacryl HR consists of covalently cross-linked allyl dextran and N,N'-Methylene bisacrylamide providing a hydrophilic resin with minimized nonspecific adsorption, typically > 95% yield for proteins.

Sephacryl HR is chemically stable in commonly used aqueous buffers and additives such as detergents, denaturing agents (8 M urea or 6 M guanidine hydrochloride), and 30% acetonitrile. A packed column should be used at the temperature range 4°C to 40°C. Further characteristics of the resins are found in Table 4.8 and in the product instructions.

Table 4.8 Sephacryl resin characteristics

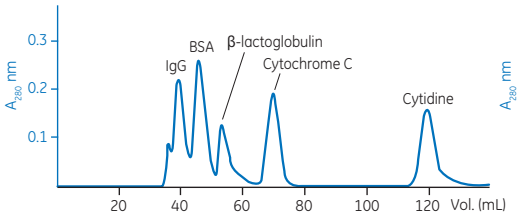
		pH stability		
Product	**Efficiency[1]**	**Operational[2]**	**Cleaning-in-place (CIP)[3]**	**Particle size, d_{50v} (µm)[4]**
Sephacryl S-100 HR, S-200 HR, S-300 HR, S-400 HR, and S-500 HR resins	≥ 5000	3 to 11	2 to 13	~ 50

1 *Theoretical plates per meter (prepacked columns only).*
2 *pH range where resin can be operated without significant change in function.*
3 *pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.*
4 *Median particle size of the cumulative volume distribution.*

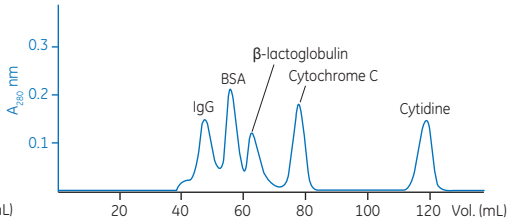
Figure 4.19 shows comparisons of the selectivity of the five Sephacryl HR resins. Typical selectivity curves for Sephacryl are shown in Figure 4.20.

Column: (A) HiPrep 16/60 Sephacryl S-100 HR
(B) HiPrep 16/60 Sephacryl S-200 HR
(C) HiPrep 16/60 Sephacryl S-300 HR
Sample: 500 µl of a mixture comprising IgG (M_r 160 000), BSA (M_r 67 000), β-lactoglobulin (M_r 35 000), cytochrome C (M_r 12 400), and cytidine (M_r 240)
Buffer: 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0
Flow rate (flow velocity): 0.8 mL/min (24 cm/h)

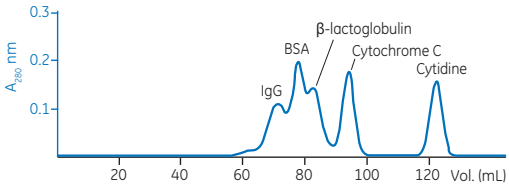
(A) HiPrep 16/60 Sephacryl S-100 HR



(B) HiPrep 16/60 Sephacryl S-200 HR

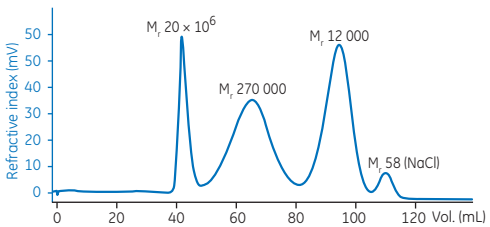


(C) HiPrep 16/60 Sephacryl S-300 HR



Column: (D) HiPrep 16/60 Sephacryl S-400 HR
(E) HiPrep 16/60 Sephacryl S-500 HR
Sample: (D) 1.2 mL of a sample containing three dextrans; $M_r > 20 \times 10^6$, M_r 270 000, and M_r 12 000
(E) 1.2 mL of a sample containing three dextrans, $M_r > 20 \times 10^6$, M_r 1.8×10^6, and M_r 25 000
Buffer: 250 mM sodium chloride
Flow rate (flow velocity): 0.5 mL/min (15 cm/h)

(D) HiPrep 16/60 Sephacryl S-400 HR



(E) HiPrep 16/60 Sephacryl S-500 HR

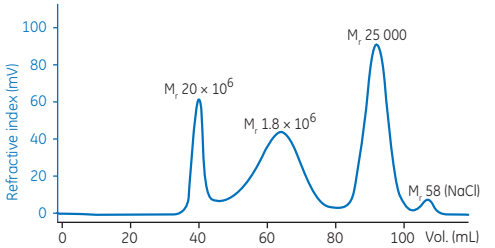
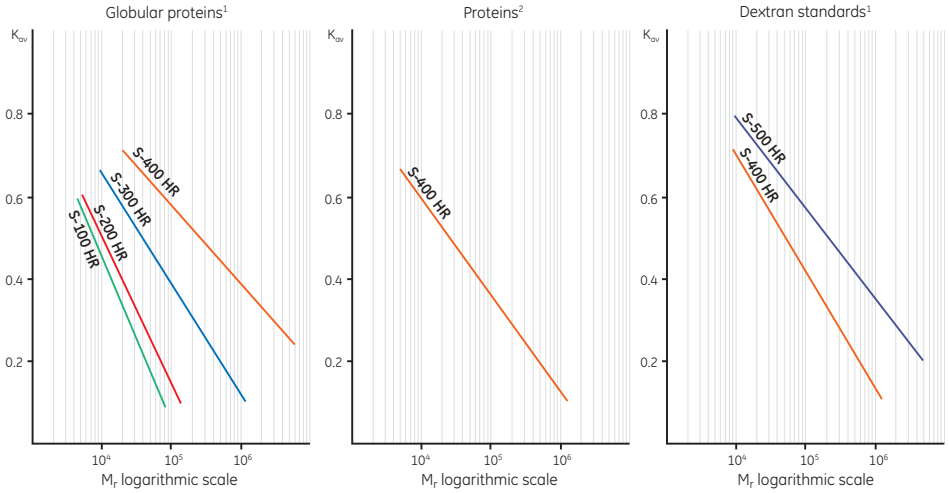


Fig 4.19. Comparison of selectivity of the five Sephacryl HR resins in prepacked HiPrep 16/60 columns. The same sample mix was used for Sephacryl S-100 HR, S-200 HR, and S-300 HR (A to C), while two different dextran mixes were used for Sephacryl S-400 HR and S-500 HR (D and E).



[1] In 50 mM phosphate buffer, 150 mM sodium chloride, pH 7.0
[2] In 6 M guanidine hydrochloride

Fig 4.20. Selectivity curves for Sephacryl HR resins.

Product selection

Sephacryl HR is available in five different fractionation ranges (Fig 4.18). The resins are available as bulk resin and prepacked in two different column formats; HiPrep 16/60 and HiPrep 26/60 (see Fig 4.21 and Table 4.9).

Fig 4.21. Sephacryl is available as bulk resin and in prepacked columns.

Table 4.9. Separation options with Sephacryl resins

Product	Fractionation range, M_r (globular proteins)	Fractionation range, M_r (dextrans)	Sample volume capacity	Maximum pressure drop over the packed bed*	Recommended operating flow rate/flow velocity†
HiPrep 16/60 Sephacryl S-100 HR	1×10^3 to 1×10^5		≤ 5 mL	0.15 MPa	0.5 mL/min
HiPrep 26/60 Sephacryl S-100 HR	1×10^3 to 1×10^5		≤ 13 mL	0.15 MPa	1.3 mL/min
Sephacryl S-100 HR (Bulk resin)	1×10^3 to 1×10^5		0.5% to 4% of total column volume	Column-dependent	10 to 35 cm/h
HiPrep 16/60 Sephacryl S-200 HR	5×10^3 to 2.5×10^5	1×10^3 to 8×10^4	≤ 5 mL	0.15 MPa	0.5 mL/min
HiPrep 26/60 Sephacryl S-200 HR	5×10^3 to 2.5×10^5	1×10^3 to 8×10^4	≤ 13 mL	0.15 MPa	1.3 mL/min
Sephacryl S-200 HR (Bulk resin)	5×10^3 to 2.5×10^5	1×10^3 to 8×10^4	0.5% to 4% of total column volume	Column-dependent	10 to 35 cm/h
HiPrep 16/60 Sephacryl S-300 HR	1×10^4 to 1.5×10^6	2×10^3 to 4×10^5	≤ 5 mL	0.15 MPa	0.5 mL/min
HiPrep 26/60 Sephacryl S-300 HR	1×10^4 to 1.5×10^6	2×10^3 to 4×10^5	≤ 13 mL	0.15 MPa	1.3 mL/min
Sephacryl S-300 HR (Bulk resin)	1×10^4 to 1.5×10^6	2×10^3 to 4×10^5	0.5% to 4% of total column volume	Column-dependent	10 to 35 cm/h
HiPrep 16/60 Sephacryl S-400 HR	2×10^4 to 8×10^6	1×10^4 to 2×10^6	≤ 5 mL	0.15 MPa	0.5 mL/min
HiPrep 26/60 Sephacryl S-400 HR	2×10^4 to 8×10^6	1×10^4 to 2×10^6	≤ 13 mL	0.15 MPa	1.3 mL/min
Sephacryl S-400 HR (Bulk resin)	2×10^4 to 8×10^6	1×10^4 to 2×10^6	0.5% to 4% of total column volume	Column-dependent	10 to 35 cm/h
HiPrep 16/60 Sephacryl S-500 HR		4×10^4 to 2×10^7	≤ 5 mL	0.15 MPa	0.5 mL/min
HiPrep 26/60 Sephacryl S-500 HR		4×10^4 to 2×10^7	≤ 13 mL	0.15 MPa	1.3 mL/min
Sephacryl S-500 HR (Bulk resin)		4×10^4 to 2×10^7	0.5% to 4% of total column volume	Column-dependent	10 to 35 cm/h

* *See Appendix 4 to convert between different pressure units.*

† *Do not exceed maximum flow rate limits. Note that the maximum flow rate will depend on the temperature and the viscosity of the solution. For further information, see Appendix 4.*

Performing a separation

Buffer: 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.2 or select the buffer in which the sample should be stored or solubilized for the next step.

To avoid ionic interactions with the resin, it is recommended to include at least 150 mM sodium chloride, or to use a buffer with equivalent ionic strength.

The sample should be fully dissolved. Centrifuge or filter to remove particulate material. Always use degassed buffers and maintain a constant temperature during the run to avoid introducing air into the column.

Ensure that the pressure limits of the column are not exceeded and that an appropriate pressure limit is set on the chromatography system. This is particularly important when working at low temperatures, such as in a cold room, or when the column is used with 20% ethanol or other viscous solutions. Use lower flow rates for high viscosity solutions and at low temperature (see Appendix 4).

Purification protocol

1. Remove the storage solution by applying at least 0.5 CV of distilled water at room temperature. Use a low flow velocity of 15 cm/h to avoid gap formation (0.5 mL/min for 16/60 column or 1.3 mL/min for 26/60 column).
2. Perform a column performance test (see Appendix 2).
3. Equilibrate with 2 CV of buffer at 30 cm/h (1.0 mL/min for 16/60 column or 2.6 mL/min for 26/60 column).
4. Reduce the flow to 15 cm/h and apply a sample volume equivalent to between 0.5% and 4% of the bed volume. Smaller sample volumes generally lead to improved resolution.
5. Elute with 1 CV of buffer.
6. Before applying a new sample, re-equilibrate the column with buffer at 30 cm/h until the baseline of the monitored signal is stable. Proteins can be monitored at 280 nm.

Note: To detect any column changes, the column performance should be checked at regular intervals (see Appendix 2).

For further information about performing a separation and for optimization, see Chapter 2 *Size exclusion chromatography in practice* and the product instructions

Cleaning

Routine cleaning of the SEC column should be performed to prolong column lifetime. The frequency of cleaning depends mainly on the sample, but once every 20 runs is a guideline.

1. Wash with 0.5 CV of 0.2 M sodium hydroxide at a flow velocity of 15 cm/h (0.5 mL/min for 16/60 column or 1.3 mL/min for 26/60 column).
2. Immediately wash with 2 CV of buffer or until the baseline of the monitored signal and the pH of the eluent are stable. Further equilibration might be necessary if the buffer contains detergent.

Note: For other cleaning solutions and for extreme cases of contamination check the instructions supplied with each product.

Reversing flow through a column packed with Sephacryl resin should only be considered under cases of severe contamination. Reversing the flow can cause channeling through the packed bed leading to poor resolution, reduced efficiency, and the need to repack the column. Prepacked columns (HiPrep) are less likely to be affected, but extreme care must be taken as they may not be repacked.

In special cases, it might be necessary to change the top filter or to remove and discard the top 2 to 3 mm of the resin. These operations must be done with extreme care to avoid serious loss of resolution. Note that this is not possible for prepacked HiPrep columns as they should not be opened.

Sephacryl HR may be autoclaved at 121°C in 0.15 M NaCl, pH 7 for 20 min (5 cycles) without significantly affecting its chromatographic properties. The resin should be removed from the column prior to autoclaving. Note that HiPrep columns cannot be repacked.

Storage

Store bulk resin at 4°C to 30°C in 20% ethanol. Do not freeze.

For long-term storage of columns, wash with 4 CV of distilled water followed by 4 CV of degassed 20% ethanol at low flow rate. Connect a storage/shipping device to prevent air from entering the column during storage. Store the column at 4°C to 30°C. Avoid changes in temperature, which can cause air bubbles in the packing.

Applications

Figures 4.22 and 4.23 show examples of separations performed on Sephacryl resins.

Column: HiPrep 26/60 Sephacryl S-100 HR
Sample: 1 mL of a mixture containing bovine insulin chain A (M_r 2532) and chain B (M_r 3496), 0.5 mg/mL of each
Buffer: 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0
Flow rate (flow velocity): 2.0 mL/min (22 cm/h)

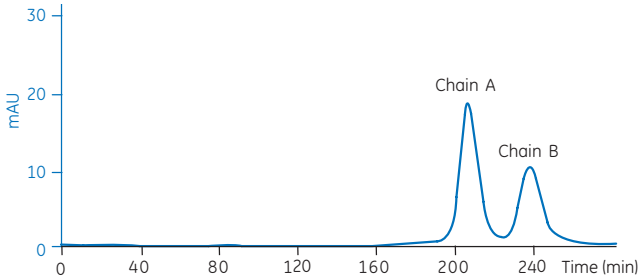


Fig 4.22. Separation of insulin chains on HiPrep 26/60 Sephacryl S-100 HR.

Column: HiPrep 26/60 Sephacryl S-200 HR
Sample: (A) 1 mL PHA-M, 2 mg/mL in acetic acid
(B) 1 mL PHA-P, 2 mg/mL in acetic acid
Buffer: 50 mM sodium phosphate, 150 mM NaCl, pH 7.0
Flow rate (flow velocity): 2 mL/min (22 cm/h)

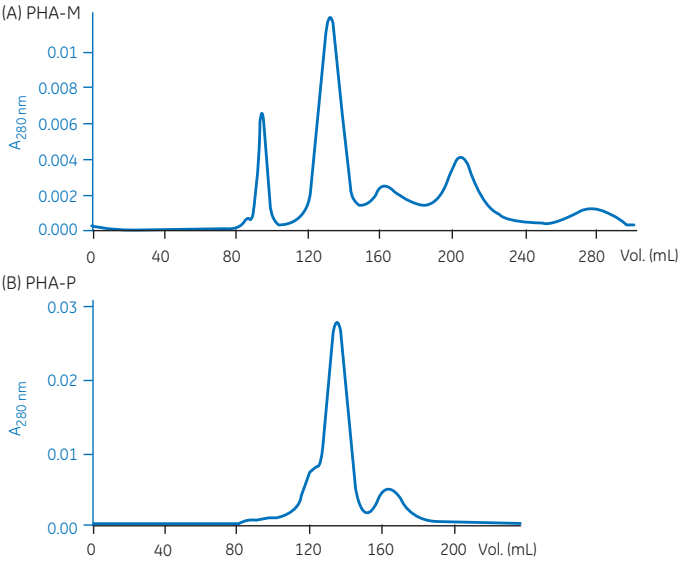


Fig 4.23. Purification of phytohemagglutinin on HiPrep 26/60 Sephacryl S-200 HR.

Chapter 5
Sephadex resins: desalting and buffer exchange

Desalting and other types of buffer exchange are examples of group separations on SEC resins where the purpose is to separate small molecules, such as salts, from large biomolecules such as proteins. Although prepacked columns designated for this purpose generally are termed desalting columns the application area is much wider. 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 [α-^{32}P] adenosine triphosphate (ATP) from nucleic acid labeling reactions

Desalting provides several advantages over dialysis, which is generally a slow technique that requires large volumes of buffer and carries the risk of losing material and activity of the target molecule during handling. When desalting, sample volumes of up to 30% of the total column volume can be processed. SEC resins with large particles packed in broad, short columns can be used, which enables fast separations using high flow rates. A sample can be processed in less than 5 min with a high recovery. The recovery depends on the biomolecule, the column format, and the purification protocol but is often > 95%. Low target molecule concentrations often result in lower recovery.

The high speed and capacity of the separation allows even relatively large sample volumes to be processed rapidly and efficiently, as illustrated in Figure 5.1 and 5.2. Figure 5.1 shows that the resolution between the protein peak and the salt peak remains high when increasing the flow rate. The position, shape, and width of the peaks are similar in all runs irrespective of flow rate.

Column:	HiTrap Desalting
Sample:	Bovine serum albumin, 2 mg/mL in 500 mM NaCl, 50 mM sodium phosphate, pH 7.0
Sample volume:	0.8 mL
Buffer:	50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0
Flow rates:	1.7, 3.3, 6.7, 10.0, 13.3, 16.7, 20.0 mL/min

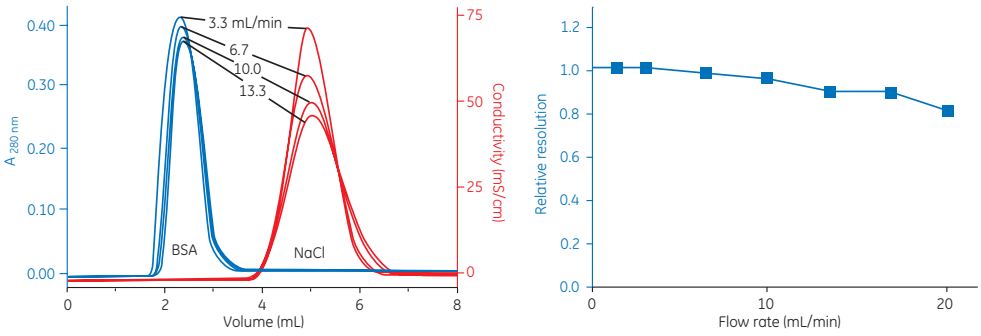


Fig 5.1. Influence of flow rate on resolution.

Figure 5.2 shows the effect of increasing sample volumes on resolution. The peaks become wider when the sample volume increases, which leads to a larger overlap of the protein peak and the NaCl peak. The effect on resolution is illustrated by the NaCl concentration in the collected protein fraction. The size of the collected volume will affect the NaCl concentration in the protein fraction and also the target protein yield.

Column: HiTrap Desalting
Sample: Bovine serum albumin, 2 mg/mL in 50 mM sodium phosphate, 500 mM sodium chloride, pH 7.0
Buffer: 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0
Sample volumes: 0.8, 1.3, 1.7, 2.2 mL
Flow rate: 5 mL/min

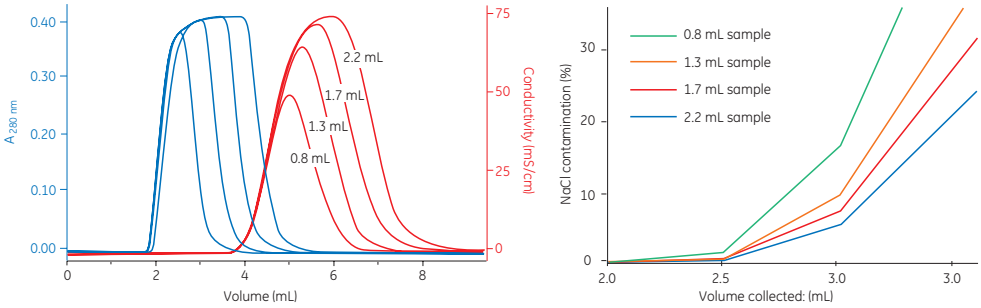


Fig 5.2. Influence of sample volume on resolution.

Generally, sample concentration does not significantly influence the separation providing viscosity does not become too high in relation to the viscosity of the eluent. Also, the target molecule needs to be stable and soluble at the concentration used.

Use desalting/buffer exchange when needed, before purification, between purification steps, and/or after purification. Note that each extra step might reduce the yield and that desalting often dilutes the sample (except for spin protocols).

Sephadex resin characteristics

SEC based on Sephadex chromatography resins enables desalting and buffer exchange of biomolecules. Sephadex is prepared by cross-linking dextran with epichlorohydrin. The exclusion limit and the fractionation range are controlled by varying the degree of cross-linking of the resin. Biomolecules with sizes above the exclusion limit of the resin can be separated from contaminants such as salts, dyes, and radioactive labels that have excess to the pores.

Different types of Sephadex resins with selectivity for specific molecular sizes are available. The characteristics of Sephadex resins are summarized in Table 5.1 and Figure 5.3

Sephadex G-25 is recommended for the majority of group separations involving globular proteins. Sephadex G-10 is suitable for desalting smaller biomolecules (M_r > 700), and Sephadex G-50 can be used to separate large molecules (M_r > 30 000) from molecules with M_r < 1500.

Table 5.1 Sephadex characteristics and typical applications

Resin	Particle size (µm)	Swelling factor§	Separation range (M_r) globular proteins	Typical applications
Sephadex G-10 Medium	40 to 120*	1.4	$< 7 \times 10^2$	Separation of small biomolecules such as peptides ($M_r > 700$) from smaller molecules ($M_r < 100$).
Sephadex G-25 Superfine	20 to 50†	1.7	1×10^3 to 5×10^3	Recommended for most group separations involving globular proteins, excellent for removing salts and other small contaminants from molecules with $M_r > 5000$.
Sephadex G-25 Fine	20 to 80†	1.7	1×10^3 to 5×10^3	
Sephadex G-25 Medium	50 to 150‡	1.7	1×10^3 to 5×10^3	
Sephadex G-25 Coarse	100 to 300‡	1.7	1×10^3 to 5×10^3	
Sephadex G-50 Fine	20 to 80†	2.1	1×10^3 to 3×10^4	Suitable for separation of molecules with $M_r > 30\,000$ from molecules $M_r < 1500$ such as labeled protein or DNA from unconjugated dyes. Often used for removal of small nucleotides from longer chain nucleic acids.

* *≥ 85% of the volume of particles are found within the range shown.*
† *≥ 80% of the volume of particles are found within the range shown.*
‡ *≥ 90% of the volume of particles are found within the range shown.*
§ *Suitable swelling solutions are water, buffers, or salt solutions. Note that Sephadex particles shrink in alcohol solutions.*

Group separation/desalting

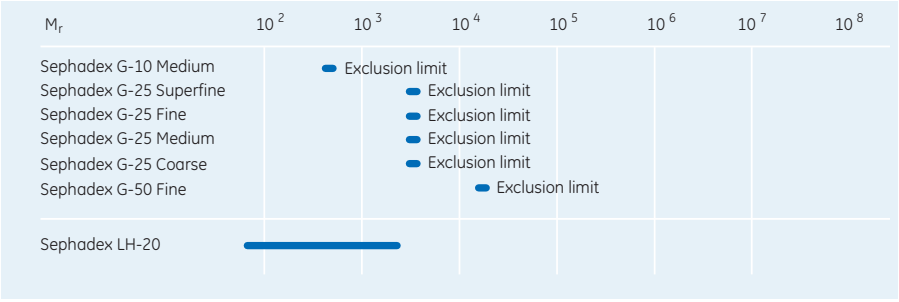


Fig 5.3. Exclusion limits of Sephadex resins. Sephadex LH-20 is for use with organic solvents, see Appendix 7.

Sephadex G-25 is available in different particle sizes. Sephadex G-25 Superfine, the resin with the smallest particles, is used for highest efficiency and resolution. This resin is suitable for small-scale separations in short columns since the small particles generate high operating pressure. Sephadex G-25 Fine particles are also relatively small and are best suited for laboratory-scale separations. Sephadex Medium and Coarse can be used when high flow rate at low operating pressure is essential, for example in large-scale applications.

Sephadex is stable in commonly used aqueous buffers and additives such as ionic and nonionic detergents, as well as denaturing agents. Most Sephadex resins are operational in the pH range 2 to 13 (for Sephadex G-50 Fine, pH 2 to 10). Cleaning-/sanitization-in-place may be performed in the pH range 2 to 13.

Further characteristics of the resins are found in the product literature for the respective resins, see *gelifesciences.com*

Product selection

Select the resin so that the size of the high molecular weight substances exceeds the exclusion limit of the resin (see Fig 5.3).

For convenience and reliable performance, GE offers a wide range of prepacked Sephadex columns for desalting and buffer exchange in different scales by chromatography systems or manual purifications using a syringe, by gravity flow, or by centrifugation (Fig 5.5 and Table 5.2). Sephadex G-25 resins are used in most prepacked columns.

Sephadex resins with different selectivities and particle size are available as dry powder in bulk packs for packing your own column. See Appendix 1 for recommendations on swelling and column packing.

Table 5.2. Columns prepacked with Sephadex for desalting and buffer exchange

Columns and 96-well plates	Loaded volume (mL)	Eluted volume (mL)	Dilution factor	Operation
PD SpinTrap™ G-25[1]	0.07 to 0.13	0.07 to 0.13	No dilution	Centrifuge
PD MultiTrap™ G-25	0.07 to 0.13	0.07 to 0.13	No dilution	Centrifuge
PD MiniTrap™ G-25[1]	0.2 to 0.5	0.2 to 0.5	No dilution	Centrifuge
	0.1 to 0.5	1.0	2 to 10	Gravity flow
PD MidiTrap™ G-25[1]	0.75 to 1.0	0.75 to 1.0	No dilution	Centrifuge
	0.5 to 1.0	1.5	1.5 to 3	Gravity flow
PD-10 Desalting columns[1]	1.75 to 2.5	1.75 to 2.5	No dilution	Centrifuge
	1.0 to 2.5	3.5	1.5 to 3.5	Gravity flow
PD MiniTrap G-10	0.1 to 0.3	0.5	1.7 to 5	Gravity flow
PD MidiTrap G-10	0.4 to 1.0	1.2	1.2 to 3.0	Gravity flow
HiTrap Desalting	0.25	1.0	4 (approx.)	Syringe/pump/system
	0.5	1.5	3 (approx.)	Syringe/pump/system
	1.0	2.0	2 (approx.)	Syringe/pump/system
	1.5 (max.)	2.0	1.3 (approx.)	Syringe/pump/system
HiPrep 26/10 Desalting	10	10 to 15	1.0 to 1.5	Pump/system
	15 (max.)	15 to 20	1.0 to 1.3	Pump/system

Contains Sephadex G-25 Medium
Contains Sephadex G-10 Medium
Contains Sephadex G-25 Superfine
Contains Sephadex G-25 Fine

[1] *Also available with Sephadex G-25 DNA grade within the illustra™ product family.*

When working with small-scale purification, several samples may be run in parallel manually by gravity flow, or by centrifugation.

If the volume of the sample exceeds the sample volume capacity, several prepacked HiTrap Desalting or HiPrep 26/10 Desalting columns can be connected in series.

Performing desalting and buffer exchange

Buffer: Use 10 to 50 mM sodium phosphate buffer, with at least 25 mM NaCl, pH 7.0 to 7.4, or select an appropriate buffer depending on the purpose of the separation. Avoid salt concentrations > 1.0 M.

To prevent potential ionic interactions the presence of a low salt concentration (25 mM sodium chloride) is recommended in the buffer during desalting. Volatile buffers such as 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate can be used if it is necessary to avoid the presence of sodium chloride.

The sample should be fully dissolved. Centrifuge or filter to remove particulate material. Always use degassed buffers to avoid introducing air into the column.

Protein concentrations up to 70 mg/mL should not influence the separation when using normal aqueous buffers.

When using a chromatography system, ensure that the pressure limits of the column are not exceeded. This is particularly important when working at low temperatures, such as in a cold room, or when the column is used with 20% ethanol or other viscous solutions. Use lower flow rates for high viscosity solutions and at low temperature (see Appendix 4).

Using a chromatography system equipped with monitors facilitates the possibility to follow the separation and simplifies optimization of the separation. Elution of biomolecules can be monitored by UV absorption (280 nm for proteins) and the appearance of salt can be followed exactly by a conductivity monitor (Fig 5.4).

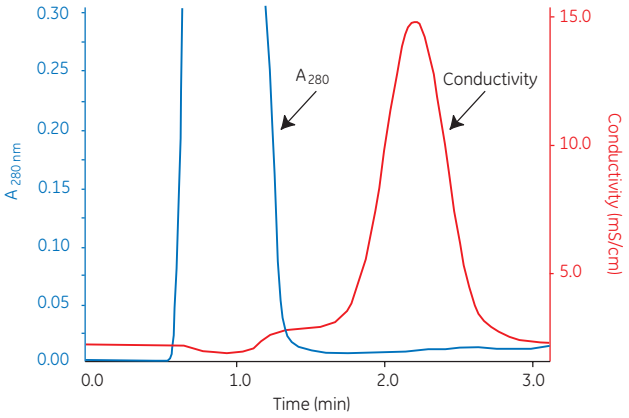


Fig 5.4. Buffer exchange of mouse plasma on HiPrep 26/10 Desalting column.

Desalting generally causes dilution of the sample (except for spin protocols). Minimal dilution is obtained when the maximum desalting capacity of the column is utilized. When possible, select a prepacked column that is suited to the volume of sample that needs to be desalted. As the sample volume increases the dilution factor decreases. For most applications, up to 30% of the total bed volume may be used. The final dilution factor also depends on the collected elution volume. Maximizing the recovery of the target molecule by collecting a large volume will increase the dilution of the sample. Note that large sample volumes and collection of large elution volumes can increase the amount of salt remaining in the sample after elution.

Use a large sample volume (≤ 30% of the total bed volume) for minimal sample dilution. Application of sample volumes larger than 30% of the bed volume will give less efficient desalting. Reduce sample volume (< 30% of the column volume) when highest resolution is required for the separation.

If conductivity cannot be monitored and recovery of completely desalted sample is the major requirement, apply sample volumes of between 15% and 20% of the total bed volume.

For the majority of separations, the instructions supplied with each product ensure satisfactory results and very little optimization should be necessary. Ensure that buffer conditions are optimal for the separation and that the maximum sample volume for the column is not exceeded.

For sample and buffer preparation, see *General advice* in Chapter 2.

Re-equilibration is not necessary between runs with the same buffer. Extended equilibration might be needed with buffers containing detergents. Equilibrate with at least 5 CV.

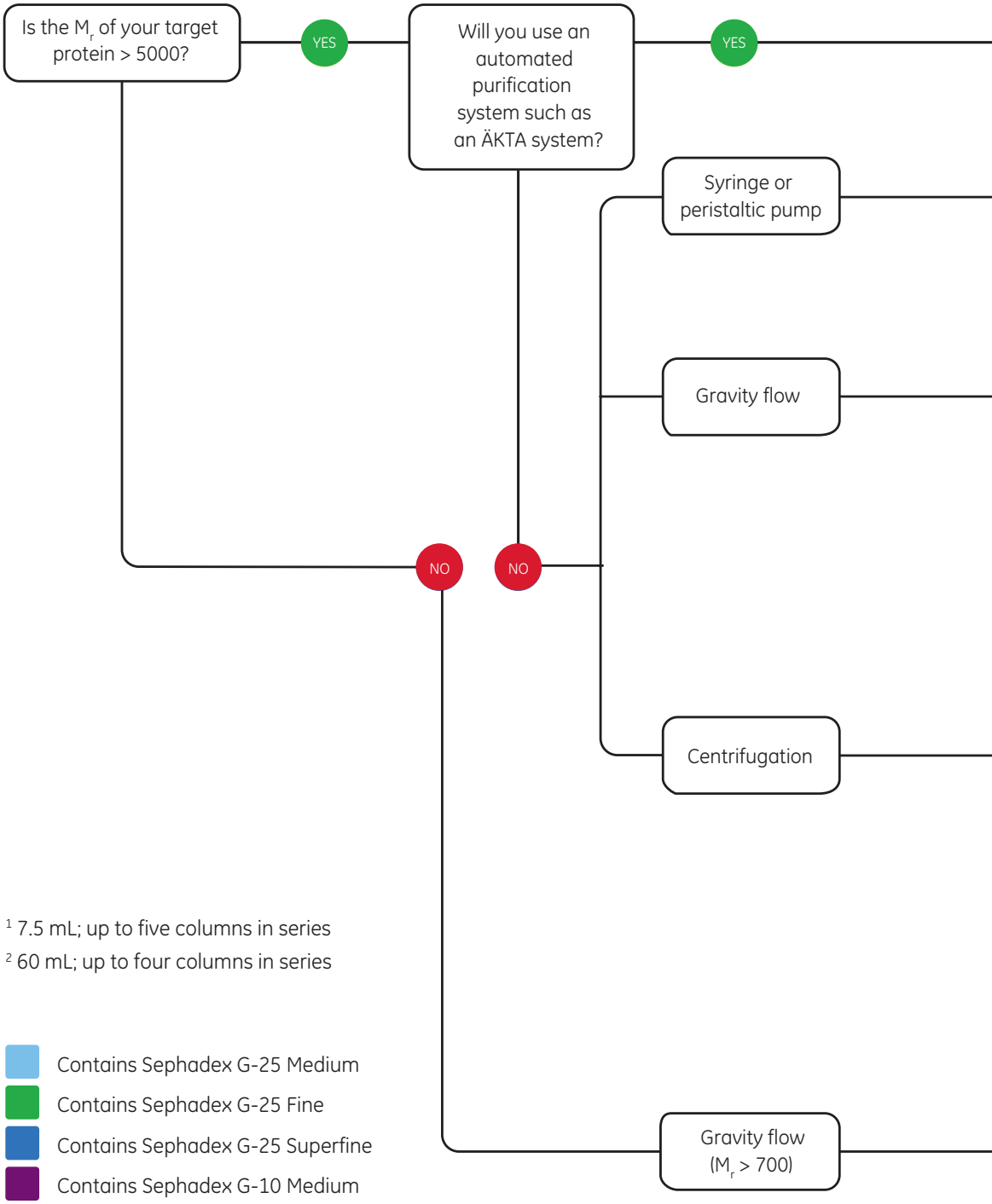
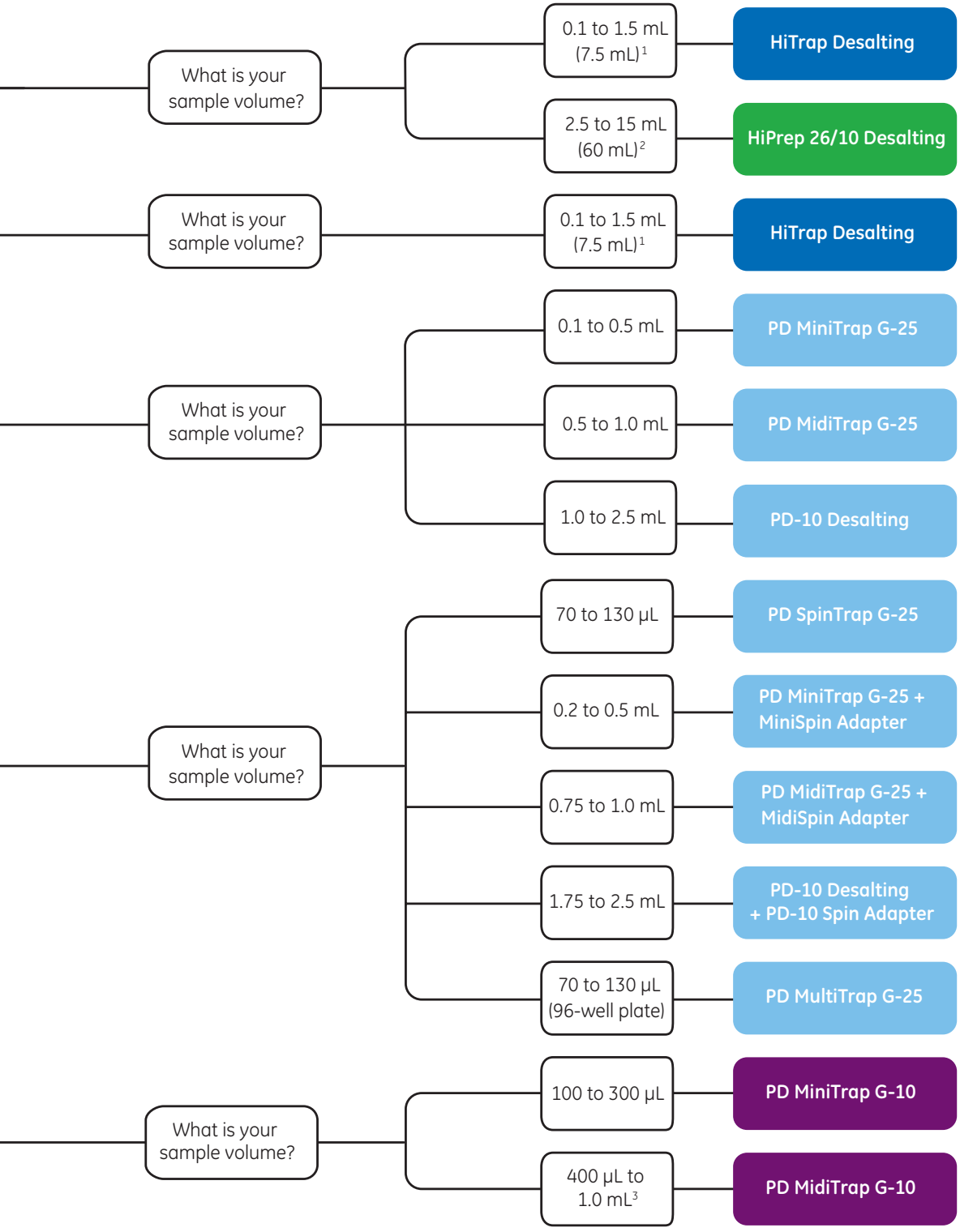


Fig 5.5. Selecting prepacked columns for desalting and buffer exchange.


What is your sample volume?
0.1 to 1.5 mL (7.5 mL)[1]
HiTrap Desalting
2.5 to 15 mL (60 mL)[2]
HiPrep 26/10 Desalting
What is your sample volume?
0.1 to 1.5 mL (7.5 mL)[1]
HiTrap Desalting
What is your sample volume?
0.1 to 0.5 mL
PD MiniTrap G-25
0.5 to 1.0 mL
PD MidiTrap G-25
1.0 to 2.5 mL
PD-10 Desalting
What is your sample volume?
70 to 130 µL
PD SpinTrap G-25
0.2 to 0.5 mL
PD MiniTrap G-25 + MiniSpin Adapter
0.75 to 1.0 mL
PD MidiTrap G-25 + MidiSpin Adapter
1.75 to 2.5 mL
PD-10 Desalting + PD-10 Spin Adapter
70 to 130 µL (96-well plate)
PD MultiTrap G-25
What is your sample volume?
100 to 300 µL
PD MiniTrap G-10
400 µL to 1.0 mL[3]
PD MidiTrap G-10

HiTrap Desalting and HiPrep 26/10 Desalting columns

Fig 5.6. Prepacked Sephadex G-25 columns: left HiPrep 26/10 Desalting, right HiTrap Desalting.

HiTrap Desalting and HiPrep 26/10 Desalting columns (Fig 5.6) are designed for fast, reproducible and convenient desalting and buffer exchange of high (M_r > 5000 for globular proteins) from low molecular weight substances (M_r < 1000). Separations are easily performed with a standalone pump, or a chromatography system such as ÄKTA. HiTrap Desalting may also be operated with a syringe. The characteristics of these prepacked columns are given in Table 5.3.

HiTrap Desalting is packed with Sephadex G-25 Superfine. The bed volume is 5 mL and the void volume 1.5 mL. Small sample volumes of 0.1 to 1.5 mL may be applied in one run. The recommended operating flow rate is in the range 1 to 10 mL/min and the maximum flow rate 15 mL/min. To avoid cross-contamination, the column should only be used with the same type of sample.

HiPrep 26/10 Desalting is packed with Sephadex G-25 Fine. The bed volume is 53 mL and the void volume 15 mL; 2.5 to 15 mL sample may be applied in one run. The recommended operating flow rate is in the range 9 to 31 mL/min (100 to 350 cm/h) and the maximum flow rate 40 mL/min (450 cm/h).

Table 5.3. Characteristics of HiTrap Desalting and HiPrep 26/10 Desalting columns

Column	Matrix	Bed volume	Recommended operational flow rate	Maximum operational flow rate
HiTrap Desalting	Sephadex G-25 Superfine	5 mL	1 to 10 mL/min	15 mL/min
HiPrep 26/10 Desalting	Sephadex G-25 Fine	53 mL	9 to 31 mL/min	40 mL/min

High molecular weight compounds start to elute after approximately 1.5 mL and low molecular weight components after 3.5 mL. Figure 5.7 shows the effect of varying the sample volume applied to the column.

Column: HiTrap Desalting
Sample: 2 mg/mL bovine serum albumin in 50 mM sodium phosphate buffer, 500 mM sodium chloride, pH 7.0
Sample volume: 0.3 mL, 1.3 mL, 2.2 mL
Eluent: 50 mM sodium phosphate buffer, 150 mM sodium chloride, pH 7.0
Flow rate: 5 mL/min

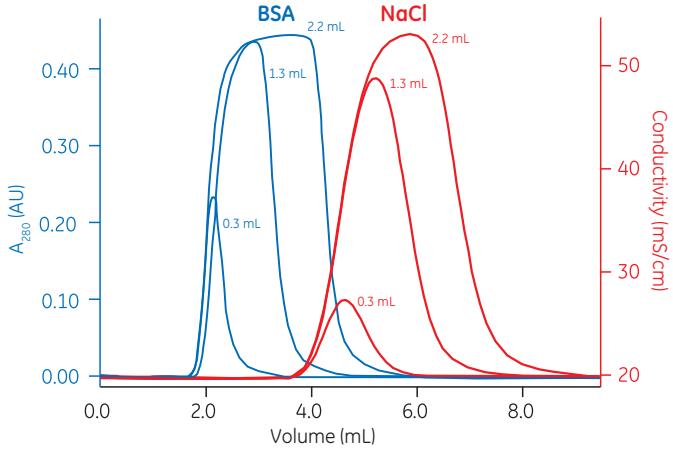


Fig 5.7. The effect of different sample volumes on desalting of bovine serum albumin using HiTrap Desalting.

HiTrap Desalting: operation with pump or chromatography system

1. **Preparation**
   Ensure that the appropriate pressure limits have been set and fill the pump tubing with buffer. Remove the stop plug and the snap-off end at the column outlet. Connect the column drop-to-drop to the pump tubing to avoid introducing air into the column.
2. **Equilibration**
   First-time use: Equilibrate the column with 25 mL buffer at 5 mL/min.
3. **Sample application**
   Apply up to 1.5 mL of sample. Use a flow rate of 5 mL/min.
4. **Elution**
   Elute the column with 10 mL buffer and collect fractions.

If air is trapped in the column, wash with degassed buffer until the air disappears. Inverting the column while washing enables the air to escape more easily through the column outlet. Small amounts of air introduced into the column by accident during sample application do not influence the separation.

HiTrap Desalting: operation with a syringe

Fig 5.8. HiTrap Desalting column operated by syringe.

Desalting with HiTrap Desalting column can be performed easily with a syringe (Fig 5.8) according to the following steps:

1. **Preparation**
   Fill the syringe with buffer and remove the stop plug. Use the supplied Luer connector and connect the column to the syringe drop-to-drop to avoid introducing air into the column. Remove the snap-off end at the column outlet.
2. **Equilibration**
   First-time use: Equilibrate the column with 25 mL of buffer at 5 mL/min[1].
3. **Sample application**
   Apply up to 1.5 mL sample using a syringe fitted to the Luer connector on the column. Use a flow rate of 5 mL/min[1]. If the sample volume is less than 1.5 mL, add buffer (stacker volume) until a total volume of 1.5 mL buffer is eluted. Discard the eluted buffer.
4. **Elution**
   Elute the high molecular weight components with the volumes listed in Table 5.4. Collect the eluate.

[1] *5 mL/min corresponds to approximately 120 drops/min*

Table 5.4. Recommended sample and elution volumes using a syringe; examples of typical yields and remaining salt in the desalted sample

Sample load (mL)	Stacker volume (mL)	Elution volume (mL)	Remaining yield (%)	Salt (%)	Dilution factor
0.25	1.25	1.0	> 95	0.0	4.0
0.50	1.0	1.5	> 95	< 0.1	3.0
1.00	0.5	2.0	> 95	< 0.2	2.0
1.50	0.0	2.0	> 95	< 0.2	1.3

Protocol for HiPrep 26/10 Desalting

1. **Preparation**
   Ensure that appropriate pressure limits have been set and fill the pump tubing with buffer. Connect the column drop-to-drop to the pump tubing to avoid introducing air into the column.
2. **Equilibration**
   First-time use or after long-term storage: Equilibrate the column with 5 CV buffer at 15 mL/min.
3. **Sample application**
   Apply up to 15 mL of sample. Use a flow rate of 20 mL/min.
4. **Elution**
   Elute the column with 2 CV of buffer and collect fractions. High molecular weight compounds start to elute after approximately 15 mL.

Scale-up and processing larger sample volumes

For separation of larger sample volumes or for increased resolution between high and low molecular weight components, up to five HiTrap Desalting or four HiPrep 26/10 Desalting columns may easily be connected in series (Fig 5.9). Connecting columns in series increases the effective bed volume and thereby increases the sample loading capacity. Five HiTrap Desalting columns allow up to 7.5 mL to be processed in one run whereas 60 mL sample can be processed using four HiPrep 26/10 Desalting columns.

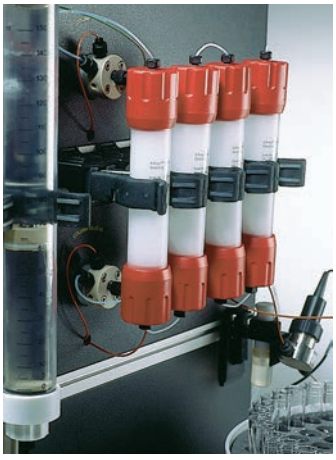


Fig 5.9. Four HiPrep 26/10 Desalting columns connected in series.

Figure 5.10 A to C shows the results obtained when one, three, and five HiTrap Desalting columns were connected in series. A sample volume corresponding to 28% of the total bed volume was applied.

Columns:	HiTrap Desalting, 1 × 5 mL, 3 × 5 mL, 5 × 5 mL
Sample:	2 mg/mL bovine serum albumin in 50 mM sodium phosphate, 500 mM sodium chloride, pH 7.0
Sample volume:	28% of column volume (1.4, 4.3, and 7.1 mL, respectively)
Buffer:	50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0
Flow rate:	5 mL/min

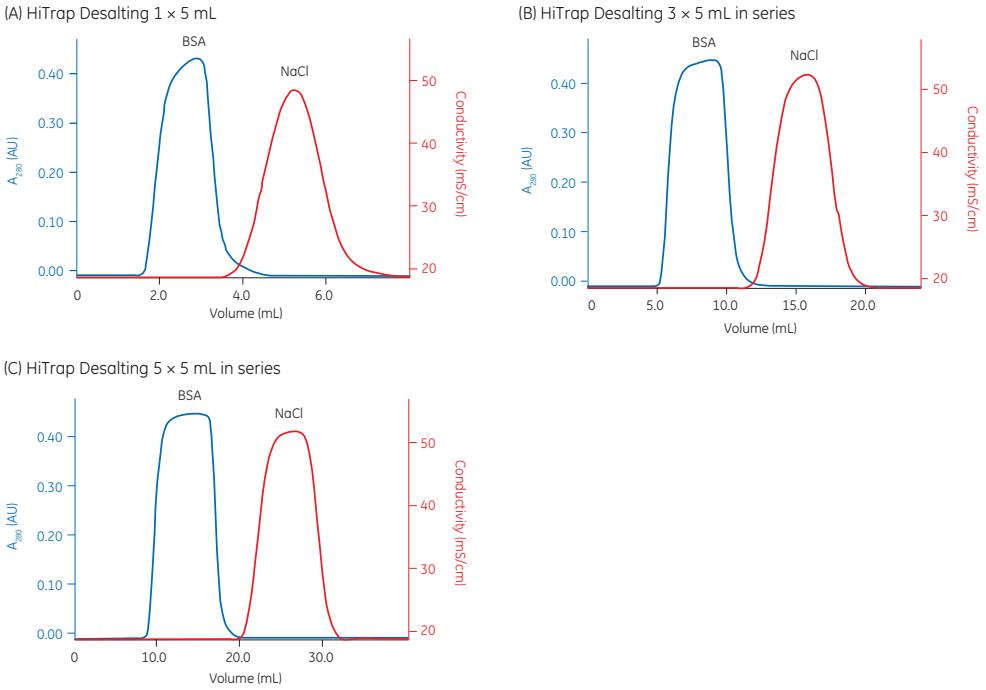


Fig 5.10. Desalting scale-up of bovine serum albumin using HiTrap columns connected in series.

For sample volumes greater than 60 mL, larger columns may be packed. Select a suitable particle size of Sephadex G-25, swell and pack into a short, wide column to facilitate high flow rates and rapid recovery of desalted materials. See Appendix 1 for details on column packing.

The maximum flow rate and sample volume is related to the particle size, as shown in Figure 5.11. Use Sephadex G-25 Medium or Coarse grades packed in columns < 50 cm in bed height to enable high flow rates. The Coarse grade can also be used for batch procedures.

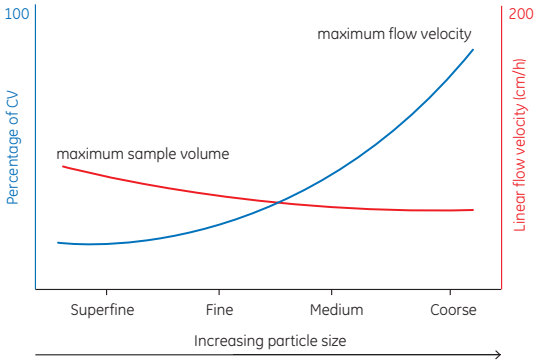


Fig 5.11. Sephadex G-25: recommended sample volumes and flow velocities vary with particle size.

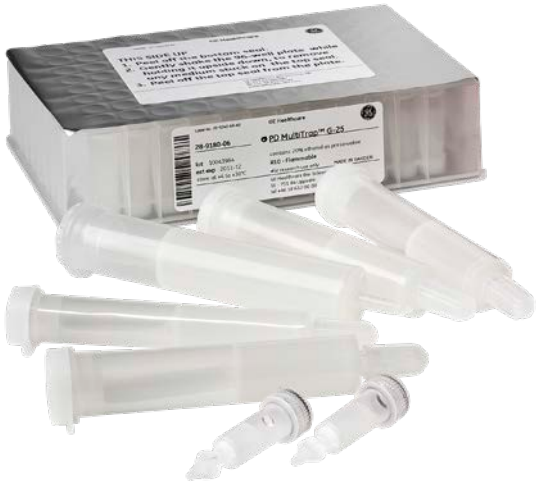


Fig 5.12. Clean-up products for small-scale manual desalting applications.

GE provides an assortment of products for convenient small-scale desalting and buffer exchange, suitable for processing multiple samples in parallel. Columns are manufactured from biocompatible polypropylene. A special bed support of polyethylene protects the resin from running dry during use. The different formats and recommended volumes for sample loading and elution are listed in Table 5.2.

PD MiniTrap, PD MidiTrap and PD-10 Desalting (Fig 5.12) are prepacked, single-use columns designed for manual separations with protocols for gravity flow and centrifugation. PD MiniTrap and PD MidiTrap columns are available packed with Sephadex G-25 and Sephadex G-10. PD-10 Desalting is packed with Sephadex G-25. Sephadex G-10 may also be packed into empty PD-10 columns and run in a similar manner as described in the gravity-flow protocol below.

PD SpinTrap G-25 and PD MultiTrap G-25 are prepacked single-use products for convenient desalting and buffer exchange of small sample volumes. PD SpinTrap columns are designed for use in standard laboratory microcentrifuges. PD MultiTrap is a 96-well filter plate format for high-throughput applications and is designed for use by centrifugation (or vacuum). PD MultiTrap can be used with common automated liquid-handling stations equipped with a centrifuge.

PD columns are also available with Sephadex G-25 DNA grade for desalting and buffer exchange of oligonucleotides and small DNA fragments. The product names are illustra NAP 5, illustra NAP-10, illustra NAP-25, and illustra MicroSpin™ G-25.

Protocols for PD-10 Desalting, PD MiniTrap, and PD MidiTrap

There are two protocols for PD-10 Desalting, PD MiniTrap, and PD MidiTrap columns prepacked with Sephadex G-25. Use the gravity protocols for highest recovery and desalting capacity and use the spin protocols to avoid dilution of the sample.

The gravity and spin protocols for PD-10 Desalting columns are shown below. PD MiniTrap and PD MidiTrap columns are operated similarly. Protocols for these products are found in the product instructions. Gravity-flow protocols are also available for PD MiniTrap and PD MidiTrap prepacked with Sephadex G-10.

Gravity-flow protocol for PD-10 Desalting

1. **Preparation**
   Remove the top cap and pour off the storage solution. Cut the sealed bottom end of the column at the notch. Place the column in a holder such as the supplied Desalting Workmate, above a plastic tray.
2. **Equilibration**
   Equilibrate the column with 25 mL of buffer. Use LabMate PD-10 Buffer Reservoir for convenience.
3. **Sample application**
   Apply up to 2.5 mL of sample. For sample volumes less than 2.5 mL, add buffer up to 2.5 mL (stacker volume) after the sample has entered the packed bed completely. Let the buffer enter the packed bed completely.
4. **Elution**
   Place a test tube for sample collection under the column. Add 3.5 mL buffer to elute the high molecular weight components and collect the eluate. A typical elution profile is shown in Figure 5.13

Column: PD-10 Desalting column
Sample: 2.5 mL of 1 mg/mL bovine serum albumin
Eluent: 1 M NaCl
Separation method: Gravity flow

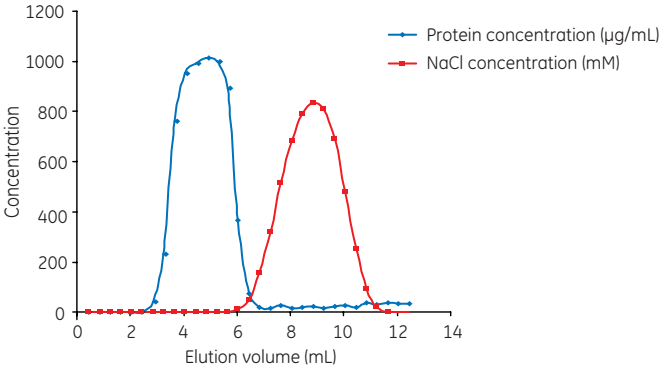


Fig 5.13. Removal of NaCl from bovine serum albumin solution with PD-10 Desalting column.

Spin protocol for PD-10 Desalting

For optimal recovery when applying sample volumes < 2.5 mL, add buffer up to 2.5 mL (stacker volume), when the sample has entered the packed bed.

1. **Preparation**
   Remove the top cap and pour off the column storage solution. If using a fixed-angle rotor, carefully remove the top filter using forceps. This is not necessary when using a swing-out rotor. A higher precision in recovery is obtained with a swing-out rotor. Cut the sealed end of the column at the notch.
2. **Equilibration**
   Equilibrate the column with 25 mL buffer by gravity. Use LabMate PD-10 Buffer Reservoir for convenience. Use a spin adapter (Fig 5.14) and place the PD-10 Desalting column in a 50 mL collection tube. Fill the column with buffer and centrifuge at 1000 × g for 3 min. Discard the flowthrough.
3. **Sample application**
   Use a clean 50 mL collection tube for sample collection. Apply up to 2.5 mL sample. If the top filter is removed, apply the sample slowly in the middle of the packed bed.
4. **Elution**
   Elute by centrifugation 1000 × g for 4 min. Collect the eluate.

Fig 5.14. Spin adapters enable the use of PD-10 Desalting column, as well as PD MidiTrap G-25 and PD MiniTrap G-25 columns in a standard centrifuge.

Protocol for PD SpinTrap G-25

Note: For optimal recovery when applying sample volumes < 100 µL, add buffer up to 100 µL (stacker volume) when the sample has entered the packed bed.

1. **Preparation**
   Suspend the resin by vortexing. Loosen screw cap lid and twist off bottom closure. Place the column in an appropriate size collection tube. Remove the storage solution by centrifugation at 800 × g for 1 min.
2. **Equilibration**
   Apply 400 µL of buffer to equilibrate the column. Centrifuge at 800 × g for 1 min. Discard the flowthrough. Repeat this procedure an additional four times.
3. **Sample application**
   Use a clean collection tube for sample collection. Apply up to 130 µL slowly and in the middle of the packed bed.
4. **Elution**
   Elute by centrifugation at 800 × g for 2 min. Collect the eluate.

Protocol for PD MultiTrap G-25

For optimal recovery when applying sample volumes < 100 µL, add buffer up to 100 µL (stacker volume), when the sample has entered the packed bed.

Note: PD MultiTrap G-25 can also be used with vacuum, but this is not recommended due to reduced reproducibility compared to centrifugation.

1. **Preparation**
   Suspend the resin by gently shaking the plate upside down. Remove the top and bottom seals and place the plate on the collection plate. Remove the storage solution by centrifugation at 800 × g for 1 min.
2. **Equilibration**
   Apply 300 µL buffer per well. Centrifuge at 800 × g for 1 min. Discard the flowthrough. Repeat this procedure an additional four times.
3. **Sample application**
   Use a new clean collection plate for sample collection. Apply up to 130 µL sample slowly in the middle of the packed bed.
4. **Elution**
   Elute by centrifugation at 800 × g for 2 min. Collect the eluate.

Cleaning

HiTrap Desalting, PD-10 Desalting, PD SpinTrap, PD MultiTrap, PD MiniTrap, and PD MidiTrap are single-use products and do not have recommendations for cleaning. They may however be reused a few times depending on the type of sample providing that cross-contamination is not a concern. Cleaning of HiPrep 26/10 Desalting is performed as follows:

1. Wash the column with 2 CV of 0.2 M sodium hydroxide, 0.1 M acetic acid or a solution of a nonionic detergent dissolved in distilled water. Use a flow rate of 10 mL/min. Ensure that the pressure drop does not exceed 0.15 MPa (21.8 psi, 1.5 bar).
2. Immediately wash the column with 5 CV of distilled water at a flow rate of 15 mL/min.
3. Re-equilibrate the column with at least 5 CV of buffer until the UV baseline and pH are stable.

Note: For other cleaning solutions and for extreme cases of contamination check the instructions supplied with the column.

Storage

Store dry Sephadex resin at 4°C to 30°C.

After use, wash the resin with 2 CV of distilled water followed by 2 CV of 20% ethanol before storage. Alternatively, wash with 2 CV of distilled water followed by 2 CV of 10 mM sodium hydroxide. Store swollen resin and columns at 4°C to 30°C. Do not freeze.

Applications

Figures 5.15 to 5.17 show examples of buffer exchange performed on prepacked Sephadex columns.

Sample: $(His)_6$-tagged protein eluted from HiTrap Chelating HP with 20 mM sodium phosphate, 500 mM sodium chloride, 500 mM imidazole, pH 7.4
Column: HiTrap Desalting
Buffer: 20 mM sodium phosphate, 150 mM sodium chloride, pH 7.0
Flow rate: 2.5 mL/min
System: ÄKTAprime

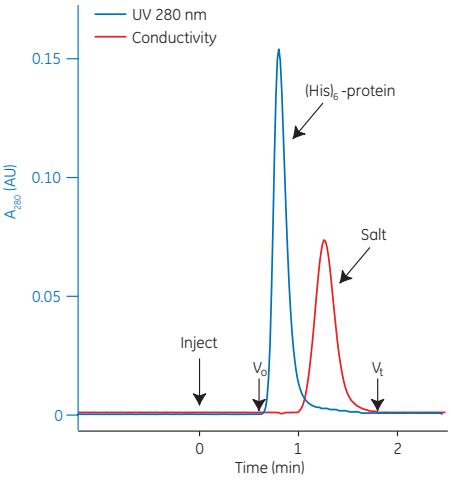


Fig 5.15. Desalting a $(His)_6$-tagged protein using HiTrap Desalting. The UV (protein) and conductivity (salt) traces enable pooling of the desalted fractions and facilitate optimization of the separation.

Column: HiPrep 26/10 Desalting
Sample: 2 mg/mL bovine serum albumin (BSA), 0.07 mg/mL N-hydroxysuccinimide (NHS) in 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0. Filtered through a 0.45 µm filter
Sample volume: 13 mL
Buffer: 50 mM sodium phosphate, 150 mM sodium chloride, pH 7.0
Flow rate: 31 mL/min

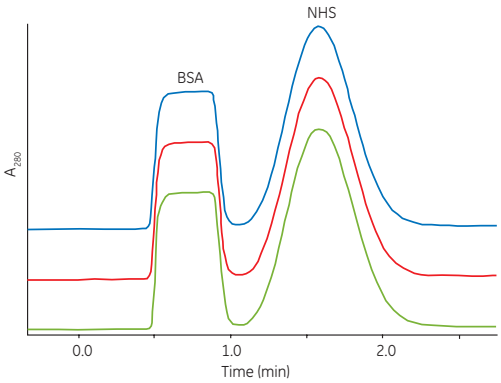


Fig 5.16. Reproducible removal of N-hydroxysuccinimide from bovine serum albumin.

PD products are also excellent for desalting biomolecules other than proteins. Figure 5.17 shows the desalting of tritium-labeled intestinal heparan sulfate ([^{3}H]-HS) after elution from an IEX column using high salt concentration. An elution pool of 1.1 mL was collected. The recovery of [^{3}H]-HS was 87% and > 98% salt was removed.

Column:	PD MidiTrap G-25
Sample:	18 600 cpm bovine intestinal ^{3}H-labeled heparan sulfate ([^{3}H]HS) in 1.5 M NaCl
Sample volume:	0.5 mL
Eluent:	Distilled water

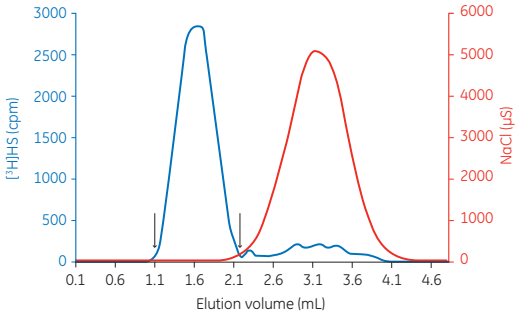


Fig 5.17. Removal of NaCl from [^{3}H]-HS on a PD MidiTrap G-25 column.

Appendix 1
Column packing and preparation

A well-packed column is essential for SEC, especially for high-resolution separations. Prepacked columns from GE will ensure reproducible results and the highest performance. Contact your local GE sales representative to inquire about our column packing services if your selected resin is not available in your required format.

Packing a column is a very critical stage in any SEC experiment. A poorly packed column can give uneven flow, peak broadening, and loss of resolution. It can also affect the maximum flow rate. If you decide to pack a SEC column yourself, follow the guidelines in this Appendix.

SEC is simple to perform once a well-packed column has been obtained. Provided that a column is used and maintained carefully it can be expected to give reproducible, high-resolution results for a long time.

Always use a flow rate for column packing that is higher than the flow rate used for separation. A guideline that works well for many resins is to never exceed 75% of the packing flow rate during the separation.

The maximum flow rate will depend on column diameter and buffer viscosity. Due to wall support, narrow columns support the bed more than wider columns.

Do not exceed the maximum recommended values for pressure or linear flow for the resin. Exceeding these values might cause the resin to compress and reduce the flow rate and resolution during the separation.

Ensure sufficient buffer for the entire run. SEC columns that run completely dry must be repacked.

Columns for packing SEC resins

A broad range of empty columns is available from GE for column packing. Ordering information for empty columns can be found at the back of this handbook.

XK, HiScale™, and Tricorn™ empty columns are optimal for SEC with a design that ensures a uniform liquid flow and minimal dead volumes to minimize dilution and to prevent remixing of separated peaks. All columns are manufactured from materials providing minimized nonspecific adsorption. They are easily dismantled and reassembled for thorough cleaning, which is particularly important when handling biological samples.

Tricorn and XK empty columns are delivered with one adjustable end piece, that is, adapter, and one fixed end piece. A second adapter can be used instead of the fixed end piece if a shorter bed height is required. HiScale columns are equipped with dual adapters. A range of accessories and spare parts are available for all empty columns.

Ensure that the column and all components are clean and in good condition and that no part is damaged. Use well degassed buffers and equilibrate all materials to the temperature at which the separation will be performed. Keep a packed column away from locations that are exposed to drafts or direct sunlight that can cause temperature changes and thereby formation of bubbles.

Longer columns (50 cm and more) can be difficult to pack under normal laboratory conditions. Alternatively, use our column packing services or connect two or more shorter columns (20 or 30 cm bed height) in series to achieve the required bed height.

Table A1.1. Bed heights and volumes for XK columns

		With one adapter		With two adapters	
Column	**Column i.d. (mm)**	**Volume (mL)**	**Bed height (cm)**	**Volume (mL)**	**Bed height (cm)**
XK 16/20	16	5 to 31	2.5 to 15.5	0 to 31	0 to 15.5
XK 16/40	16	45 to 70	22.5 to 35	16 to 70	8 to 35
XK 16/70	16	105 to 130	53 to 65	76 to 130	38 to 65
XK 16/100	16	165 to 190	82.5 to 95	136 to 190	68 to 95
XK 26/20	26	5 to 66	1 to 12.5	0 to 66	0 to 12.5
XK 26/40	26	122 to 186	23 to 35	45 to 186	8.5 to 35
XK 26/70	26	281 to 344	53 to 65	204 to 344	38.5 to 65
XK 26/100	26	440 to 504	83 to 95	365 to 504	68.5 to 95
XK 50/20	50	0 to 274	0 to 14	0 to 274	0 to 14
XK 50/30	50	265 to 559	14 to 28	0 to 559	0 to 28
XK 50/60	50	794 to 1090	40 to 56	500 to 1090	26 to 56
XK 50/100	50	1590 to 1860	81 to 95	1270 to 1860	65 to 95

Table A1.2. Maximum bed heights and volumes for HiScale columns

		With two adapters	
Column	**Column i.d. (mm)**	**Volume (mL)**	**Bed height (cm)**
HiScale 16/20	16	40	20
HiScale 16/40	16	80	40
HiScale 26/20	26	106	20
HiScale 26/40	26	212	40
HiScale 50/20	50	393	20
HiScale 50/40	50	785	40

Table A1.3. Bed volumes and heights for Tricorn columns

		With one adapter		With two adapters	
Column	**Column i.d. (mm)**	**Volume (mL)**	**Bed height (cm)**	**Volume (mL)**	**Bed height (cm)**
Tricorn 10/150	10	10.1 to 12.5	12.9 to 15.9	7.6 to 12.3	9.6 to 15.6
Tricorn 10/200	10	14.1 to 16.4	17.9 to 20.9	11.5 to 16.2	14.6 to 20.6
Tricorn 10/300	10	21.9 to 24.3	27.9 to 30.9	19.4 to 24.1	24.6 to 30.6
Tricorn 10/600	10	45.5 to 47.8	57.9 to 60.9	42.9 to 47.6	54.6 to 60.6

Checking column efficiency

A column performance test should be done before first-time use of a column. Column performance should also be checked at regular intervals by determining the theoretical plate number and the peak symmetry. Note that the measured result for column efficiency is dependent on the system used, including the capillaries and dead volumes. See Appendix 2. *Determination of column efficiency and asymmetry factor*, for further details.

Column packing for preparative SEC

Superdex prep grade, Superose prep grade and Sephacryl High Resolution (HR) resins can easily be packed in XK or similar columns. For preparative SEC, it is recommended to use bed heights between 30 and 60 cm. The volume of settled resin should be ~ 1.15-fold that of the required packed bed volume.

The following packing protocol works well for packing most columns. Further guidelines can be found in the individual instructions for the resins and in the instructions for empty columns.

General packing protocol

1. The resins are supplied in a suspension containing 20% ethanol. Resuspend the resin by shaking gently. Avoid using magnetic stirrers, spatulas or glass rods since they can damage the resins.
2. Wash the resin with 5 to 10 CV of distilled water on a glass filter and resuspend in distilled water to a final concentration of 50% settled resin. The resin must be thoroughly washed to remove the 20% ethanol storage solution. The shipping solution for Superdex 30 prep grade and Superdex 75 prep grade also contains 0.2 M sodium acetate. These resins need to be more thoroughly washed before packing. See Superdex prep grade Instructions 18106029 for further information.

   To produce a more evenly dispersed slurry, 0.05% Tween 20 may be added to reduce surface tension.
3. Prepare a suitable empty column for packing according to the column instructions. Mount a bottom filter if this is not part of the end piece. Wet the filter. If the slurry volume exceeds the column volume, attach a packing reservoir or packing tube.

   Using a second column tube and a packing connector instead of using a packing reservoir makes it easier to obtain a well-packed column.
4. Fill the column with distilled water to a height of approximately 2 cm.
5. Resuspend and pour the well-degassed slurry down the inside of the column in one continuous step. Fill up the column or column reservoir with distilled water. Avoid introducing air bubbles.
6. Attach a top adapter to the column tube (or the cap on the packing reservoir). Connect to a pump and open the column outlet.

   To achieve satisfactory column efficiency, pack Superdex prep grade and Superose prep grade resins in two steps: Step 1 for 2 h or until the bed has reached a constant height and Step 2 for 60 min. For packing flow rates in XK columns, see Table A1.4.

   Sephacryl HR can usually be packed satisfactorily using only the higher flow rate given in Step 2 of Table A1.4. Use the two-step process if the column efficiency is unsatisfactory after the first attempt.
7. Stop the pump and remove the packing reservoir or packing tube if used. Fill up the column with distilled water and mount a wetted adapter onto the column as described in the column instructions. Also, mount a wetted filter if this is not part of the adapter. Ensure no air bubbles are trapped in the column. Avoid introducing air bubbles.
8. Continue packing the column at the flow rate used in Step 2 for approximately 10 min. Mark the position of the bed surface, stop the pump, close the column outlet, adjust the adapter down to the mark and then a further 3 mm into the resin. The column is now ready for use.

Do not exceed maximum pressures during packing:

- 0.2 MPa (2 bar, 29 psi) to 0.4 MPa (4 bar, 58 psi) for Sephacryl S-100 HR, S 200 HR, and S-300 HR
- 0.15 MPa (1.5 bar, 21 psi) for Sephacryl S-400 HR and S-500 HR
- 0.4 to 0.5 MPa (5 bar, 72.5 psi) for Superdex prep grade (30, 75, and 200).
- 0.4 MPa (4 bar, 58 psi) for Superose 6 prep grade.
- 0.7 MPa (7 bar, 101.5 psi) for Superose 12 prep grade.

Table A1.4. Maximum bed heights and recommended flow rates during column packing of different SEC resins in XK columns

		Sephacryl HR		Superdex and Superose prep grade	
Column	**Bed height (cm)**	**Step 1 (mL/min)**	**Step 2 (mL/min)**	**Step 1 (mL/min)**	**Step 2 (mL/min)**
XK 16/40	35	1 to 2	2 to 4	1 to 2	4 to 6
XK 16/70	65	1 to 2	2 to 4	1 to 2	4 to 6
XK 16/100	95	1 to 2	2 to 4	1 to 2	4 to 6
XK 26/40	35	2 to 4	4 to 8	2 to 4	10 to 14
XK 26/70	65	2 to 4	4 to 8	2 to 4	10 to 14
XK 26/100	95	2 to 4	4 to 8	2 to 4	10 to 14
XK 50/20	15	8 to 10	4 to 8	9 to 11	19 to 21
XK 50/30	25	8 to 10	10 to 14	9 to 11	19 to 21
XK 50/60	55	8 to 10	10 to 14	9 to 11	19 to 21
XK 50/100	95	8 to 10	10 to 14	9 to 11	19 to 21

Column packing for buffer exchange using Sephadex resin

Sephadex G-10, G-25, and G-50 resins can easily be packed in for example XK or HiScale columns. For buffer exchange, a column bed height of 10 cm is recommended. Sephadex is supplied as a dry powder and must be allowed to swell in excess packing solution before use.

Accelerate the swelling process by using a boiling water bath (Table A1.5). This also serves to degas the suspension. Allow the suspension to cool before use.

Table A1.5. Bed volume and swelling times for Sephadex resins

Resin	**Approx. bed volume (mL/g resin)**	**Swelling time (h), 20°C**	**Swelling time (h), 90°C**
Sephadex G-10 Medium	2 to 3	3	1
Sephadex G-25 (all grades)	4 to 6	3	1
Sephadex G-50 Fine	9 to 11	3	1

The following packing protocol works well for packing most columns. Further guidelines can be found in the individual instructions for the resins and in the instructions for empty columns:

General packing protocol

1. Weigh out the correct amount of dry Sephadex and allow the resin to swell as described above. Avoid using magnetic stirrers, spatulas, or glass rods since they can damage the resin. After swelling, adjust with buffer to form a thick slurry, apply vacuum to remove air bubbles. Approximately 75% settled resin is suitable. Fine particles can be decanted.
2. Prepare a suitable empty column for packing according to the column instructions. Mount a bottom filter if this is not part of the end piece. Wet the filter. If the slurry volume is greater than the volume of the column, attach a packing reservoir or packing tube.
3. Fill the column with the packing solution to a height of approximately 2 cm.
4. Resuspend and pour the well-degassed slurry down the inside of the column in one continuous step. Fill the column or the packing reservoir to the top with packing solution. Avoid introducing air bubbles.
5. Attach a top adapter to the column tube (or the cap on the packing reservoir). Connect to a pump, open the column outlet and start the flow. Pump 2 to 3 CV of packing solution through the column to stabilize the bed and equilibrate completely. Use a slightly higher flow rate than the flow rate to be used during separations. Maintain the packing flow rate for at least 3 CV after a constant bed height is obtained.
6. Close the column outlet and remove the packing reservoir or packing tube if used. Fill up the column with packing solution and mount a wetted adapter onto the column as described in the column instructions. Also, mount a wetted filter if this is not part of the adapter. Ensure no air bubbles are trapped in the column and that no air bubbles are in the flow path.
7. Open the column outlet and continue packing the column for approximately 10 min. Mark the position of the bed surface, stop the pump, close the column outlet, adjust the adapter down to the mark and then a further 3 mm into the resin. The column is now ready for use.

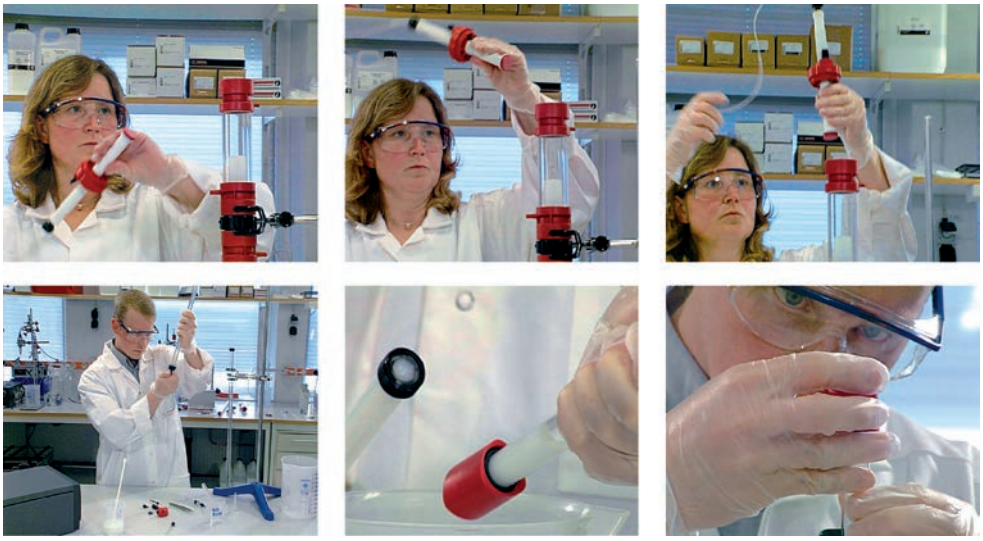


Fig A1.1. A well-packed column is essential for SEC, especially for high-resolution separations.

Appendix 2
Determination of column efficiency and asymmetry factor

Column performance is essential for all SEC separations, particularly for high-resolution separations. Two parameters are generally used to characterize the column performance:

- **Efficiency** (theoretical plate number per meter, N/m) describes the column ability to produce narrow peaks.
- **Peak asymmetry factor (A_s)** describes the symmetry of the peak.

To detect any column changes, column performance should be checked at regular intervals. A column performance test should also be carried out before first-time use of a column to evaluate the column packing and to create a baseline for the column performance. Likewise, a column performance test is recommended for prepacked columns. Note that the measured column efficiency and peak asymmetry are affected by the chromatography system used, including the capillaries and other dead volumes. This means that the measured column efficiency can deviate from values given in the specification for the column. Keeping the internal volumes of the chromatography system low is particularly important working with small columns for high-resolution separations. See Chapter 2 section *System configuration* for further details.

Column efficiency test

The efficiency and peak asymmetry can be determined as follows:

1. Equilibrate the packed column in distilled water or buffer at the flow rate recommended in the product instructions.
2. Inject 2% acetone (20 mg/mL in water) in a volume equivalent to 0.2% to 0.4% of the geometrical bed volume.
3. Monitor the UV absorbance at 280 nm and determine the elution volume for acetone.
4. Calculate column efficiency, that is, the number of theoretical plates per meter (N/m):

$$\frac{N}{m} = 5.54 \times \left(\frac{V_e}{W_{1/2}}\right)^2 \times \frac{1}{L}$$

where:

V_e = peak elution (retention) volume

$W_{1/2}$ = peak width at half peak height

L = bed height (m)

V_e and $W_{1/2}$ are in the same units

5. Calculate the asymmetry factor (A_s):

where:

a = first half peak width at 10% peak height
b = second half peak width at 10% peak height

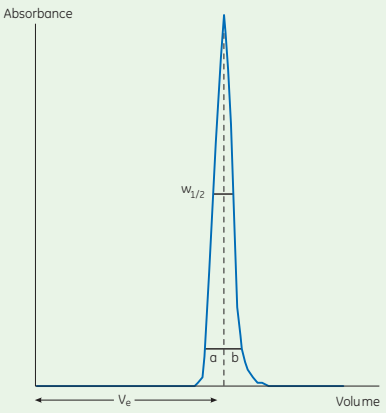


Fig A2.1. Determination of column efficiency by number of theoretical plates and peak asymmetry.

Typical values for column performance:

Superdex prep grade:	Efficiency, N/m > 10 000, peak asymmetry, A_s = 0.70 to 1.30
Superdex Increase:	Efficiency, N/m > 40 000, peak asymmetry, A_s = 0.70 to 1.20

The column performance may also be checked by running a function test using a suitable sample. This test is an alternative to the above efficiency test and will also provide information regarding resolution. Descriptions for how a function test can be done are included in many of the instructions for prepacked columns, see for example Instructions *Superdex 75 Increase 10/300 GL and Superdex 75 Increase 5/150 GL*, 29163059.

Appendix 3
Selection of purification equipment

Accurate, reproducible control of the separation is critical for good results, but also for reliability in routine preparative work and repeated experiments. Use a liquid chromatography system (rather than a peristaltic pump or gravity flow) to fully utilize the potential of the column. When working with small prepacked columns such as HiTrap Desalting, a syringe or standalone pump may also be used. For buffer exchange of small sample volumes, gravity and spin columns are also available.

Note that the chromatography system used might affect the resolution of the separation. For highest resolution, it is important that the chromatography system has as small internal volumes as possible. This is especially important working with small columns for high-resolution separation.

System recommendations for high-resolution SEC columns

The small bed volume of 3.2/300, and 5/150 columns makes them extra sensitive to dead volumes in the system. For these columns, systems such as ÄKTA pure 25 and older systems such as ÄKTA micro and ÄKTApurifier 10 are recommended.

Depending on the chromatography system setup, the contributions from dead volumes to band broadening will vary. Use short, narrow tubing and avoid unnecessary components in the flow path. The injection technique used can also affect the resolution and needs to be considered when working with high-resolution separations. ÄKTA pure may be configured for microscale purification. For optimal configuration of AKTA pure 25, see *Optimal configuration of ÄKTA pure 25 for small scale SEC*, 29181181. The 10/300 SEC column has a larger bed volume and may also be used in systems such as ÄKTA avant 25 and older systems such as ÄKTAexplorer 10. See Chapter 2, section *System configuration* for further details.

Note that ÄKTA start chromatography system is not compatible with Superdex Increase and Superose Increase columns (3.2/300, 5/150, or 10/300), see *Prepacked chromatography columns for ÄKTA systems*, 28931778.

Table A3.1. Selection of purification equipment for buffer exchange and high-resolution separations

	ÄKTA protein purification system				
Application	**ÄKTA start**	**ÄKTAprime plus**	**ÄKTAxpress**	**ÄKTA pure**	**ÄKTA avant**
Simple, one-step desalting, buffer exchange	×	×	×	×	×
Automated and reproducible protein purification using all common techniques including support for gradient elution	×[1]	×	×	×	×
Software compatible with regulatory requirements, e.g., good laboratory practice (GLP)			×	×	×
Method development and optimization using DoE				(×)	×
Automatic buffer preparation					×
Automatic buffer scouting				(×)	×
Automatic column scouting				(×)	×
Automatic multistep purification		(×)	×	(×)	(×)
Scale-up, process development				(×)	×
Software[2] for system control and data handling	UNICORN™ start	PrimeView[3]	UNICORN 5	UNICORN 6 or later	UNICORN 6 or later

[1] *For SEC, ÄKTA start is not compatible with Superdex Increase and Superose Increase columns (10/300 GL, 5/150 GL, and 3.2/300).*

[2] *A specific software version might be needed for the chosen system. See the web page for each respective system at gelifesciences.com*

[3] *With PrimeView, you can monitor results and evaluate data but not create method nor control the system.*

× = *included*
(×) = *optional configuration*
DoE = Design of experiments

ÄKTA start

ÄKTAprime plus

ÄKTAxpress

ÄKTA pure

ÄKTA avant

Fig A3.1. Selection of chromatography systems from GE that are suitable for size exclusion chromatography, buffer exchange, or desalting.

Appendix 4
Column pressure limits and maximum flow rate

Pressure is generated by the flow through the column and the chromatographic system. The pressure may be expressed in megapascal (MPa), bar, or pounds per square inch (psi) and can be converted as follows: 1 MPa = 10 bar = 145 psi.

Column pressure limits

To protect the column hardware and the bed of the packed resin, it is important to set limits that must not be exceeded during the run. There are two important pressures that need to be considered:

1. **Column hardware pressure:** The column hardware pressure limit is the maximum pressure the hardware can withstand without being damaged (Fig A4.1). This value is fixed for each column type. The column hardware pressure limit for each column type is included in the column instructions and in the UNICORN column list, in ÄKTA systems.

   The pressure on the column hardware equals the sum of the back pressure generated by the column itself and the back pressure generated by the system components located after the column. This sum must never exceed the column hardware pressure limit.

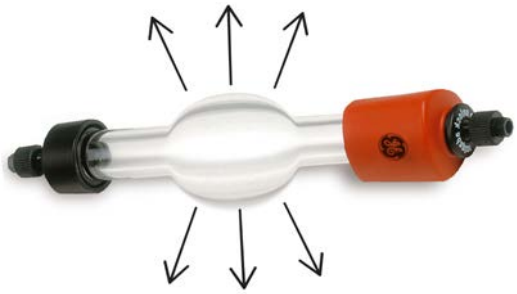


Fig A4.1. The column hardware pressure limit is the maximum pressure the column hardware can withstand without damage.

2. **Pressure drop over the packed bed:** The pressure drop over the packed bed is caused by flow resistance when a solution is passed through the column. The pressure over the packed bed can be described by the Hagen-Poiseuille equation[1] and depends on several parameters including:

   - Flow rate
   - Viscosity of sample and eluent including running temperature
   - Bed height
   - Resin particle size and shape
   - The resin void fraction (V_o/V_c)

[1] *See pp 71, equation 3.27 in Janson, J.-C. ed.* Protein Purification: Principles, High Resolution Methods, and Applications, *third edition, Wiley Online Library (2011). doi: 10.1002/9780470939932*

Note that several of these factors are related to how the column has been packed.

The maximum pressure drop over the packed bed or the maximum delta pressure (Δp), is the maximum pressure the packed bed can withstand without risking gap formation (Fig A4.2) or bed collapse. This value is strongly dependent on how the column has been packed.

For many columns, the maximum Δp provided in the product instructions and in the UNICORN column list is a typical value (see Tables 4.2 and 4.7). The actual value is individual for each column and needs to be determined. The procedure for doing this is described in Instructions for the column at hand, for example *Superdex 200 Increase columns*, 29027271. Note that most prepacked columns are designed to withstand a maximum flow, not a maximum pressure. Consequently, the packed bed is best protected by controlling the flow rate. However, insufficient column cleaning can increase column back pressure over time. Determine the column specific pressure limit as well as the initial column back pressure at first-time-use and monitor the back pressure regularly.

Fig A4.2. The maximum pressure over the packed bed is the maximum pressure the packed bed of chromatography medium can withstand without gap formation. This is not a fixed value.

The pressure drop over different parts of a chromatography system and how it affects the column is described in Figure A4.3.

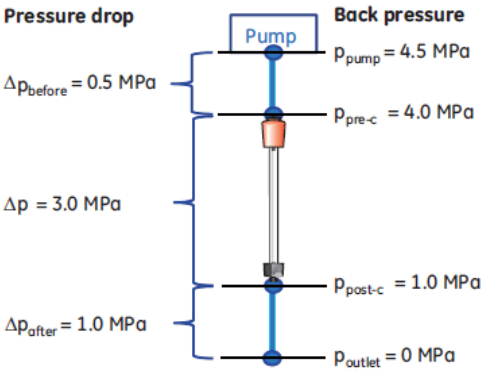


Fig A4.3. Example of the pressure in different parts of a system during run of a column. Note that the pressure values are only used to illustrate the principle. All ÄKTA systems measure pressure at the system pump, P_{pump}. Some systems have additional pressure sensors located before and after the column, $P_{pre\text{-}c}$ and $P_{post\text{-}c}$, which enables direct measurement of the pressure drop over the packed bed, Δp.

Δp_{before} does not affect the column hardware or the packed bed.

The pressure on the column hardware is the sum of Δp_{after} and Δp. Do not exceed the column hardware limit!

For many columns, Δp is individual and needs to be determined for each column.

For more information, refer to the *ÄKTA Laboratory-scale Chromatography Systems Instrument Management Handbook*, 29010831.

Maximum flow rate

The maximum flow rate of a prepacked column is found in the product instructions and the UNICORN column list. The value listed is generally valid for water at room temperature.

When packing your own column, the maximum flow rate will depend on the properties of the resin, the packing procedure as well as the column dimensions. Due to wall support, narrow columns support the bed more than wider columns, which allows higher linear flow. A guideline that works well for many resins is to never exceed 75% of the packing flow rate during the separation.

When changing running conditions such as temperature or viscosity, the maximum flow rate is affected (Table A4.1). It is therefore important to adjust the flow rate according to the running conditions used. Use lower flow rates for high-viscosity solutions and/or low temperature.

Table A4.1. Example of maximum flow rate limits at different viscosity and temperature, Superdex 30 Increase 10/300 GL

Temperature	Buffer	Flow rate (mL/min)
20°C to 25°C	Water	1.20
	20% ethanol	0.60
	10% glycerol	0.60
	30% acetonitrile	1.20
	40% methanol	0.60
4°C to 8°C	Water	0.60
	20% ethanol	0.30
	10% glycerol	0.30
	30% acetonitrile	0.60
	40% methanol	0.30

The viscosity of the sample also needs to be considered. When running viscous samples such as samples containing glycerol, it is important to dilute the sample or lower the flow rate, see example above. High protein concentration or additives in the sample can cause instability of the separation and the back pressure might increase.

Note that the viscosity of liquids increases with high salt concentrations, some additives, and low temperature. Mixing organic and aqueous solution will also affect the viscosity. Figure A4.4 shows some examples of how the viscosity varies for commonly used liquids and temperatures in chromatography.

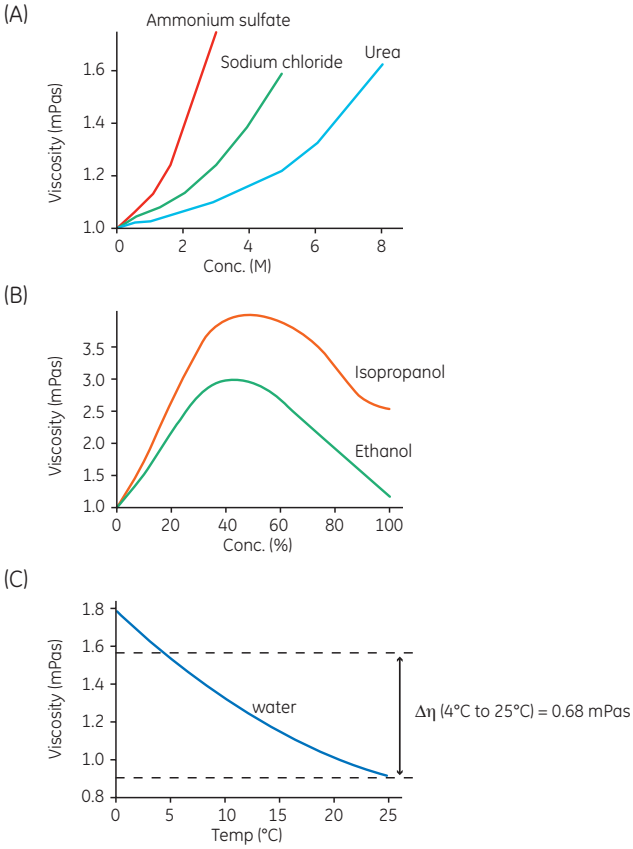


Fig. A4.4 Effect of type of salt, salt concentration (A), content of organic solvents in water (B), and temperature (C) on viscosity. 1 mPas = 1 cP.

Appendix 5
Converting flow rates and velocities; other conversion data

Converting between volumetric flow rate (mL/min) and linear flow velocity (cm/h)

When comparing results for columns of different sizes, it is convenient to express flow as linear flow velocity (cm/h). However, flow is usually measured in volumetric flow rate (mL/min). To convert between linear flow velocity and volumetric flow rate use one of the formulas below:

From volumetric flow rate (mL/min) to linear flow velocity (cm/h)

$$\text{Linear flow velocity (cm/h)} = \frac{\text{Volumetric flow rate (mL/min)} \times 60}{\text{column cross-sectional area (cm}^2\text{)}}$$

$$= Z \times 60 \times \frac{4}{\pi \times d^2}$$

where

Z = volumetric flow rate in mL/min
d = column inner diameter in cm

Example:

What is the linear flow velocity in a Tricorn 5/50 column (i.d. 0.5 cm) when the volumetric flow rate is 1 mL/min?

Z = volumetric flow rate = 1 mL/min
d = column inner diameter = 0.5 cm

$$\text{Linear flow velocity (cm/h)} = 1 \times 60 \times \frac{4}{\pi \times 0.5 \times 0.5} \text{ cm/h}$$

$$= 306 \text{ cm/h}$$

From linear flow velocity (cm/h) to volumetric flow rate (mL/min)

$$\text{Volumetric flow rate (mL/min)} = \frac{\text{Linear flow velocity (cm/h)}}{60} \times \text{column cross-sectional area (cm}^2\text{)}$$

$$= \frac{Y}{60} \times \frac{\pi \times d^2}{4}$$

where

Y = linear flow velocity in cm/h
d = column inner diameter in cm

Example:

What is the volumetric flow rate in an XK 16/70 column (i.d. 1.6 cm) when the linear flow velocity is 150 cm/h?

Y = linear flow velocity = 150 cm/h
d = column inner diameter = 1.6 cm

$$\text{Volumetric flow rate (mL/min)} = \frac{150 \times \pi \times 1.6 \times 1.6}{60 \times 4} \text{ mL/min}$$

$$= 5.0 \text{ mL/min}$$

From volumetric flow rate (mL/min) to using a syringe

5 mL/min = approximately 120 drops/min on a HiTrap Desalting 5 mL column

Protein weight to molar quantity

Protein size and amount conversion

M_r	1 µg protein	1 nmol protein
10 000	100 pmol; 6×10^{13} molecules	10 µg
50 000	20 pmol; 1.2×10^{13} molecules	50 µg
100 000	10 pmol; 6.0×10^{12} molecules	100 µg
150 000	6.7 pmol; 4.0×10^{12} molecules	150 µg

Nucleic acid molecular weight

Approximate molecular weights (M_r) of nucleic acids

M_r of ssRNA = (no. of nucleotides × 320.5) + 159.0
M_r of ssDNA = (no. of nucleotides × 303.7) + 79.0
M_r of dsDNA = (no. of nucleotides × 607.4) + 157.9

Pressure units

Pressure units may be expressed in megapascal (MPa), bar, or pounds per square inch (psi) and can be converted as follows: 1 MPa = 10 bar = 145 psi.

Appendix 6
Molecular weight estimation

Unlike electrophoretic techniques, SEC provides a means of determining the molecular weight or size (Stokes radius) of native or denatured proteins under a wide variety of conditions of pH, ionic strength, and temperature.

To fully understand and follow the procedures outlined, it is important to have read Chapter 1, section *Size exclusion chromatography in theory*. Refer also to the list of *Common acronyms and abbreviations* at the beginning of this handbook for chromatography acronyms and definitions described in this Appendix.

For molecular weight determination, several theoretical models have been proposed to describe the behavior of solutes during SEC. Most models assume that the partition of molecules between the particles and surrounding liquid is an entirely steric effect. In practice, a homologous series of compounds demonstrate a sigmoidal relationship between their various elution volume parameters and the logarithm of their molecular weights. Thus, molecular weight determination by SEC can be made by comparing an elution volume parameter, such as the distribution coefficient (K_{av}) of the substance of interest, with the values obtained for several known calibration standards.

A calibration curve is prepared by measuring the elution volumes of several standards, calculating their corresponding K_{av} values (or similar parameter), and plotting their K_{av} values vs the logarithm of their molecular weight. The molecular weight of an unknown substance can be determined from the calibration curve once its K_{av} value is calculated from its elution volume.

Various elution parameters, such as V_e (elution volume), V_e/V_0 (elution volume/void volume), K_d (distribution coefficient), and K_{av} have been used in the literature for the preparation of calibration curves. However, the use of K_{av} is recommended since it is:

1) less sensitive to errors caused by variations in column preparation and column dimension
2) does not require determination of the internal volume (V_i) as is required with K_d.

For good estimation of molecular weight, calibration standards must have the same relationship between molecular weight and molecular shape as the substance of interest. Calibration kits from GE provide well-characterized, globular protein standards for molecular weight estimation in the low molecular weight (LMW) and high molecular weight (HMW) range.

The Gel Filtration LMW Calibration Kit contains five individually lyophilized proteins with molecular weights in the range 6 500 to 75 000 and Blue Dextran 2000, which is used to determine the V_0 of the column (Table A6.1). The Gel Filtration HMW Calibration Kit contains five individually lyophilized proteins with molecular weights in the range 43 000 to 669 000 together with Blue Dextran 2000 (Table A6.2). These well-defined protein standards show excellent behavior in SEC and enable simple, reliable calibration of SEC columns.

Note that the molecular weight determinations of glycoproteins, lipoproteins, nonglobular proteins, or other polymers may not correlate well to calibration curves with globular proteins. For such compounds, useful information can be obtained by relating the elution volume data to a molecular size parameter, such as Stokes radius (R_{St}), rather than to molecular weight. The calibration kit proteins may be used for these plots as well.

Table A6.1. Characteristics of Gel Filtration LMW Calibration Kit

Protein (weight per vial)	Molecular weight (M_r)	Source
Aprotinin (10 mg)	6500	Bovine lung
Ribonuclease A (50 mg)	13 700	Bovine pancreas
Carbonic anhydrase (15 mg)	29 000	Bovine erythrocytes
Ovalbumin (50 mg)	43 000	Hen egg
Conalbumin (50 mg)	75 000	Chicken egg white
Blue Dextran 2000 (50 mg)	2 000 000	

Table A6.2. Characteristics of Gel Filtration HMW Calibration Kit

Protein (weight per vial)	Molecular weight (M_r)	Source
Ovalbumin (50 mg)	43 000	Hen egg
Conalbumin (50 mg)	75 000	Chicken egg white
Aldolase[1] (50 mg)	158 000	Rabbit muscle
Ferritin[1] (15 mg)	440 000	Horse spleen
Thyroglobulin (50 mg)	669 000	Bovine thyroid
Blue Dextran 2000 (50 mg)	2 000 000	

[1] *These proteins are supplied mixed with sucrose or mannitol to maintain stability and aid their solubility.*

Typical elution profiles from calibrations performed on prepacked Superdex columns and corresponding calibration curves are shown in Figures A6.1 and A6.2.

Sample: Proteins from Gel Filtration Calibration Kits LMW and HMW: aprotinin (Apr), RNase A (R), carbonic anhydrase (CA), ovalbumin (O), conalbumin (C), aldolase (Ald), ferritin (F), and thyroglobulin (T)
Sample vol.: Figures A10.1 and A10.2, 100 µL
Buffer: 50 mM phosphate buffer, 150 mM sodium chloride, pH 7.2
Flow rate: Figure A10.1, 0.5 mL/min
Figure A10.2, 0.6 mL/min

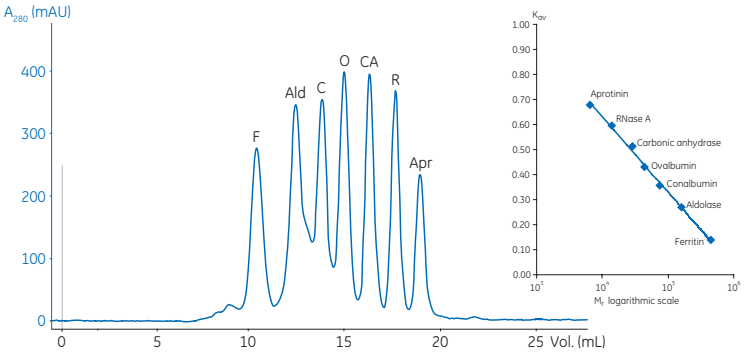


Fig A10.1. Chromatographic separation and calibration curve for the standard proteins on Superdex 200 10/300 GL column.

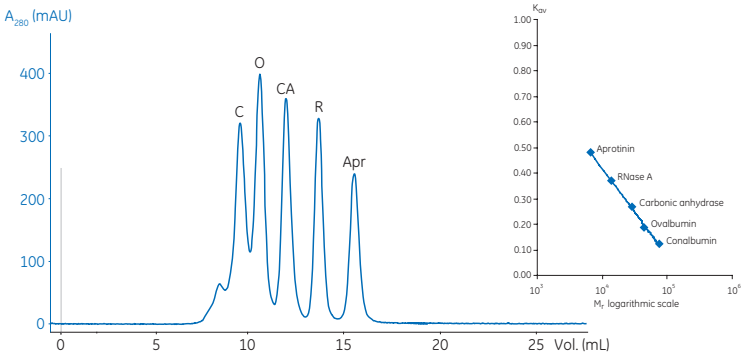


Fig A10.2. Chromatographic separation and calibration curve for the standard proteins on Superdex 75 10/300 GL column.

Many of the parameters important for a successful molecular weight determination are the same as for any high-resolution separation:

Use a resin with the correct fractionation range for the molecules of interest. The expected molecular weight values should fall in the linear part of the selectivity curve. If the molecular weight is unknown, use a resin with a wide fractionation range such as Superose 6 Increase. Use a prepacked column whenever possible.

Use freshly prepared calibration standards, selected so that the expected molecular weight values are covered by the calibration range. Always filter Blue Dextran 2000 before use.

Use the same buffer for the separation of calibrants and sample.

Performing a molecular weight determination in the presence of urea, guanidine hydrochloride, or SDS transforms polypeptides and proteins to a random coil configuration and reduces structural differences. Differences will be seen in the resulting molecular weight values when compared to values acquired under nondenaturing conditions.

Deviations from a K_{av}:log M_r calibration curve can occur if the molecule of interest does not have the same molecular shape as the standards. Under certain conditions, factors other than the size and shape of the molecules being studied can also influence the separation. These nonspecific interactions can usually be avoided and are generally only significant when performing chromatography of highly charged substances at low ionic strength; or aromatic molecules on dense resins with high matrix content (higher dry weight of resin).

The behavior of thyroglobulin is nonlinear on Superose 6 Increase and may be excluded from the calculation of K_{av} on Superose 6 Increase columns. Thyroglobulin should however be included in a plot of $\sqrt{(-\log K_{av})}$ vs Stokes radius (R_{St}).

Performing a molecular weight determination

1. Prepare a fresh, filtered solution of Blue Dextran 2000 (1 mg/mL) in the running buffer. Apply Blue Dextran to the column, using a sample volume < 2% of the total column volume (V_c).
2. Dissolve the selected calibration standards in the running buffer (at concentrations recommended by the manufacturer). If necessary, filter the calibration solution.
3. Apply the calibration solution to the column, in a sample volume < 2% of V_c.
4. Determine the elution volumes (V_e) for the standards.

5. Calculate the K_{av} values for the standards and prepare a calibration curve of K_{av} vs the logarithm of their molecular weights, as follows:

$$K = \frac{V_e - V_0}{V_c - V_0}$$

where

V_e = elution volume for the standard
V_0 = column void volume = elution volume for Blue Dextran 2000
V_c = total column volume

6. Plot the K_{av} value for each standard against the corresponding logarithmic molecular weight and calculate the regression line.

7. Apply the sample in the same volume as used for the standards and determine the elution volume (V_e) for the molecule of interest.

8. Calculate the corresponding K_{av} for the molecule of interest and determine its molecular weight from the calibration curve.

A calibrated column may be used for extended periods if the column is kept in good condition.

Appendix 7
Sephadex LH-20

Sephadex LH-20 is a special resin designed for separation and purification of natural substances, which require the presence of organic solvents to maintain their solubility. This makes the resin useful for purification of molecules such as steroids, terpenoids, lipids, and low molecular weight peptides (up to 35 amino acid residues).

In addition to SEC applications, Sephadex LH-20 is often used for other types of chromatography. The hydrophilic and hydrophobic properties of the resin make it useful for liquid/liquid partition chromatography. Moreover, the resin can have a strong affinity for aromatic compounds enabling adsorption chromatography. The aromatic adsorption is most pronounced when lower alcohols such as isopropanol are used as solvent.

Due to its special physicochemical properties, Sephadex LH-20 offers an alternative selectivity to other SEC resins and is often used for preparation of closely related substances. Sephadex LH-20 has found widespread use in the isolation of active components from herbal extract used in traditional chinese medicine (TCM). It can be used either during initial purification prior to polishing by ion exchange or reversed phase chromatography, or as the final polishing step, for example, during the preparation of diastereomers.

Resin characteristics

Sephadex LH-20 consists of hydroxypropylated cross-linked dextran particles with both hydrophilic and hydrophobic character. The resin is made from Sephadex G-25 and can be swollen in water or organic solvents. Table A7.1 summarizes the characteristics of Sephadex LH-20.

Table A7.1. Characteristics of Sephadex LH-20

Matrix	Hydroxypropylated, cross-linked dextran
Exclusion limit (M_r) globular proteins	4000 to 5000 (depends on solvent)
Sample loading:	
adsorption mode	Depends on resolution required
molecular sizing	< 2% of total bed volume
partition mode	< 1% of total bed volume
Particle form	Spherical, porous
Average diameter (dry)	70 µm
Recommended max. operating flow velocity	720 cm/h
Rec. operating flow velocity	60 cm/h
pH stability	
operational[1]	2 to 13
CIP[2]	2 to 13
Chemical stability	Stable in most aqueous and organic eluent systems. The resin should not be exposed to strong oxidizing agents.
Autoclavable	20 min at 121°C
Operating temperature	4°C to 40°C

[1] *pH range where resin can be operated without significant change in function.*
[2] *pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.*

Packing a column

General packing protocol

The following packing protocol works well for packing most columns. Further guidelines can be found in the individual instructions for the resins and in the instructions for empty columns.

1. Sephadex LH-20 is supplied as a dry powder and must be swollen before use. The degree of swelling depends on the solvent used (see Table A7.2). Swell the resin for at least 3 h at room temperature in an excess of the solvent to be used for the separation. Avoid using magnetic stirrers, spatulas, or glass rods since they can damage the resin.
2. Prepare a slurry in a ratio 75% settled resin to 25% solvent. Decant any fine particles.
3. Prepare a suitable empty column for packing according to the column instructions. Make sure that the column tolerates the solvent used. Mount a bottom filter if this is not part of the end piece. Wet the bottom filter. If the slurry volume exceeds the column volume, attach a packing reservoir or packing tube.
4. Equilibrate all materials to room temperature.
5. Fill the column with the solvent to a height of approximately 2 cm.
6. Resuspend and pour the slurry down the inside of the column in one continuous step. Fill the column or column reservoir to the top with solvent. Avoid air bubbles.
7. Attach a top adapter to the column tube (or the cap on the packing reservoir). Connect to a pump, and open the column outlet. Pack at 300 cm/h until the bed has reached a constant height and stop the flow. Remove the packing reservoir or packing tube if used.
8. Fill the column with solvent and mount a wetted adapter onto the column as described in the column instructions. Also, mount a wetted filter if this is not part of the adapter. Ensure no air bubbles are trapped in the column and that no air bubbles are in the flow path.
9. Open the column outlet and continue packing until the packed bed is stable. A final adjustment of the top adapter might be necessary.

In some solvents such as chloroform, Sephadex LH-20 is less dense than the solvent and the resin will float. In this case, a column with two adapters should be used. Pour the resin into the column and drain until the second adapter can be inserted. Lock the adapter in position at the surface of the resin and direct the flow of solvent upwards. The bed will be packed against the top adapter and the lower adapter can be pushed slowly upwards towards the lower surface of the resin. Close the column outlet when moving the adapter to avoid compressing the bed.

Table A7.2. Approximate values for packed bed volumes of Sephadex LH-20 swollen in different solvents

Solvent	Approx. bed volume (mL/g dry Sephadex LH-20)
Dimethyl sulfoxide	4.4 to 4.6
Pyridine	4.2 to 4.4
Water	4.0 to 4.4
Dimethylformamide	4.0 to 4.4
Methanol	3.9 to 4.3
Saline	3.8 to 4.2
Ethylene dichloride	3.8 to 4.1
Chloroform[1]	3.8 to 4.1
Propanol	3.7 to 4.0
Ethanol[2]	3.6 to 3.9
Isobutanol	3.6 to 3.9
Formamide	3.6 to 3.9
Methylene dichloride	3.6 to 3.9
Butanol	3.5 to 3.8
Isopropanol	3.3 to 3.6
Tetrahydrofuran	3.3 to 3.6
Dioxane	3.2 to 3.5
Acetone	2.4 to 2.6
Acetonitrile[3]	2.2 to 2.4
Carbon tetrachloride[3]	1.8 to 2.2
Benzene[3]	1.6 to 2.0
Ethyl acetate[3]	1.6 to 1.8
Toluene[3]	1.5 to 1.6

[1] *Containing 1% ethanol.*
[2] *Containing 1% benzene.*
[3] *Bed volumes < 2.5 mL/g dry Sephadex LH-20 are generally not useful.*

Transferring Sephadex LH-20 from aqueous solution to organic solvent

Transfer Sephadex LH-20 from an aqueous solution to an organic solvent by moving through a series of solvent mixtures of increasing concentration. This will ensure efficient replacement of the water by the required solvent.

To transfer from aqueous solution or organic solvent (100% A) to a new organic solvent (100% B), proceed as follows:

1. Transfer to 70% A:30% B
2. Transfer to 30% A:70%, B
3. Finally transfer to 100% B. If A and B are not mutually miscible, make the transfer via an intermediate solvent, for example from water to chloroform via acetone, as shown in Figure A7.1.

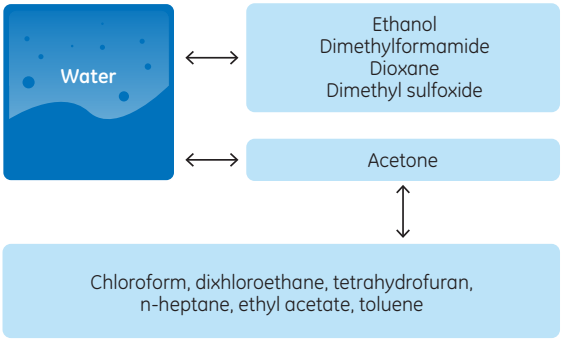


Fig A7.1. Suggested methods for transferring Sephadex LH-20 to organic solvents.

1. Transfer the required amount of resin to a sintered glass Buchner funnel and remove the excess aqueous solution by gentle suction.
2. Add the next solvent and resuspend the resin by stirring gently.
3. Using suction, remove excess solvent and resuspend in the same solvent.
4. Repeat the process with the next solvent in the series. Perform at least two resuspensions for each change of solvent conditions until the final solvent composition is reached.
5. Pack the resin into a suitable column.

Applications

Isolation of an HIV-1 reverse transcriptase (HIV-1 RT) inhibitor from P. niruri

Phyllanthus niruri is a widespread tropical plant that has been used as a natural medicine against edema and jaundice for many years. Aqueous extracts of this plant contain an inhibitor of HIV-1 reverse transcriptase (RT), which has been identified as repandusinic acid A monosodium salt (RA), a small tannin-like molecule (1). Purification results of RA from *P. niruri* using a purification scheme including Sephadex LH-20 are found in *Sephadex LH-20 data file*, 18110722. The structure of the free acid form of repandusinic acid A is shown in Figure A7.2.

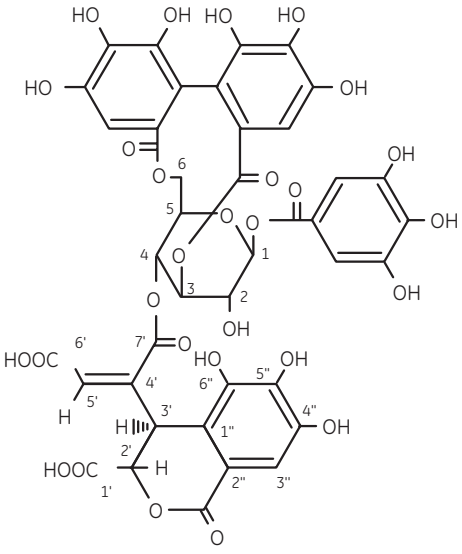


Fig A7.2. Structure of free acid form of repandusinic acid A.

Preparative separation of 2-acetamidobenzoic acid from 4-acetamidobenzoic acid

The dual hydrophilic-hydrophobic characteristics of Sephadex LH-20 offers an alternative selectivity to other SEC resins, which enables high resolution of closely related molecular species. Sephadex LH-20 was used in a preparative-scale separation of 2-acetamidobenzoic acid from 4-acetamidobenzoic acid, which are molecules that differ only by the position of the acetamide moiety on the benzene ring. The separation of the molecules was made possible only because of the selectivity the resin, see *Data file Sephadex LH-20* 18110722 for details.

Reference

1. Ogata, T. *et al.* HIV-1 reverse transcriptase inhibitor from *Phyllanthus niruri*. *AIDS Res. Hum. Retroviruses* **8**, 1937–1944 (1992). https://doi.org/10.1089/aid.1992.8.1937

Product index

Related literature

Document	Code number
Affinity Chromatography Handbook Vol. 1: Antibodies	18103746
Affinity Chromatography Handbook Vol. 2: Tagged proteins	18114275
Affinity Chromatography Handbook Vol. 3: Specific Groups of Biomolecules	18102229
Strategies for Protein Purification Handbook	28983331
Purifying Challenging Proteins, Principles and Methods	28909531
Ion Exchange Chromatography, Principles and Methods	11000421
Hydrophobic Interaction and Reversed Phase Chromatography, Principles and Methods	11001269
Multimodal Chromatography Handbook	29054808
Protein Sample Preparation Handbook	28988741
Size exclusion chromatography columns resins, Selection guide	18112419
Columns and resins for antibody purification and immunoprecipitation, Selection guide	28935197
Ion exchange columns and media, Selection guide	18112731
Affinity chromatography columns and media, Selection guide	18112186
Hydrophobic interaction chromatography, Selection guide	29022223
Prepacked chromatography columns for ÄKTA systems, Selection guide	28931778
XK empty columns, Data file	28997659
HiScale columns, Data file	28975523
Tricorn empty high-performance columns, Data file	18114736

Ordering information

High-resolution SEC

Product	Quantity	Code number
Superdex		
Superdex 30 Increase 10/300 GL	1 × 24 mL column	29219757
Superdex 30 Increase 3.2/300	1 × 2.4 mL column	29219758
Superdex 75 Increase 10/300 GL	1 × 24 mL column	29148721
Superdex 75 Increase 5/150 GL	1 × 3 mL column	29148722
Superdex 75 Increase 3.2/300	1 × 2.4 mL column	29148723
Superdex 200 Increase 10/300 GL	1 × 24 mL column	28990944
Superdex 200 Increase 5/150 GL	1 × 3 mL column	28990945
Superdex 200 Increase 3.2/300	1 × 2.4 mL column	28990946
HiLoad 16/600 Superdex 30 prep grade	1 × 120 mL column	28989331
HiLoad 26/600 Superdex 30 prep grade	1 × 320 mL column	28989332
HiLoad 16/600 Superdex 75 prep grade	1 × 120 mL column	28989333
HiLoad 26/600 Superdex 75 prep grade	1 × 320 mL column	28989334
HiLoad 16/600 Superdex 200 prep grade	1 × 120 mL column	28989335
HiLoad 26/600 Superdex 200 prep grade	1 × 320 mL column	28989336
Superdex 30 prep grade	150 mL	17090501
Superdex 30 prep grade	1 L	17090503
Superdex 75 prep grade	150 mL	17104401
Superdex 75 prep grade	1 L	17104402
Superdex 75 prep grade	5 L	17104404
Superdex 200 prep grade	150 mL	17104301
Superdex 200 prep grade	1 L	17104302
Superdex 200 prep grade	5 L	17104304
Superose		
Superose 6 Increase 10/300 GL	1 × 24 mL column	29091596
Superose 6 Increase 5/150 GL	1 × 3 mL column	29091597
Superose 6 Increase 3.2/300	1 × 2.4 mL column	29091598
Superose 6 prep grade	125 mL	17048901
Superose 6 prep grade	1 L	17048903
Superose 6 prep grade	5 L	17048904
Superose 12 prep grade	125 mL	17053601
Superose 12 prep grade	1 L	17053603

Product	Quantity	Code number
Sephacryl		
HiPrep 16/60 Sephacryl S-100 HR	1 × 120 mL column	17116501
HiPrep 26/60 Sephacryl S-100 HR	1 × 320 mL column	17119401
HiPrep 16/60 Sephacryl S-200 HR	1 × 120 mL column	17116601
HiPrep 26/60 Sephacryl S-200 HR	1 × 320 mL column	17119501
HiPrep 16/60 Sephacryl S-300 HR	1 × 120 mL column	17116701
HiPrep 26/60 Sephacryl S-300 HR	1 × 320 mL column	17119601
HiPrep 16/60 Sephacryl S-400 HR	1 × 120 mL column	28935604
HiPrep 26/60 Sephacryl S-400 HR	1 × 320 mL column	28935605
HiPrep 16/60 Sephacryl S-500 HR	1 × 120 mL column	28935606
HiPrep 26/60 Sephacryl S-500 HR	1 × 320 mL column	28935607
Sephacryl S-100 HR	150 mL	17061210
Sephacryl S-100 HR	750 mL	17061201
Sephacryl S-100 HR	10 L	17061205
Sephacryl S-200 HR	150 mL	17058410
Sephacryl S-200 HR	750 mL	17058401
Sephacryl S-200 HR	10 L	17058405
Sephacryl S-300 HR	150 mL	17059910
Sephacryl S-300 HR	750 mL	17059901
Sephacryl S-300 HR	10 L	17059905
Sephacryl S-400 HR	150 mL	17060910
Sephacryl S-400 HR	750 mL	17060901
Sephacryl S-400 HR	10 L	17060905
Sephacryl S-500 HR	150 mL	17061310
Sephacryl S-500 HR	750 mL	17061301
Sephacryl S-500 HR	10 L	17061305
Calibration Kits		
Gel Filtration Calibration Kit LMW	1	28403841
Gel Filtration Calibration Kit HMW	1	28403842

Desalting and group separations

HiTrap Desalting	1 × 5 mL column	29048684
HiTrap Desalting	5 × 5 mL columns	17140801
HiPrep 26/10 Desalting	1 × 53 mL column	17508701
HiPrep 26/10 Desalting	4 × 53 mL column	17508702
PD-10 Desalting Column	30 gravity-fed columns	17085101
Empty PD-10 Desalting Column	50 gravity-fed empty columns	17043501
PD MiniTrap G-10	50 columns	28918010
PD MidiTrap G-10	50 columns	28918011
PD Spin Trap G-25	50 columns	28918004
PD MultiTrap G-25	4 × 96-well plates	28918006
PD MiniTrap G-25	50 columns	28918007
PD MidiTrap G-25	50 columns	28918008
MiniSpin Adapter	10	28923243

Product	Quantity	Code number
MidiSpin Adapter	10	28923244
PD-10 Spin Adapter	10	28923245
Collection plate 500 µl V-bottom	5 × 96-well plates	28403943
LabMate PD-10 Buffer Reservoir	10	18321603
Sephadex G-10	100 g	17001001
Sephadex G-10	500 g	17001002
Sephadex G-10	5 kg	17001003
Sephadex G-25 Coarse	100 g	17003401
Sephadex G-25 Coarse	500 g	17003402
Sephadex G-25 Coarse	5 kg	17003403
Sephadex G-25 Fine	100 g	17003201
Sephadex G-25 Fine	500 g	17003202
Sephadex G-25 Fine	5 kg	17003203
Sephadex G-25 Medium	100 g	17003301
Sephadex G-25 Medium	500 g	17003302
Sephadex G-25 Medium	5 kg	17003303
Sephadex G-25 Superfine	100 g	17003101
Sephadex G-25 Superfine	5 kg	17003103
Sephadex G-50 Fine	100 g	17004201
Sephadex G-50 Fine	500 g	17004202
Sephadex G-50 Fine	5 kg	17004203
Sephadex G-50 Superfine	100 g	17004101
Sephadex G-50 Superfine	5 kg	17004103
Sephadex G-50 Medium	100 g	17004301
Sephadex G-50 Medium	500 g	17004302
Sephadex G-50 Medium	5 kg	17004303
Separation in organic solvents		
Sephadex LH-20	25 g	17009010
Sephadex LH-20	100 g	17009001
Sephadex LH-20	500 g	17009002
Sephadex LH-20	5 kg	17009003

Empty columns

XK columns		
XK 16/20 column	1	28988937
XK 16/40 column	1	28988938
XK 16/70 column	1	28988946
XK 16/100 column	1	28988947
XK 26/20 column	1	28988948
XK 26/40 column	1	28988949
XK 26/70 column	1	28988950
XK 26/100 column	1	28988951
XK 50/20 column	1	28988952
XK 50/30 column	1	28988953
XK 50/60 column	1	28988964
XK 50/100 column	1	28988965

Product	Quantity	Code number
Tricorn columns		
Tricorn 10/150	1	28406416
Tricorn 10/200	1	28406417
Tricorn 10/300	1	28406418
Tricorn 10/600	1	28406419
HiScale columns		
HiScale 16/20	1	28964441
HiScale 16/40	1	28964424
HiScale 26/20	1	28964514
HiScale 26/40	1	28964513
HiScale 50/20	1	28964445
HiScale 50/40	1	28964444

More details and products can be found at *proteins.gelifesciences.com*

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