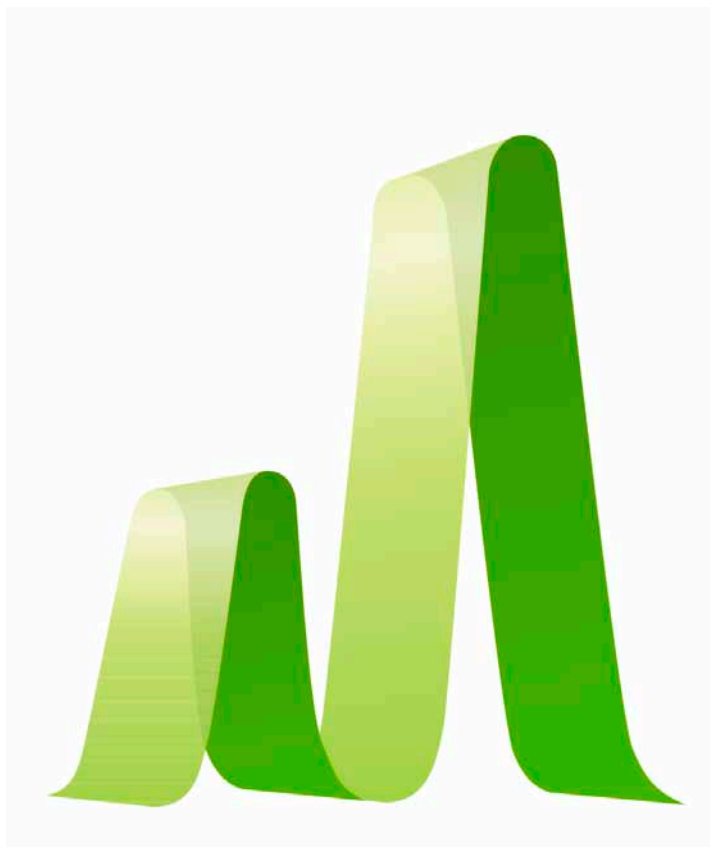




Gel filtration

Principles and Methods



Handbooks from GE Healthcare



GST Gene Fusion System

Handbook
18-1157-58

Affinity Chromatography

Principles and Methods
18-1022-29

Antibody Purification

Handbook
18-1037-46

Ion Exchange Chromatography and Chromatofocusing

Principles and Methods
11-0004-21

Cell Separation Media

Methodology and Applications
18-1115-69

Purifying Challenging Proteins

Principles and Methods
28-9095-31

Isolation of mononuclear cells

Methodology and Applications
18-1152-69

High-throughput Process Development with PreDictor Plates

Principles and Methods
28-9403-58

Gel Filtration

Principles and Methods
18-1022-18

Recombinant Protein Purification Handbook

Principles and Methods
18-1142-75

Hydrophobic Interaction and Reversed Phase Chromatography

Principles and Methods
11-0012-69

2-D Electrophoresis using immobilized pH gradients

Principles and Methods
80-6429-60

Microcarrier Cell Culture

Principles and Methods
18-1140-62

Nucleic Acid Sample Preparation for Downstream Analyses

Principles and Methods
28-9624-00

Strategies for Protein Purification

Handbook
28-9833-31

Gel filtration

Principles and Methods

Contents

Introduction	7
Chapter 1	
Gel filtration in practice	9
Introduction	9
Purification by gel filtration	9
Group separation	10
High-resolution fractionation	11
Rapid purity check and screening	12
Resolution in gel filtration	12
Sample volume and column dimensions	12
Media selection	14
Sample and buffer preparation	17
Sample buffer composition	17
Sample concentration and viscosity	17
Sample volume	19
Buffer composition	19
Denaturing (chaotropic) agents and detergents	19
Column and media preparation	20
Sample application	21
Elution and flow rates	21
Controlling flow	23
Method development for high resolution fractionation	23
Maintenance of gel filtration columns	24
Equipment selection	24
Scaling up	24
BioProcess Media for large-scale production	25
Troubleshooting	26
Chapter 2	
Superdex: the first choice for high resolution, short run times, and high recovery	31
Separation options	33
Separation examples	34
Performing a separation	39
First time use or after long-term storage	39
Cleaning	40
Removing severe contamination	40
Media characteristics	41
Chemical stability	41
Storage	41
Chapter 3	
Superose: broad fractionation range for laboratory scale	43
Separation options	45
Separation examples	45
Performing a separation	46
First time use or after long-term storage	46
Cleaning	47

Media characteristics.....	47
Chemical stability	48
Storage.....	48
Chapter 4	
Sephacryl: fast, high recovery separations at laboratory and industrial scale	49
Separation options.....	52
Separation examples	53
Performing a separation	54
First time use or after long-term storage	54
Cleaning.....	55
To remove severe contamination.....	55
Media characteristics.....	55
Chemical stability	56
Storage.....	56
Chapter 5	
Sephadex: desalting, buffer exchange and sample clean up	57
Separation options.....	58
Separation examples	62
Performing a separation	63
General considerations.....	63
Small-scale desalting of samples.....	63
Desalting larger sample volumes using HiTrap and HiPrep columns.....	63
Buffer preparation	64
Sample preparation	64
Buffer exchange	64
HiTrap Desalting columns.....	64
Manual purification with a syringe	65
Simple desalting with ÄKTAprime plus.....	66
Desalting on a gravity-feed PD-10 column	67
Buffer Preparation	67
Optimization of desalting	67
Scale-up and processing larger sample volumes	68
Increasing sample loading capacity from 1.5 ml up to 7.5 ml.....	69
Increasing sample loading capacity from 15 ml up to 60 ml.....	69
For sample volumes greater than 60 ml.....	70
Media characteristics.....	70
Column Packing	71
Cleaning.....	71
Chemical stability	71
Storage.....	71
Chapter 6	
Sephadex LH-20 – gel filtration in presence of organic solvents.....	73
Media characteristics.....	73
Separation examples	73
Packing a column	75
Performing a separation	76
Cleaning	76
Chemical stability.....	77
Storage	77
Transferring Sephadex LH-20 from aqueous solution to organic solvents	77

Chapter 7	
Gel filtration in theory	79
Defining the process.....	79
Selectivity curves and media selection	81
Resolution.....	82
Chapter 8	
Gel filtration in a purification strategy.....	85
The purification strategy according to CIPP.....	85
Gel filtration as a polishing step.....	86
Purification of humanized IgG ₄ monoclonal antibody.....	87
Appendix 1	
Column packing and preparation.....	89
Columns for packing gel filtration media	89
Checking column efficiency.....	90
Column packing for high resolution fractionation using Superdex prep grade and Sephacryl High Resolution	91
Column packing for group separations using Sephadex	93
Controlling flow.....	95
Appendix 2	
Sephadex and Darcy's law	96
Appendix 3	
Sample preparation	97
Sample clarification.....	97
Centrifugation.....	97
Filtration.....	97
Desalting	97
Denaturation	98
Precipitation and resolubilization.....	98
Ammonium sulfate precipitation	99
Removal of lipoproteins.....	101
Appendix 4	
Selection of purification equipment.....	102
Appendix 5	
Converting from linear flow (cm/h) to volumetric flow rates (ml/min) and vice versa.....	103
From linear flow (cm/h) to volumetric flow rate (ml/min)	103
From volumetric flow rate (ml/min) to linear flow (cm/hour).....	103
From ml/min to using a syringe	104
Appendix 6	
Conversion data.....	105
Proteins.....	105
Nucleic Acids.....	105
Column pressures.....	105
Appendix 7	
Amino acids table	106
Appendix 8	
Analysis and characterization	108
Protein detection and quantification.....	108
Purity check and protein characterization	108

Purity	108
Characterization	109
Appendix 9	
Storage of biological samples	110
General recommendations	110
Common storage conditions for purified proteins.....	110
Appendix 10	
Molecular weight estimation and molecular weight distribution analysis	111
Performing a molecular weight determination.....	113
Product index.....	115
Related literature	116
Ordering information	117

Introduction

Biomolecules are purified using chromatography techniques that separate them according to differences in their specific properties, as shown in Figure 1.

Property	Technique
Size	Gel filtration (GF), also called size exclusion chromatography (SEC)
Hydrophobicity	Hydrophobic interaction chromatography (HIC) Reversed phase chromatography (RPC)
Charge	Ion exchange chromatography (IEX)
Biorecognition (ligand specificity)	Affinity chromatography (AC)
Isoelectric point	Chromatofocusing (CF)

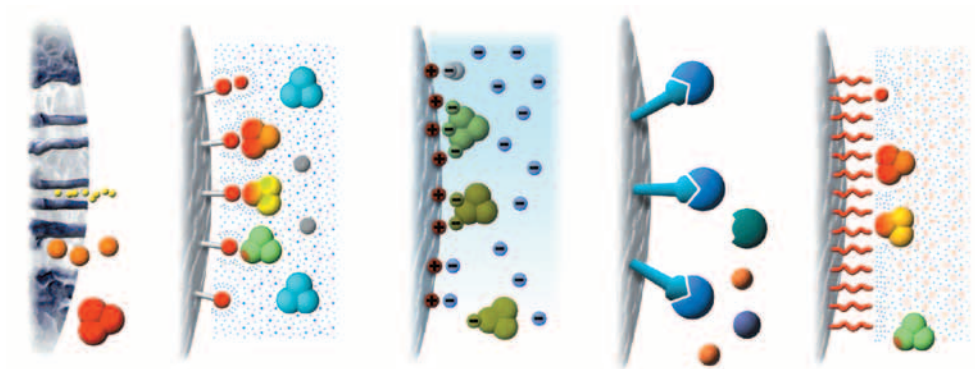


Fig 1. Schematic drawing of separation principles in chromatography purification. From left to right: GF, HIC, IEX, AC, and RPC.

Since the introduction of Sephadex™ more than 50 years ago, gel filtration has played a key role in the purification of proteins and enzymes, polysaccharides, nucleic acids and other biological macromolecules. Gel filtration is the simplest and mildest of all the chromatography techniques and separates molecules on the basis of differences in size. The technique can be applied in two distinct ways:

1. Group separations: the components of a sample are separated into two major groups according to size range. A group separation can be used to remove high or low molecular weight contaminants (such as phenol red from culture fluids) or for desalting and buffer exchange.
2. High resolution fractionation of biomolecules: the components of a sample are separated according to differences in their molecular size. High resolution fractionation can be used to isolate one or more components, to separate monomers from aggregates, or to perform a molecular weight distribution analysis.

Gel filtration can also facilitate the refolding of denatured proteins by careful control of changing buffer conditions.

This handbook describes the use of gel filtration for the purification and separation of biomolecules, with a focus on practical information for obtaining the best results. The media available, selection criteria and examples with detailed instructions for the most common applications are included, as well as the theoretical principles behind the technique. The first step towards a successful separation is to select the correct medium and this handbook focuses on the most up-to-date gel filtration media and prepacked columns.

Symbols



this symbol indicates general advice to improve procedures or recommend action under specific situations.



this symbol denotes mandatory advice and gives a warning when special care should be taken.



highlights chemicals, buffers and equipment.



outline of experimental protocol.

Chapter 1

Gel filtration in practice

Introduction

Gel filtration (also referred to as size exclusion chromatography, SEC) separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution (the degree of separation between peaks). Consequently, a significant advantage of gel filtration 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.

Gel filtration is well suited for biomolecules that may be sensitive to changes in pH, concentration of metal ions or co-factors and harsh environmental conditions. Separations can be performed in the presence of essential ions or cofactors, detergents, urea, guanidine hydrochloride, at high or low ionic strength, at 37°C or in the cold room according to the requirements of the experiment. Purified proteins can be collected in any chosen buffer.

This chapter provides general guidelines applicable to any gel filtration separation. A key step towards successful separation is selecting the correct medium; this handbook includes guides to the most up-to-date gel filtration media and prepacked columns. Application examples and product-specific information are found in Chapters 2 to 6.

Purification by gel filtration

To perform a separation, gel filtration medium is packed into a column to form a packed bed. The medium is a porous matrix of spherical particles with chemical and physical stability and inertness (lack of reactivity and adsorptive properties). 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 outside the particles, or mobile phase. Samples are eluted isocratically so there is no need to use different buffers during the separation. However, a wash step using the running buffer is usually included at the end of a separation to remove molecules that may have been retained on the column and to prepare the column for a new run.

Gel filtration can be used directly after ion exchange, chromatofocusing, hydrophobic interaction, or affinity, since the buffer composition will not generally affect the final separation. For further details on using gel filtration in a purification strategy, refer to Chapter 8.

Figure 1.1 illustrates the separation process of gel filtration and the theory for this process is described in Chapter 7.

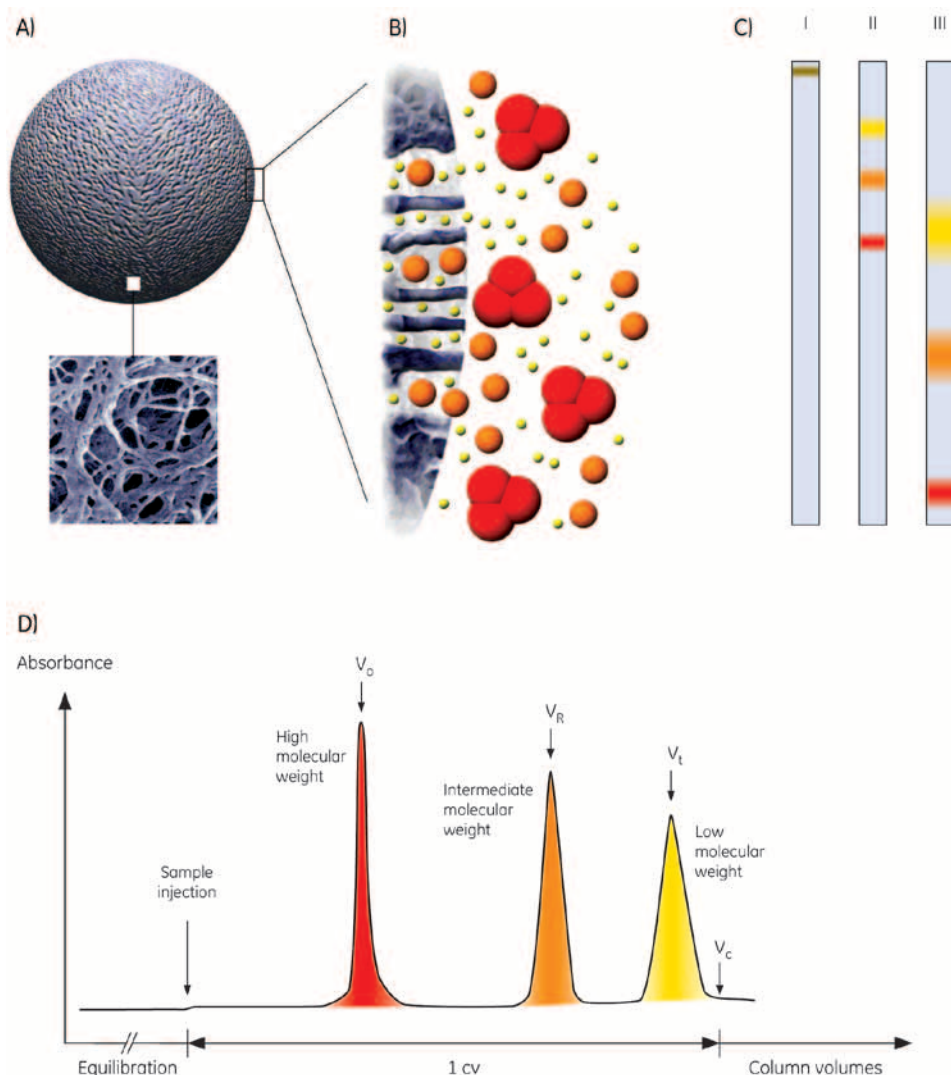


Fig 1.1. Process of gel filtration (A) Schematic picture of a bead with an electron microscopic enlargement. (B) Schematic drawing of sample molecules diffusing into bead pores. (C) Graphical description of separation I. Sample is applied on the column, II. The smallest molecule (yellow) is more delayed 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.

Group separation

Gel filtration is used in group separation mode to remove small molecules from a group of larger molecules and as a fast, simple solution for buffer exchange. Small molecules such as excess salt or free labels are easily separated from larger molecules. Samples can be prepared for storage or for other chromatography techniques and assays. Gel filtration in group separation mode is often used in protein purification schemes for desalting and buffer exchange. Sephadex G-10, G-25 and G-50 are used for group separations. Large sample volumes, up to 30% of the total column volume (packed bed), can be applied at high flow rates using broad, short columns. Figure 1.2 shows the chromatogram (elution profile) of a typical group separation. Large molecules are eluted in or just after the void volume, V_D , as they pass through the column at the same speed as the flow of buffer. For a well-packed column the void volume is equivalent to approximately 30% of the total column volume. Small molecules such

as salts that have full access to the pores move down the column, but do not separate from each other. These molecules usually elute just before one total column volume, V_t , of buffer has passed through the column. In this case the proteins are detected by monitoring their UV absorbance, usually at 280 nm, and the salts are detected by monitoring the conductivity of the buffer.

Column: HiTrap™ Desalting 5 ml
Sample: (Histidine)₆ protein eluted from HiTrap Chelating HP with sodium phosphate 20 mM, sodium chloride 0.5 M, imidazole 0.5 M, pH 7.4
Buffer: Sodium phosphate 20 mM, sodium chloride 0.15 M, pH 7.0
 void volume V_o , total column volume V_t

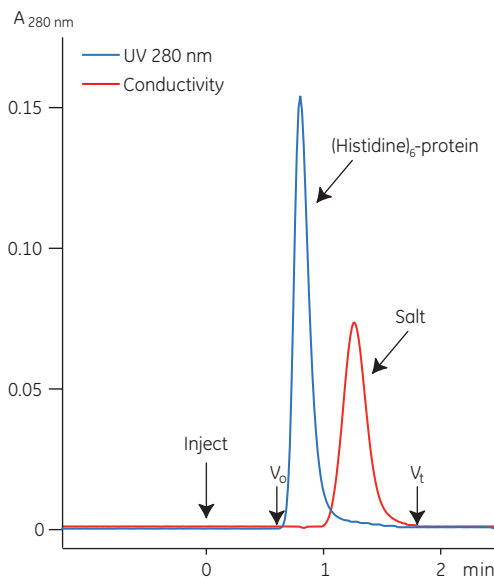


Fig 1.2. Typical chromatogram of a group separation. UV (protein) and conductivity (salt) detection enable pooling of the desalted fractions and facilitate optimization of the separation.

Refer to Chapter 5, p 57 for detailed information on how Sephadex is used in group separation of high and low molecular weight substances in applications like desalting, buffer exchange, and sample clean up.

Refer to Chapter 7 for detailed information on the theory of gel filtration.

High-resolution fractionation

Gel filtration is used in fractionation mode to separate multiple components in a sample on the basis of differences in their size. The goal may be to isolate one or more of the components, or to analyze the molecular weight distribution in the sample. The best results for high resolution fractionation will be achieved with samples that originally contain few components or with samples that have been partially purified by other chromatography techniques to eliminate most of the unwanted proteins of similar size.

High-resolution fractionation by gel filtration is well suited for the final polishing step in a purification scheme. Monomers are easily separated from aggregates. Samples can be transferred to a suitable buffer for assay or storage.

Rapid purity check and screening

Superdex™ is a high resolution gel filtration medium. Superdex 75 5/150 GL and Superdex 200 5/150 GI are short columns with small bed volumes and are suitable for rapid protein homogeneity analyses or purity checks. They save time when screening many samples, and require less buffer and sample than longer columns. However, when using the same media, shorter columns give lower resolution than longer columns.

Resolution in gel filtration

The success of gel filtration depends primarily on choosing conditions that give sufficient selectivity and counteract peak broadening effects during the separation. After selection of gel filtration medium, sample volume and column dimensions are the two most critical parameters that will affect the resolution of the separation.

The final resolution is influenced by many factors, see Table 1.1. The molecular weight range over which a gel filtration medium can separate molecules is referred to as the selectivity of the medium (see fractionation range guide for gel filtration media on page 16). Resolution is a function of the selectivity of the medium and the efficiency of that medium to produce narrow peaks (minimal peak broadening), as illustrated in Chapter 7, Figure 7.7.

Table 1.1. Factors that influence resolution

Medium-related factors	Particle size Particle uniformity Match between pore size and analyte size
Column-related factors	Bed height Column packing quality
Experimental-related factors	Flow rate Sample volume Viscosity

Sample volume and column dimensions

The sample volume can be expressed as a percentage of the total column volume (packed bed). Smaller sample volumes help to avoid overlap if closely spaced peaks are eluted. Figure 1.3 illustrates how sample volume can influence a high resolution fractionation.

-  For group separations, use sample volumes up to 30% of the total column volume.
-  For high resolution fractionation, a sample volume from 0.5% to 4% of the total column volume is recommended, depending on the type of medium used. For most applications the sample volume should not exceed 2% to achieve maximum resolution. Depending on the nature of the specific sample, it may be possible to load larger sample volumes, particularly if the peaks of interest are well resolved. This can only be determined by experimentation.

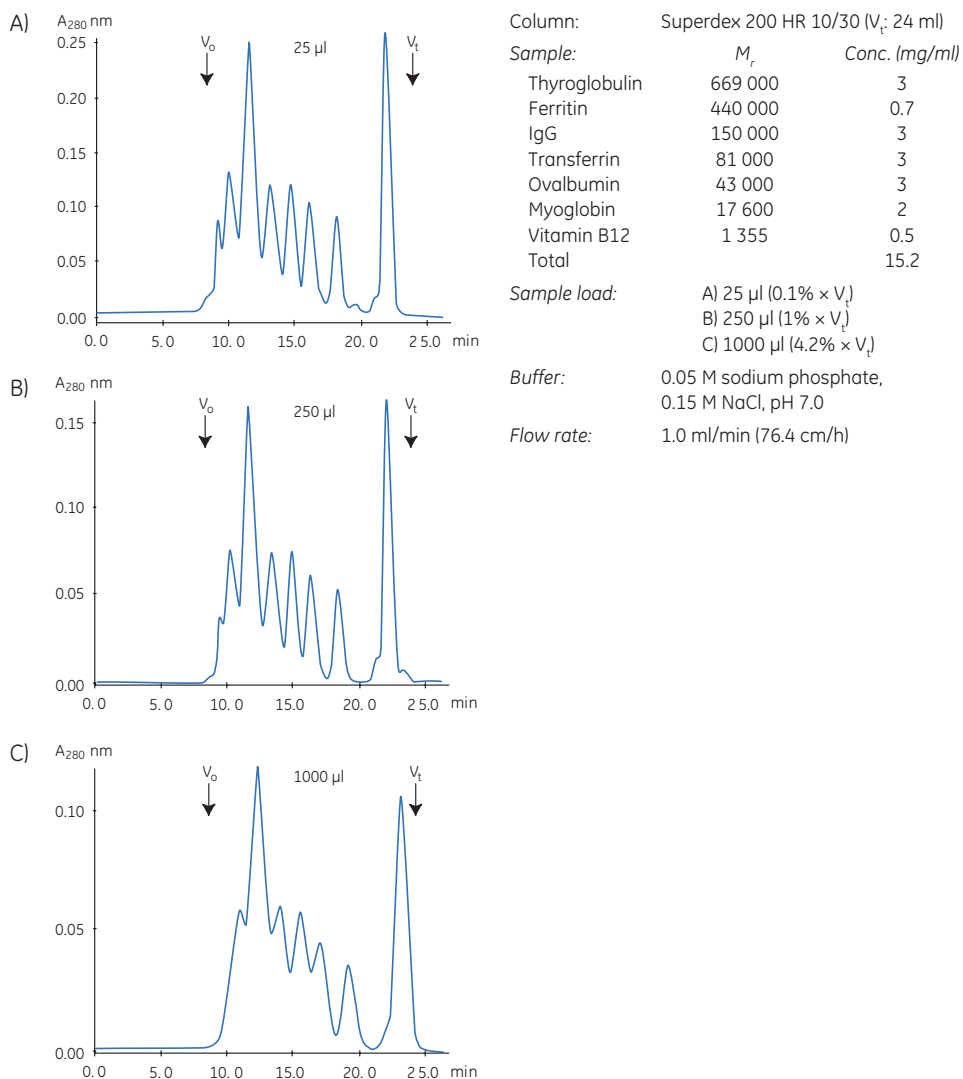


Fig 1.3. Influence of sample volume on resolution (Superdex 200 HR 10/30 is replaced with Superdex 200 10/300 GL).



The ratio of sample volume to column volume influences resolution, as shown in Figure 1.4, where higher ratios give lower resolution. Column volumes are normally selected according to the sample volumes to be processed. Since larger sample volumes may require significantly larger column volumes, it may be beneficial to repeat the separation several times on a smaller column and pool the fractions of interest or concentrate the sample (see Appendix 3 on sample preparation).



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 0.5% do not normally improve resolution.



Concentrating samples can increase the capacity of a gel filtration separation. Avoid concentrations above 70 mg/ml protein as viscosity effects may interfere with the separation.



Sample dilution is inevitable since diffusion occurs as sample passes through the column. To minimize sample dilution, use a sample volume that gives the resolution required between the peaks of interest.

Column: HiLoad™ 16/60 Superdex 200 prep grade
Sample: Solution of transferrin (M_r 81 000) and IgG (M_r 160 000) by equal weight
Sample concentration: 8 mg/ml
Buffer: 50 mM NaPO₄, 0.1 M NaCl, pH 7.2
Flow rate: 1 ml/min (30 cm/h)

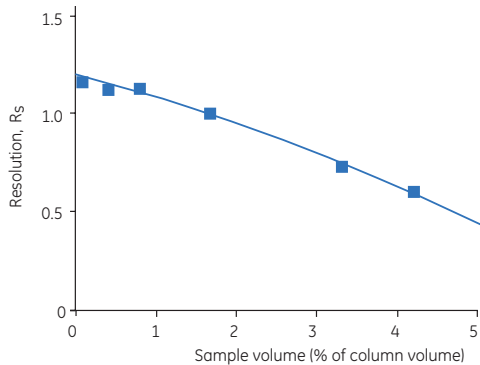


Fig 1.4. Influence of ratio of sample volume to column volume on the resolution of transferrin and IgG on prepacked HiLoad 16/60 Superdex 200 prep grade. Resolution is defined in Chapter 7.

The height of the packed bed affects both resolution and the time taken for elution. The resolution in gel filtration increases with the square root of bed height. Doubling the bed height gives an increase in resolution equivalent to $\sqrt{2} = 1.4$ (40%). For high resolution and fractionation, long columns will give the best results. Sufficient bed height together with a low flow rate allows time for 'intermediate' molecules to diffuse in and out of the matrix and give sufficient resolution.



If a very long column is necessary, the effective bed height can be increased by using columns, containing the same media, coupled in series.

Refer to Chapter 7 for detailed information on the theory of gel filtration.

Media selection

Today's gel filtration media cover a molecular weight range from 100 to 80 000 000, separating biomolecules from peptides to very large proteins and protein complexes.

The selectivity of a gel filtration medium depends solely on its pore size distribution and is described by a selectivity curve. Gel filtration media are supplied with information about selectivity, as shown for Superdex in Figure 1.5. The curve is a plot of the partition coefficient K_{av} against the log of the molecular weight for a set of standard proteins (for calculation of K_{av} , see Chapter 7 Gel filtration in theory).

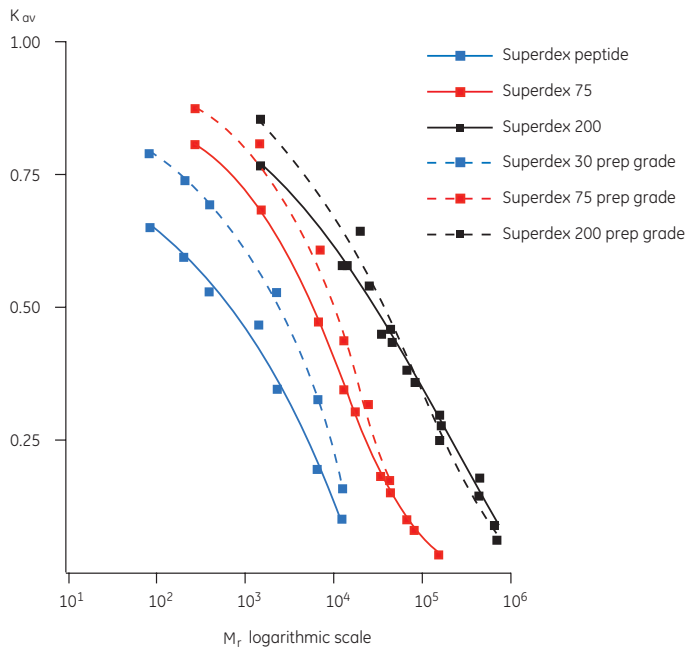


Fig 1.5. Selectivity curves for Superdex.

Selectivity curves are almost linear in the range $K_{av} = 0.1$ to $K_{av} = 0.7$ and can be used to determine the fractionation range of a gel filtration medium (Fig 1.6).

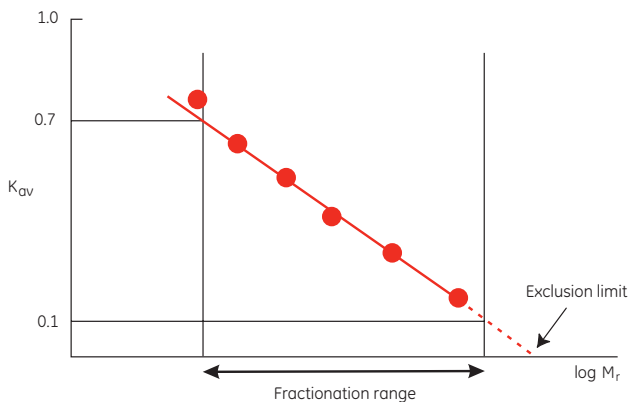


Fig 1.6. Defining fractionation range and exclusion limit from a selectivity curve.

The fractionation range defines the range of molecular weights that have partial access to the pores of the matrix; that is molecules within this range should be separable by high resolution fractionation. The exclusion limit for a gel filtration medium, also determined from the selectivity curve, indicates the size of the molecules that are excluded from the pores of the matrix and therefore elute in the void volume.



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

When choosing a medium, consider two main factors:

1. The aim of the experiment (high resolution fractionation or group separation).
2. The molecular weights of the target proteins and contaminants to be separated.

The final scale of purification should also be considered. Figure 1.7 gives some guidance to media selection. All media are available in prepacked columns, which is recommended if you have little experience in column packing.

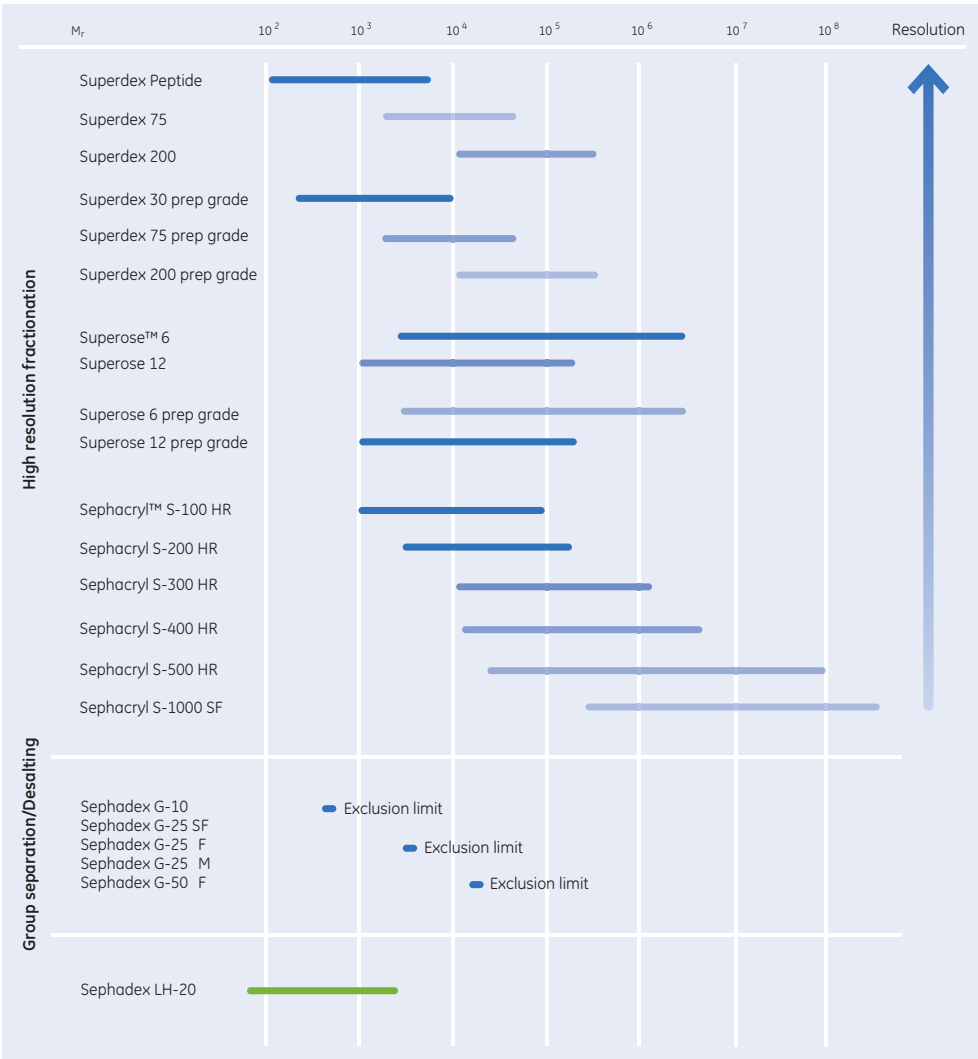


Fig 1.7. Gel filtration media fractionation range guide.

- 👉 Superdex is the first choice for high resolution, short run times, and high recovery.
- 👉 Superdex prep grade and Sephacryl are suitable for fast, high recovery separations at laboratory and industrial scale.

Superdex, Sephacryl, or Superose are high resolution media with a wide variety of fractionation ranges. In cases when two media have similar fractionation ranges, select the medium with the steepest selectivity curve (see chapter 2, 3, and 4 for the respective medium) for the best resolution of all the sample components. If a specific component is of interest, select the medium where the log of molecular weight for the target component falls in the middle of the selectivity curve.



Sephadex is recommended for rapid group separations such as desalting and buffer exchange. Sephadex is used at laboratory and production scale, before, between or after other chromatography purification steps.

For group separations, select gel filtration media that elute high molecular weight molecules at the void volume to minimize peak broadening or dilution and reduce time in the column. The lowest molecular weight substances should appear by the time one column volume of buffer has passed through the column.

Table 1.2. Sephadex media properties

Medium	Cut-off	Application examples
Sephadex G-10	700	Desalting of peptides
Sephadex G-25	1500	Desalting of proteins and oligonucleotides
Sephadex G-50	5000	Removal of free labels from labeled macromolecules

Sample and buffer preparation

Removal of particles in the sample is extremely important for gel filtration. Clarifying a sample before applying it to a column will avoid the risk of blockage, reduce the need for stringent washing procedures and extend the life of the medium.



Samples must be clear and free from particulate matter, especially when working with bead sizes of 34 μm or less.



Appendix 3 contains an overview of sample preparation techniques. For small sample volumes a syringe-tip filter of cellulose acetate or PVDF can be sufficient.

Sample buffer composition

The pH, ionic strength and composition of the sample buffer will not significantly affect resolution as long as these parameters do not alter the size or stability of the proteins to be separated and are not outside the stability range of the gel filtration medium. The sample does not have to be in exactly the same buffer as that used to equilibrate and run through the column. Sample is exchanged into the running buffer during the separation, an added benefit of gel filtration.

Sample concentration and viscosity

Gel filtration is independent of sample mass and hence sample concentration, as can be seen in Figure 1.8. High resolution can be maintained despite high sample concentration and, with the appropriate medium, high flow rates.

Column: XK 16/70 (140 ml)
Medium: Superdex 200 prep grade
Sample: Solution of transferrin (M_r 81 000) and IgG (M_r 160 000) by equal weight
Sample volume: $0.8\% \times V_r$
Buffer: 0.05 sodium phosphate, 0.1 M sodium chloride, pH 7.2
Flow rate: 1 ml/min (30 cm/h)

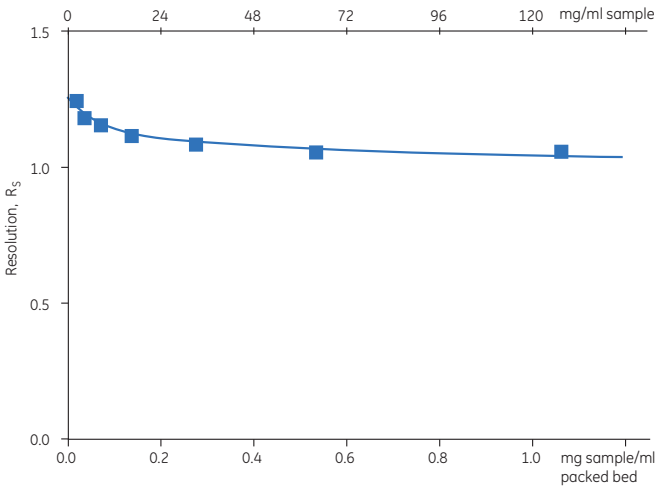


Fig 1.8. Influence of sample concentration on the resolution of transferrin and IgG on Superdex 200 prep grade.

The solubility or the viscosity of the sample may however limit the concentration that can be used. 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 NaCl at different sample viscosities in Figure 1.9. High sample viscosity causes instability of the separation and an irregular flow pattern. This leads to very broad and skewed peaks, and the back pressure might increase.

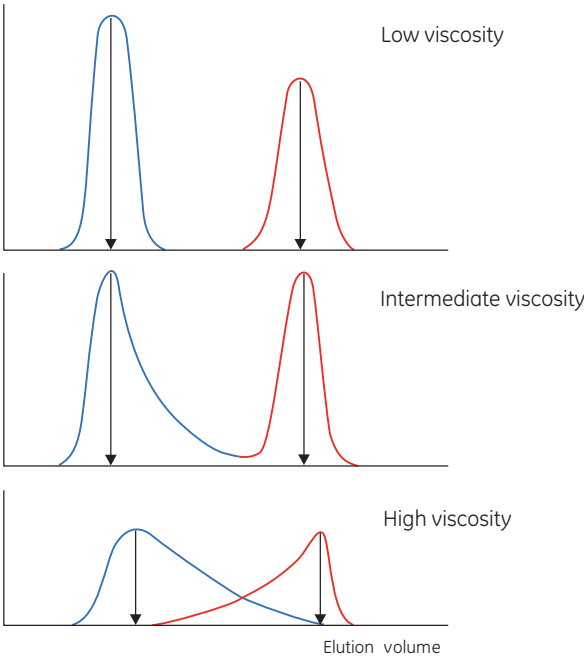


Fig 1.9. Deteriorating separation caused by increasing viscosity. Elution diagrams obtained when hemoglobin (blue) and NaCl (red) were separated. Experimental conditions were identical except that the viscosities were altered by the addition of increasing amounts of dextran. Note that lowering flow rate will not improve the separation.



Samples should generally not exceed 70 mg/ml protein. Dilute viscous samples, but not more than necessary to keep the sample volume low. Remember that viscosity varies with temperature.

Sample volume

Sample volume is one of the most important parameters in gel filtration. Refer to page 12 for more information.

Buffer composition

Buffer composition will generally not directly influence the resolution unless the buffer affects the shape or biological activity of the molecules. Extremes of pH and ionic strength and the presence of denaturing agents or detergents can cause conformational changes, dissociation or association of protein complexes.

Select buffer conditions that are compatible with protein stability and activity. The product of interest will be collected in this buffer. Use a buffer concentration that maintains buffering capacity and constant pH. Use from 25 mM up to 150 mM NaCl to avoid nonspecific ionic interactions with the matrix which can be seen as delays in peak elution. Note that some proteins may precipitate in low ionic strength solutions. Volatile buffers such as ammonium acetate, or ammonium bicarbonate should be used if the separated product will be lyophilized.



Use high quality water and chemicals. Solutions should be filtered through 0.45 μm or 0.22 μm filters before use. It is essential to degas buffers before any gel filtration separation since air bubbles can significantly affect performance. Buffers will be automatically degassed if they are filtered under vacuum.



When working with a new sample, try these conditions first: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0 or select the buffer into which the product should be eluted for the next step (e.g., further purification, analysis, or storage).



Avoid extreme changes in pH or other conditions that may cause inactivation or even precipitation. If the sample precipitates in the gel filtration column, the column will be blocked, possibly irreversibly, and the sample may be lost.

Denaturing (chaotropic) agents and detergents

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

Superdex and Sephacryl are in general more suitable than classical media such as Sepharose™ or Sephadex for working under dissociating or denaturing conditions or at extreme pH values.

Detergents are useful as solubilizing agents for proteins with low aqueous solubility, such as membrane components, and will not affect the separation. Sometimes, denaturing agents or detergents are necessary to maintain the solubility of the sample. Such additives must be present all the time, both in the running buffer and the sample buffer.



If high concentrations of additives are needed, use lower flow rates to avoid excessive pressure since they may increase the viscosity of the buffer.

If proteins precipitate, elute later than expected, or are poorly resolved during gel filtration, it is recommended to add a suitable concentration of a denaturing agent or detergent to the running buffer.



Urea or guanidine hydrochloride is 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 the calibration standards must also be run in the same buffer.



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



Gel filtration can be used to exchange the detergent environment of a protein. For example, a protein solubilized in SDS could be transferred to a milder detergent such as Triton™ X-100 without losing solubility.

Column and media preparation

To perform a separation, gel filtration medium is packed into a column 30–60 cm in height for high-resolution fractionation and up to 10 cm in height for group separations. Rapid screening experiments can be performed on 15 cm columns. The volume of the packed bed is determined by the sample volumes that will be applied.

Efficient column packing is essential, particularly for high resolution fractionation. The efficiency of a packed column defines its ability to produce narrow symmetrical peaks during elution. Column efficiency is particularly important in gel filtration in which separation takes place as only a single column volume of buffer passes through the column. The uniformity of the packed bed and the particles influences the uniformity of the flow profile and hence affects the shape and width of the peaks. High-performance gel filtration media with high bed uniformity (smaller and more uniform particles) give decreased peak widths and better resolution.

Efficiency is defined in terms of theoretical plates per meter (N).

$$N = 5.54 (V_e/W_{1/2})^2 \times 1000/L$$

where

V_e = peak elution (retention) volume

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

L = bed height (mm)

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

Refer to Chapter 7 Gel filtration in theory and Appendix 1 for further information on column efficiency and column packing.



Prepacked columns are highly recommended for the best performance and reproducible results.



Efficiency can be improved by using a smaller particle size. However, using a smaller particle size may create an increase in back pressure so that flow rate must be decreased and run time lengthened.



Buffers, media or prepacked 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 cause air bubbles in the packing and affect the separation.

Storage solutions and preservatives should be washed away thoroughly before using any gel filtration medium. Equilibrate the column with 1–2 column volumes of buffer before starting a separation.

Sample application

A liquid chromatography system should be used for high-resolution separation. For group separations it is possible to use manual purification. Samples can be applied by gravity feed to prepacked columns such as PD-10 Desalting.

Elution and flow rates

Samples are eluted isocratically from a gel filtration column, using a single buffer system. After sample application the entire separation takes place as one column volume of buffer (equivalent to the volume of the packed bed) passes through the column.

The goal for any separation is to achieve the highest possible resolution in the shortest possible time. Figures 1.10, 1.11, and 1.12 shows that resolution decreases as flow rate increases. Each separation must be optimized to provide the best balance between these two parameters. Put simply, maximum resolution is obtained with a long column and a low flow rate whereas the fastest run is obtained with a short column and a high flow rate. Suitable flow rates for high resolution fractionation or group separation are usually supplied with each product.

The advantage of a higher flow rate (and consequently a faster separation) may outweigh the loss of resolution in the separation.

Column: Superdex 200 HR 10/30 (V_t : 24 ml)

Sample:	M_r	Conc. (mg/ml)
Thyroglobulin	669 000	3
Ferritin	440 000	0.7
IgG	150 000	3
Transferrin	81 000	3
Ovalbumin	43 000	3
Myoglobin	17 600	2
Vitamin B12	1 355	0.5
Total		15.2

Buffer: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0

Flow rate:
1) 0.25 ml/min (19.1 cm/h)
2) 1.0 ml/min (76.4 cm/h)

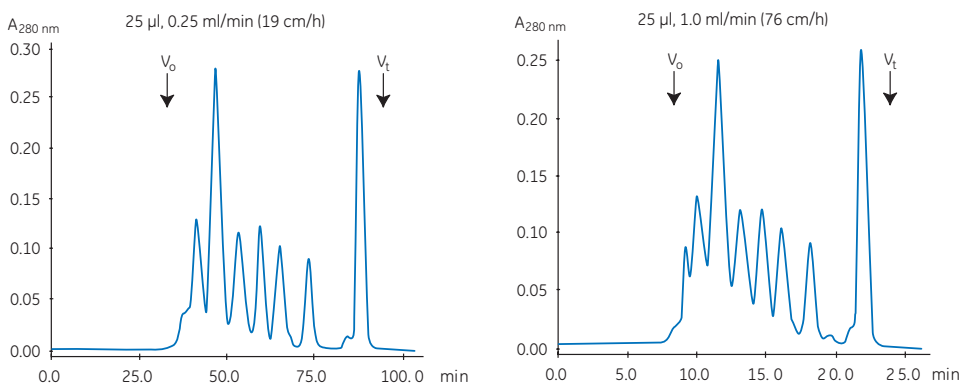


Fig 1.10. Influence of flow rate on resolution (Superdex 200 HR 10/30 is replaced with Superdex 200 10/300 GL).

Column: HiLoad 16/60 Superdex 30 prep grade
 Sample: IGF-1 containing monomers and dimers
 Sample load: 1 ml ($0.8\% \times V_t$)
 Sample concentration: a) 1.25 mg/ml
 b) 5 mg/ml
 Buffer: 50 mM sodium acetate, 0.1 M NaCl, pH 5.0

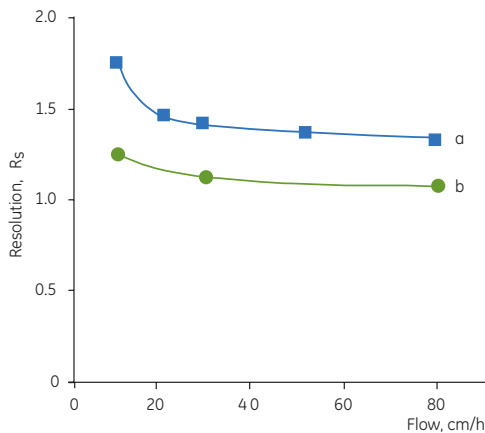


Fig 1.11. Resolution between two different concentrations of IGF-1 containing monomers and dimers at different flow rates.

Columns: HiPrep™ 16/10 Sephacryl S-100 HR
 HiPrep 16/10 Sephacryl S-200 HR
 HiPrep 16/10 Sephacryl S-300 HR
 Sample: IgG, ovalbumin, cytochrome C, 1:2:1
 Sample load: 2.4 ml ($2\% \times V_t$)
 Total sample load: 8 mg
 Buffer: 50 mM NaPO_4 , 0.15 M NaCl, 0.02% NaN_3 , pH 7.0

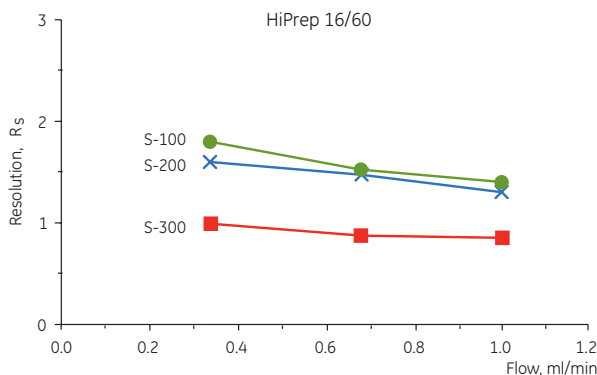


Fig 1.12. Influence on resolution between IgG, ovalbumin and cytochrome C using different gel filtration media.



If peaks are well separated at a low flow rate, increase the flow rate or shorten the column to save time. Alternatively, increase the sample volume and benefit from a higher capacity without significant loss of resolution.

For group separations such as desalting, monitor the elution of protein at A_{280} and follow the elution of the salt peak using a conductivity monitor. Adjust flow rate and sample volume to balance speed of separation against an acceptable level of salt in the final sample. Recommended flow rates are given in the instructions supplied with each product.

Flow rate is measured in volume terms, for example ml/min, but when comparing results between columns of different sizes it is useful to use the linear flow, cm/hour. A conversion table is available in Appendix 5. Results obtained at the same linear flow on different sized columns will be comparable as far as the effects of flow rate are concerned.

Selecting a smaller particle size of the same medium (if available) can also help to achieve the correct balance between flow rate and resolution. Smaller particles of the same medium can increase column efficiency, improve resolution to allow the use of higher flow rates. However, smaller particles can also result in increased back pressure and this factor may become restrictive if the intention is to scale up the separation.

Include a wash step at the end of a run to remove any molecules that may have been retained on the column, to prevent cross-contamination and to prepare the column for a new separation.

Controlling flow

Accurate, reproducible control of the flow rate is not only essential for good resolution, but also for reliability in routine preparative work and repeated experiments.



Use a chromatography system (rather than a peristaltic pump or gravity feed) to fully utilize the high rigidity and excellent flow properties of Sephacryl, Superose, or Superdex for high resolution fractionation.



Always pump the buffer to a column rather than drawing the buffer through the column with the pump below. This reduces the risk of bubble formation as a result of suction. If you have packed the column yourself, always use a lower flow rate for separation than the flow rate used for column packing.



Use a syringe or a pump for work with small prepacked columns such as HiTrap Desalting. Gravity feed with PD-10 Desalting for group separations of small sample volumes.



Gel filtration columns must not run dry. Ensure that there is sufficient buffer for long, unattended runs or that the pump is programmed to stop the flow after a suitable time. Columns that run dry must be repacked since the packed bed has been destroyed.

Method development for high resolution fractionation

Prepacked columns are delivered with standard running conditions that give satisfactory results in most situations. If optimization is needed, follow these steps (given in order of priority):

1. Select the medium that will give the best resolution of the target protein(s), see gel filtration media fractionation range guide, page 16.
2. To ensure reproducibility and high resolution, select a prepacked column that is best suited to the volume of sample that needs to be processed (see Chapters 2 to 4 for details of prepacked columns containing Superdex, Sephacryl, or Superose).
3. Select the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates supplied in the instructions for the specific medium and column.
4. Determine the maximum sample volume that can be loaded without reducing resolution.

Higher flow rates and viscous buffers yield higher operating pressures (remember that buffer viscosity increases when running at 4°C). Check the maximum operating pressure of the packed column and set the upper pressure limit on the chromatography system accordingly.



If greater resolution is required, increase the bed height by connecting two columns containing the same medium in series. Alternatively, try a medium with the same or similar fractionation range, but with a smaller particle size.

To process larger sample volumes, see scaling up below.

Maintenance of gel filtration columns

When a gel filtration medium has been in use for some time, it may be necessary to remove precipitated proteins or other contaminants that can build up on the column. Cleaning may be necessary when; a colored band can be seen at top of the column, a space occurs between the upper adaptor and the bed surface, there is a loss in resolution, or a significant increase in back pressure. Detailed cleaning procedures for each gel filtration medium are given in Chapters 2 to 6. In all cases, prevention is better than cure and routine cleaning is recommended.

If an increase in back pressure is observed, either on the pressure monitor or by seeing the surface of the medium move downwards, check that the problem is actually caused by the column before starting the cleaning procedure. Disconnect one piece of equipment at a time (starting at the fraction collector), start the pump and check the pressure after each piece is disconnected. Clogged on-line filters is a common cause of increased back pressure. Check back pressure at the same stage during each run, since the value can vary within a run, for example during sample injection or when changing to a different buffer.

-  Always use filtered buffers and samples to reduce the need for additional column maintenance. See Appendix 3 for further details on sample preparation.
-  Always use well degassed buffers to avoid the formation of air bubbles in the packed column during a run.
-  Buffers, prepacked columns and samples should be kept at the same temperature to prevent air bubbles forming in the column.
-  Filter cleaning solutions before use and always re-equilibrate the column with 2–3 column volumes of buffer before the next separation.

Equipment selection

Appendix 4 provides a guide to the selection of suitable systems for gel filtration separation.

Scaling up

After establishing a high-resolution or group separation on a small column, larger columns can be packed to process larger sample volumes in a single step. General guidelines for scaling up are shown in Table 1.3.

Table 1.3 General guidelines for scaling up gel filtration separations

Maintain	Increase
Bed height	Column diameter
Linear flow rate	Volumetric flow rate
Sample composition	Sample volume

To scale up a gel filtration separation, follow this workflow:

1. Optimize the separation at small scale (see method development page 23).
2. Maintain the sample concentration and the ratio of sample to column volume.
3. Increase the column volume by increasing the cross-sectional area of the column.
4. Maintain the bed height.
5. Run the separation at the same linear flow rate as used on the smaller column (see Appendix 5).

Refer to Appendix 1 for column selection and column packing.

Different factors related to the equipment may affect performance after scale-up. If a larger column has a less efficient flow distribution system, or a larger system introduces dead volumes, peak broadening may occur. This will cause extra dilution of the product fraction or even loss of resolution if the application is sensitive to variations in efficiency.

For media such as Superdex or Superose it is usually recommended to select a larger particle size for scale-up. For high-resolution fractionation, pack a small column containing the larger particles and repeat the separation to facilitate any optimization that may be needed to achieve the same resolution on the larger column.

Scaling up on Sephadex G-25, even to production scale, is a straightforward and well-established process. Well-known examples of commercial applications include buffer exchange in processes for removing endotoxins from albumin, and preparative steps during the production of vaccines. Figure 1.13 shows an example of a large scale buffer exchange step used during the production of albumin and IgG from human plasma.

Column: BPSS 400/600, 75 l
Sample: 10 l human plasma
1: sample application
2: eluent application
Eluent: 0.025 M sodium acetate
Flow: 240 l/h

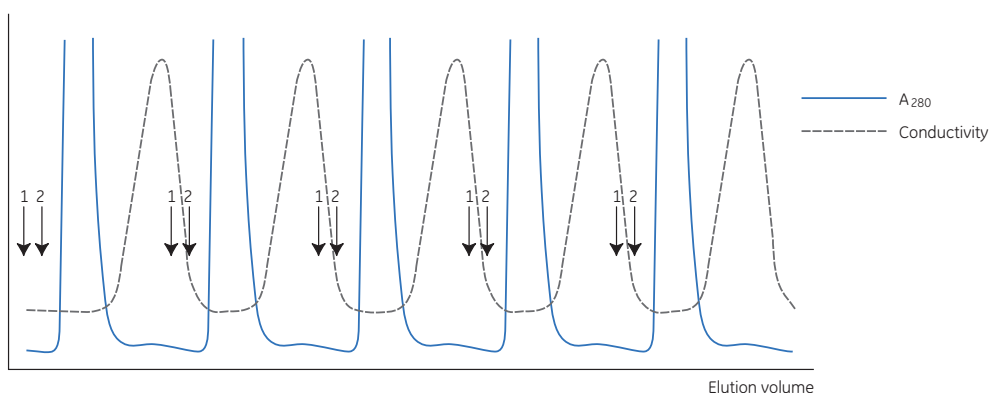


Fig 1.13. Chromatogram of the buffer exchange step on Sephadex G-25 Coarse during production of albumin and IgG from human plasma.

BioProcess™ Media for large-scale production

Specific BioProcess Media have been designed for each chromatographic stage in a process from Capture to Polishing. Use BioProcess Media for every stage results in a systematic approach to method development. Column packing methods are established for a wide range of scales. The same BioProcess Media can be used for development work, pilot studies, and routine production.

High flow rates, high capacity and high recovery contribute to the overall economy of an industrial process.

All BioProcess Media can be cleaned- and sanitized-in-place.

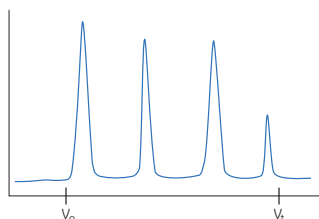
Regulatory Support Files contain details of performance, stability, extractable compounds and analytical methods available. The essential information in these files gives an invaluable starting point for process validation, as well as support for clinical and marketing applications submitted to regulatory authorities.

Large capacity production integrated with clear ordering and delivery routines mean BioProcess media are available in the right quantity, at the right place, at the right time. Future supplies of BioProcess media are assured, making them a safe investment for long-term production.

BioProcess Media is produced following validated methods and are tested under strict control, fulfilling performance specifications. A certificate of analysis is available with each order. Our media safety stock agreements offer the right quantity of media, manufactured to specified quality levels, and delivered at the right time.

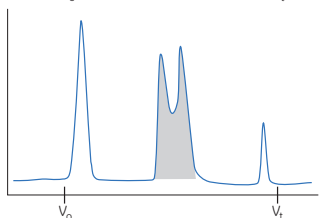
Troubleshooting

This section focuses on practical problems that may occur during gel filtration. Figure 1.14 indicate how a chromatogram may deviate from ideal behavior during a separation. Table 1.4 on the following pages contains suggestions of possible causes and their remedies.



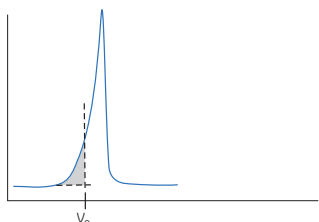
Satisfactory separation

Well resolved, symmetrical peaks.



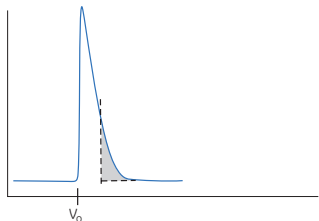
Poor resolution

Review factors affecting resolution (see page 12), including media selection, particle size, sample volume: column volume and flow rate.



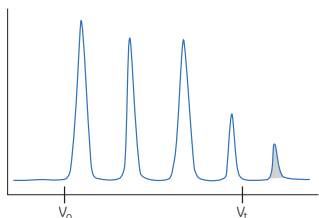
Leading peaks

Asymmetric peaks: sample elutes before void volume indicates channelling in column bed. Leading peaks can also be due an overpacked column (packed at too high a pressure or flow rate). The column may need to be repacked.



Tailing peaks

Asymmetric peaks: sample application uneven. Check the top of column if possible. Ensure the medium is evenly packed and that sample is applied without disturbing the packed bed. Tailing peaks can also be due to underpacking of the column (packed at too low a pressure or flow rate).



Late elution

Peaks seen after one column volume of buffer has passed through the column. Always include a wash step between runs to ensure removal of late eluting molecules.

Fig 1.14. Normal chromatograms and chromatograms deviating from ideal behavior.

Table 1.4. Troubleshooting guide for gel filtration

Situation	Cause	Remedy
Peak of interest is poorly resolved from other major peaks.	Sample volume is too high.	Decrease sample volume and apply 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 not mounted vertically.	Adjust column position. Column may need to be repacked'.
	Column is poorly packed.	Check column efficiency (See Appendix 1). Repack if needed*. Use prepacked columns.
	Column is dirty.	Clean and re-equilibrate.
	Incorrect medium.	Check selectivity curve. Check for adsorption effects. Consider effects of denaturing agents or detergents if present.
	Large dead volumes. Column too short. Flow rate too high.	Minimize dead volumes in tubings and connections. See Appendix 1 for recommended bed heights. Check recommended flow rates. Reduce flow.
Protein does not elute as expected.	Ionic interactions between protein and matrix.	Maintain ionic strength of buffers above 0.05 M (preferably include 0.15 M NaCl).
	Hydrophobic interactions between protein and matrix.	Reduce salt concentration to minimize hydrophobic interaction. Increase pH. Add suitable detergent or organic solvent, e.g., 5% isopropanol.
	Sample has not been filtered properly.	Clean the column, filter the sample and repeat.
	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. Column is overloaded with sample. Microbial growth has occurred in the column.	Clean the column or use a new column. Decrease the sample load. Microbial growth rarely occurs in columns during use. To prevent infection of packed columns, store in 20% ethanol when possible.
Protein elutes later than expected or even after running a total column volume.	Hydrophobic and/or ionic interactions between protein 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 150 mM) to minimize ionic interaction.
Peaks elute late and are very broad.	Column is dirty.	Clean and re-equilibrate.
Protein elutes earlier than expected (before the void volume).	Channeling in the column.	Repack column using thinner slurry of medium. Avoid introduction of air bubbles*.
Molecular weight or shape is not as expected.	Protein has changed during storage	Prepare fresh samples.
	Ionic interactions between proteins and matrix.	Maintain ionic strength of buffers above 0.05 M (preferably include 0.15 M NaCl).
	Hydrophobic interactions between protein and matrix.	Reduce salt concentration to minimize hydrophobic interaction. Increase pH. Add suitable detergent or organic solvent e.g., 5% isopropanol.
	Precipitation of protein in the column filter and/or at the top of the bed.	If possible, clean the column, exchange or clean the filter or use a new column.
Leading or very rounded peaks in chromatogram.	Column overloaded.	Decrease sample load and repeat.
Tailing peaks.	Column is 'under packed'.	Check column efficiency (see Appendix 1). Repack using a higher flow rate. Use prepacked columns*.

Situation	Cause	Remedy
Leading peaks.	Column is 'over packed'.	Check column efficiency (see Appendix 1). Repack using a lower flow rate. Use prepacked columns*.
Medium/beads appear in eluent.	Bed support end piece is loose or broken.	Replace or tighten.
	Column operated at too high pressure.	Do not exceed recommended operating pressure for medium or column.
Low recovery of activity but normal recovery of protein.	Protein may be unstable or inactive in the buffer	Determine the pH and salt stability of the protein.
	Enzyme separated from co-factor or similar.	Test by pooling aliquots from the reactions and repeat the assay.
Lower yield than expected.	Protein may have been degraded by proteases.	Add protease inhibitors to the sample and buffers to prevent proteolytic digestion. Run sample through a medium 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.	May be caused by removal of salts or unsuitable buffer conditions.
	Hydrophobic proteins.	Use denaturing agents, polarity reducing agents, or detergents.
More sample is recovered than expected.	Nonspecific adsorption.	
	Protein is co-eluting with other substances.	Optimize conditions to improve resolution. Check buffer conditions used for assay before and after the run. Check selection of medium.
More activity is recovered than was applied to the column.	Different assay conditions have been used before and after the chromatography step.	Use the same assay conditions for all the assays in the purification scheme.
	Removal of inhibitors during separation.	
Reduced or poor flow through the column.	Presence of lipoproteins or protein aggregates.	Remove lipoproteins and aggregates during sample preparation (see Appendix 3).
	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 or adaptor 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.	If possible, repack the column or use a new column*.
	Microbial growth.	Microbial growth rarely occurs in columns during use. To prevent infection of packed columns, store in 20% ethanol when possible.
	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 (see Appendix 3). 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.

Situation	Cause	Remedy
Air bubbles in the bed.	Column packed or stored at cool temperature and then warmed up.	Remove small bubbles by passing degassed buffer through the column. Take special care if buffers are used after storage in a fridge or cold-room. Do not allow column to warm up due to sunshine or heating system. Repack column if possible (see Appendix 1*).
	Buffers not properly degassed.	Buffers must be degassed thoroughly.
Cracks in the bed.	Large air leak in column.	Check all connections for leaks. Repack the column if possible (see Appendix 1).
Distorted bands as sample runs into the bed.	Air bubble at the top of the column or in the inlet adaptor.	If possible, re-install the adaptor taking care to avoid air bubbles.
	Particles in buffer or sample.	Filter or centrifuge the sample. Protect buffers from dust.
	Blocked or damaged net in upper adaptor.	If possible, dismantle the adaptor, 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 (too low packing pressure, too short equilibration). Column packed at too high pressure.

* Not all prepacked columns can be repacked.

Chapter 2

Superdex: the first choice for high resolution, short run times, and high recovery

Superdex are gel filtration media with a unique composite medium of dextran and agarose. This matrix combines the excellent gel filtration properties of cross-linked dextran with the physical and chemical stabilities of highly cross-linked agarose, to produce a separation medium with outstanding selectivity and high resolution. In addition, its low nonspecific interaction permits high recovery of biological material. Together these properties make Superdex the first choice gel filtration media for all applications from laboratory to process scale.



Fig 2.1. Fractionation ranges for Superdex.



Fig 2.2. Prepacked HiLoad Superdex prep grade columns.

Superdex is the first choice for a high resolution fractionation with short run times and good recovery. Selectivity curves and pressure-flow relationship curves for Superdex are shown in Figures 2.3 and 2.4. A typical linear flow is up to 75 cm/h. The media is available in two versions, Superdex and Superdex prep grade. The difference is the particle sizes.

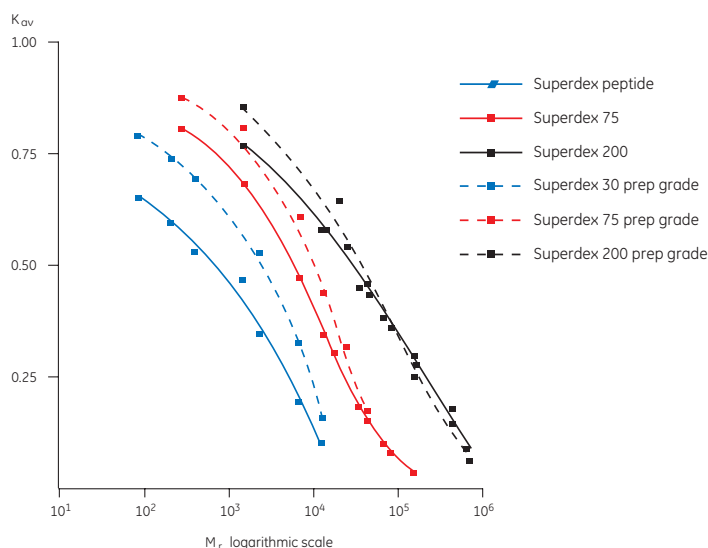


Fig 2.3. Selectivity curves for Superdex (13 μm) and Superdex prep grade (34 μm) media.

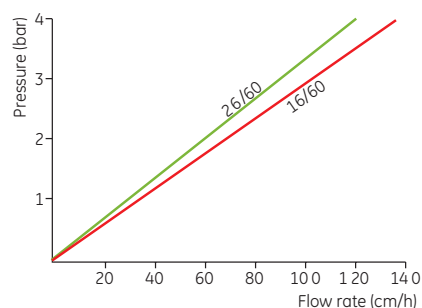


Fig 2.4. Pressure drop as a function of flow rate for HiLoad columns packed with Superdex prep grade. Bed height approximately 60 cm in distilled water at 25°C. To calculate volumetric flow rate, multiply linear flow by cross-sectional area of column (2 cm^2 for XK 16, 5.3 cm^2 for XK 26). See Appendix 5 for more information about flow rate calculations.

Superdex is a composite medium based on highly cross linked porous agarose particles to which dextran has been covalently bonded, as illustrated in Figure 2.5. The result is media with high physical and chemical stability, mainly due to the highly cross-linked agarose matrix, and excellent gel filtration properties determined mainly by the dextran chains. The mechanical rigidity of Superdex allows even relatively viscous eluents, such as 8 M urea, to be run at practical flow rates. The media can withstand high flow rates during equilibration or cleaning thereby shortening overall cycle times. This stability makes Superdex prep grade very suitable for use in industrial processes where high flow rates and fast, effective cleaning-in- place protocols are required. Under normal chromatography conditions nonspecific interactions between proteins and Superdex are negligible when using buffers with ionic strengths in the range 0.15 M to 1.5 M.

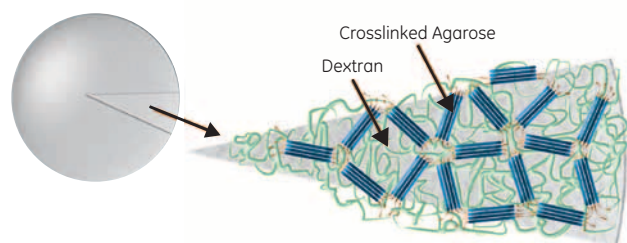


Fig 2. 5. A schematic section of a Superdex particle. In Superdex the dextran chains are covalently linked to a highly cross-linked agarose matrix.

Separation options

Superdex is produced in two different mean particle sizes (13 µm and 34 µm) and four different selectivities (Superdex Peptide, Superdex 30, Superdex 75 and Superdex 200, see Table 2.1).



Use the 13 µm particles of Superdex Peptide, Superdex 75 and Superdex 200 in prepacked columns for highest resolution analytical separations with smaller sample volumes.



Use the 34 µm particles of Superdex prep grade (available in prepacked columns or as bulk media) for preparative applications.

Table 2.1. Separation options with Superdex medium

Product*	Fractionation range, M_r (globular proteins)	Sample loading capacity†	Maximum operating back pressure	Recommended operation flow‡
Superdex Peptide PC 3.2/30	$1 \times 10^2 - 7 \times 10^3$	25–250 µl	1.8 MPa, 18 bar, 260 psi	<0.15 ml/min
Superdex Peptide 10/300 GL	$1 \times 10^2 - 7 \times 10^3$	25–250 µl	1.8 MPa, 18 bar, 260 psi	<1.2 ml/min
HiLoad 16/60 Superdex 30 pg§	$<1 \times 10^4$	≤5 ml	0.3 MPa, 3 bar, 42 psi	≤1.6 ml/min
HiLoad 26/60 Superdex 30 pg§	$<1 \times 10^4$	≤13 ml	0.3 MPa, 3 bar, 42 psi	≤4.4 ml/min
Superdex 30 pg§(Bulk media)	$<1 \times 10^4$	0.5–4% of total column volume	Column-dependent	10–50 cm/h
Superdex 75 PC 3.2/30	$3 \times 10^3 - 7 \times 10^4$	<50 µl	2.4 MPa, 24 bar, 350 psi	<0.1 ml/min
Superdex 75 5/150 GL	$3 \times 10^3 - 7 \times 10^4$	4–50 µl	1.8 MPa, 18 bar, 260 psi	<0.6 ml/min
Superdex 75 10/300 GL	$3 \times 10^3 - 7 \times 10^4$	25–250 µl	1.8 MPa, 18 bar, 260 psi	<1.5 ml/min
HiLoad 16/60 Superdex 75 pg§	$3 \times 10^3 - 7 \times 10^4$	≤5 ml	0.3 MPa, 3 bar, 42 psi	≤1.6 ml/min
HiLoad 26/60 Superdex 75 pg§	$3 \times 10^3 - 7 \times 10^4$	≤13 ml	0.3 MPa, 3 bar, 42 psi	≤4.4 ml/min
Superdex 75 pg§(Bulk media)	$3 \times 10^3 - 7 \times 10^4$	0.5–4% of total column volume	Column-dependent	10–50 cm/h
Superdex 200 PC 3.2/20	$1 \times 10^4 - 6 \times 10^5$	<50 µl	1.5 MPa, 15 bar, 220 psi	<0.1 ml/min
Superdex 200 5/150 GL	$1 \times 10^4 - 6 \times 10^5$	4–50 µl	1.5 MPa, 15 bar, 220 psi	<0.6 ml/min
Superdex 200 10/300 GL	$1 \times 10^4 - 6 \times 10^5$	25–250 µl	1.5 MPa, 15 bar, 220 psi	0.25–0.75 ml/min
HiLoad 16/60 Superdex 200 pg§	$1 \times 10^4 - 6 \times 10^5$	≤5 ml	0.3 MPa, 3 bar, 42 psi	≤1.6 ml/min
HiLoad 26/60 Superdex 200 pg§	$1 \times 10^4 - 6 \times 10^5$	≤13 ml	0.3 MPa, 3 bar, 42 psi	≤4.4 ml/min
Superdex 200 pg§(Bulk media)	$1 \times 10^4 - 6 \times 10^5$	0.5–4% of total column volume	Column-dependent	10–50 cm/h

* PC and 10/300 GL columns are packed with Superdex and HiLoad are packed with Superdex prep grade.

† For maximum resolution, apply as small sample volume as possible. Note that sample volumes less than 0.5% normally do not improve resolution.

‡ See Appendix 5 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa.

§ prep grade.



Start with Superdex 200 when the molecular weight of the protein of interest is unknown. Superdex 200 or Superdex 200 prep grade are especially suitable for the separation of monoclonal antibodies from dimers and from contaminants of lower molecular weight, for example albumin and transferrin.



Start with Superdex Peptide or Superdex 30 prep grade for separations of peptides, oligonucleotides and small proteins below M_r 10 000.



Exposure to temperatures outside the range 4°C to 40°C will destroy the efficiency of the packed bed and the column will need to be repacked.

Separation examples

Figures 2.6 to 2.15 illustrate examples of separations performed on Superdex Peptide, Superdex 75 and Superdex 200 run on ÄKTA™ design systems. Detection can also be made by light scattering (Fig 2.9). This method reveals the molecular mass in an unambiguous way.

Column: Superdex Peptide 10/300 GL
 Sample: 1. Cytochrome c (M_r 12 384) 1 mg/ml
 2. Aprotinin (M_r 6 512) 2 mg/ml
 3. Vitamin B12 (M_r 1 355) 0.1 mg/ml
 4. (Gly)₃ (M_r 189) 0.1 mg/ml
 5. Gly (M_r 75) 7.8 mg/ml
 Sample load: 50 µl
 Buffer: 50 mM phosphate, 0.15 M NaCl, pH 7.0
 Flow rate: 0.5 ml/min, room temperature

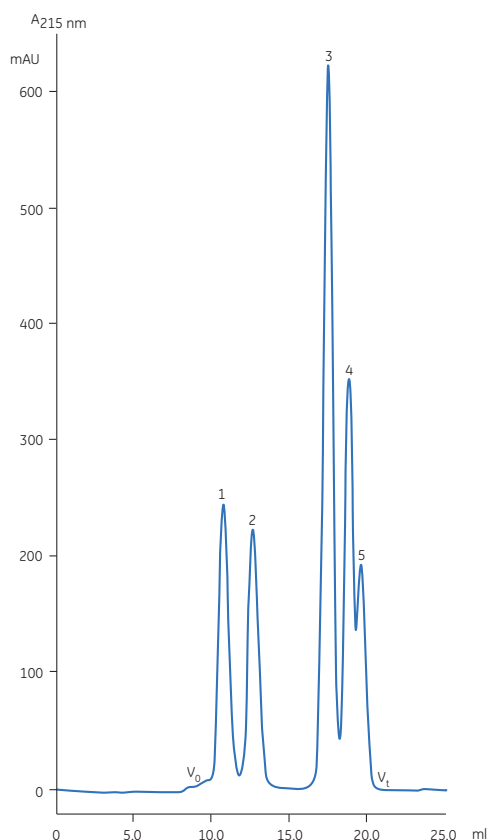


Fig 2.6. Separation of standard peptides on Superdex Peptide 10/300 GL.

Column: Superdex 200 HR 10/30
Sample: M_r Conc. (mg/ml)
 Thyroglobulin 669 000 3
 Ferritin 440 000 0.7
 IgG 150 000 3
 Transferrin 81 000 3
 Ovalbumin 43 000 3
 Myoglobin 17 600 2
 Vitamin B12 1 355 0.5
 Total 15.2
Total sample amount: 0.38 mg
Sample load: 25 ml
Buffer: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0
Flow rate: 0.25 ml/min (19 cm/h)

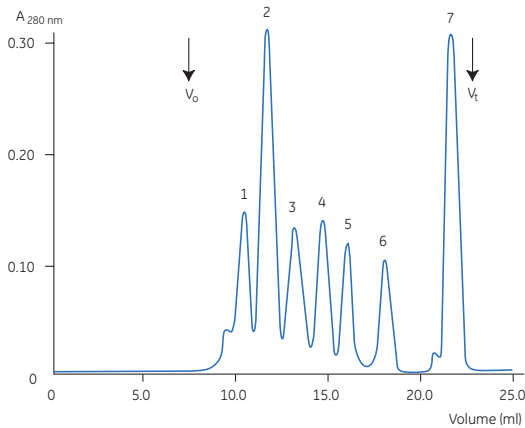
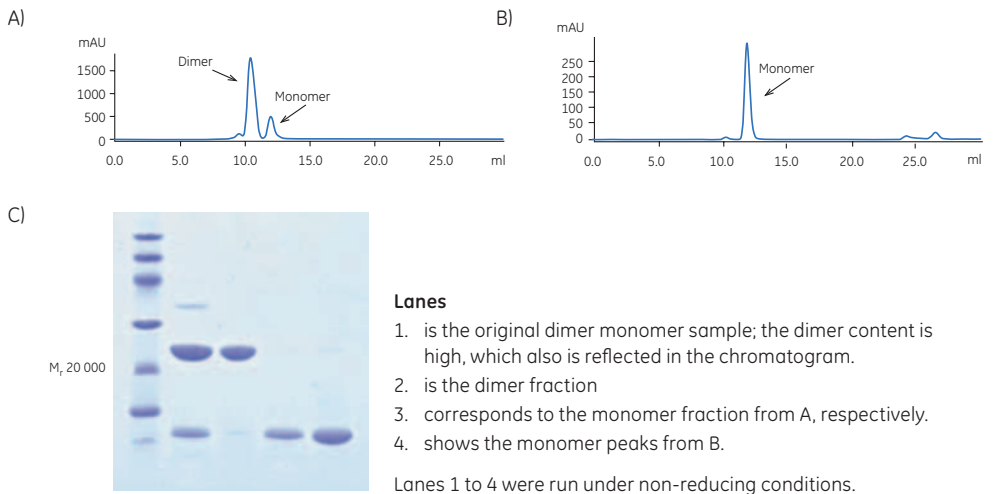


Fig 2.7. Separation of standard proteins (Superdex 200 HR 10/30 is replaced with Superdex 10/300 GL).

Column: Superdex 75 10/300 GL
Sample: rec Cys-prot
Sample load: 200 μ l
Buffer: 0.05 M Tris HCl, 1 mM EDTA, 0.15 M NaCl, pH 8.4
Flow rate: 0.5 ml/min



Lanes

1. is the original dimer monomer sample; the dimer content is high, which also is reflected in the chromatogram.
2. is the dimer fraction
3. corresponds to the monomer fraction from A, respectively.
4. shows the monomer peaks from B.

Lanes 1 to 4 were run under non-reducing conditions.

Fig 2.8. (A) Dimer-monomer separation of a recombinant cysteine-containing protein (recCys-prot) on Superdex 75 10/300 GL. (B) purification of the dimer fraction reduced with DTE. (C) Coomassie™ stained SDS-PAGE gel. Lane S is LMW-SDS Marker Kit (17-0446-01),

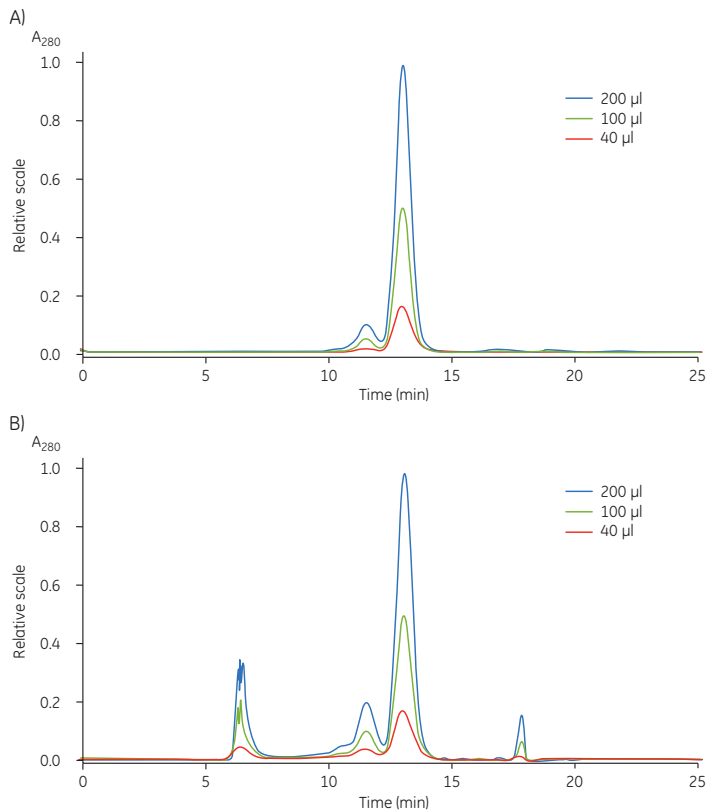


Fig 2.9. Analysis of ovalbumin aggregation on 2 Superdex 200 5/150 columns in series. (A) Detection with A_{280} . (B) Detection with light scattering. Peak 1 is dimer/trimer, peak 2 is the monomer. Note the strong scattering signal from minute amounts of impurities in the void fraction.

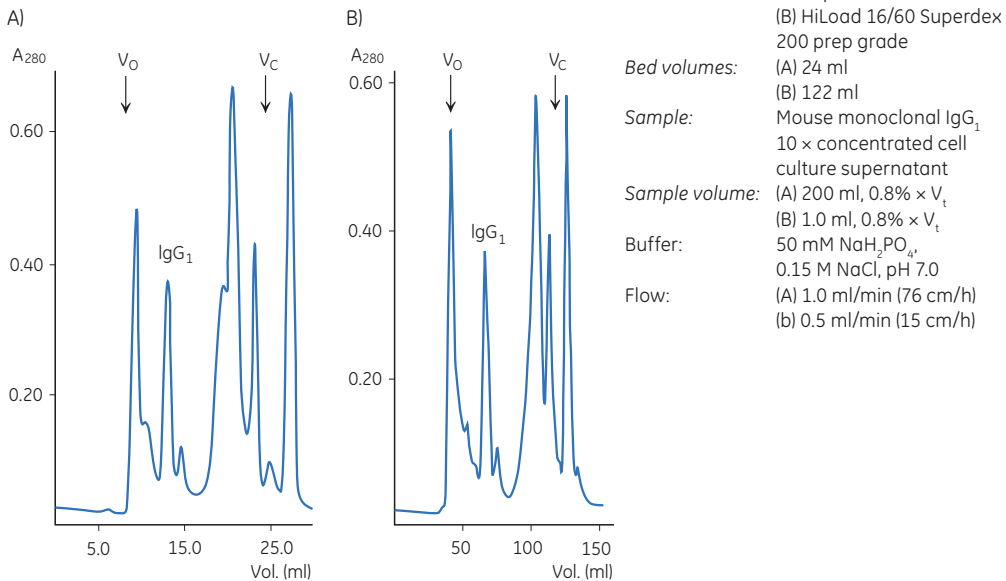


Fig 2.10. Scale-up (five times) of a mouse monoclonal IgG₁ separation from (A) Superdex 200 HR 10/30 (replace by Superdex 200 10/300 GL) to (B) HiLoad 16/60 Superdex 200 prep grade. Superdex 200 prep grade medium (mean particle size 34 µm) has a similar selectivity to Superdex 200 (mean particle size 13 µm).

Column: HiLoad 16/60 Superdex 30 prep grade
Sample: 50 ml mix of five synthetic peptides in 1% TFA
1. M_r 3 894
2. M_r 3 134
3. M_r 2 365
4. M_r 1 596
5. M_r 827
Buffer: 20 mM Tris-HCl, 0.25 M NaCl, pH 8.5
Flow rate: 1 ml/min (30 cm/h)

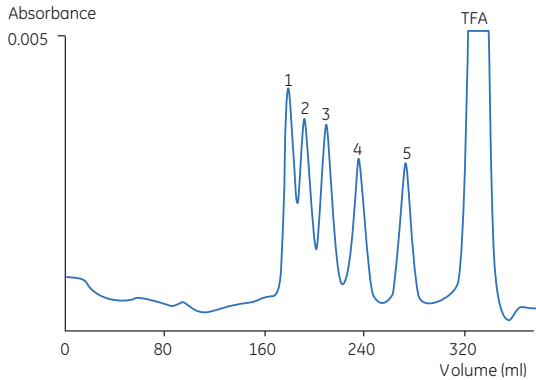


Fig 2.11. Separation of test substances on HiLoad 16/60 Superdex 30 prep grade.

Columns: A) HiLoad 16/60 Superdex 75 prep grade
B) HiLoad 16/60 Superdex 200 prep grade
Sample: 1. Myoglobin 1.5 mg/ml, M_r 17 000
2. Ovalbumin 4 mg/ml, M_r 43 000
3. Albumin 5 mg/ml, M_r 67 000
4. IgG 0.2 mg/ml, M_r 158 000
5. Ferritin 0.24 mg/ml, M_r 440 000
Sample load: 0.5 ml
Buffer: 0.05 M phosphate buffer, 0.15 M NaCl, 0.01% sodium azide, pH 7.0
Flow rate: 1.5 ml/min (45 cm/h)

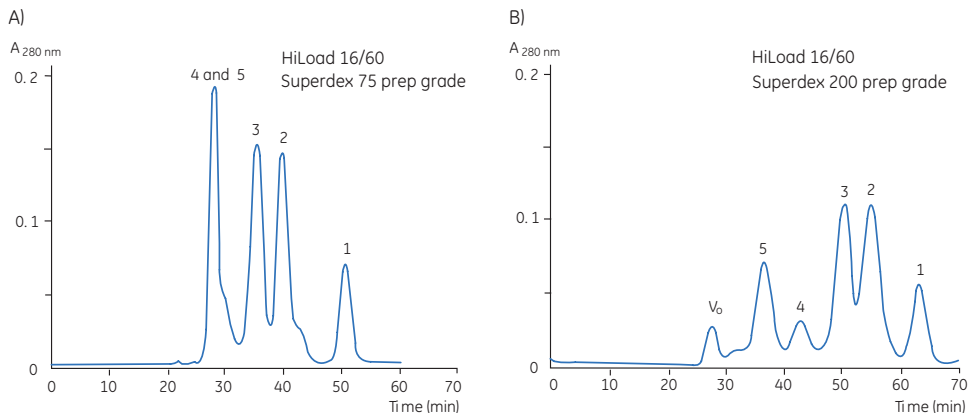


Fig 2.12. Comparison of the selectivity of Superdex 75 prep grade and Superdex 200 prep grade for model proteins. Superdex 75 prep grade (A) gives excellent resolution of the three proteins in the M_r range 17 000 to 67 000 while the two largest proteins elute together in the void volume. Superdex 200 prep grade (B) resolves the two largest proteins completely. The three smaller proteins are not resolved quite as well as the larger ones or as in (A). The void volume (V_0) peak at 28 minutes in (B) is caused by protein aggregates.

Columns: HiLoad Superdex 200 prep grade
 Column volumes, V_t : A) ≈ 120 ml (16/60)
 B) ≈ 320 ml (26/60)
 Sample: Mouse monoclonal cell supernatant, IgG_{2b} incl. 1% Fetal Calf Serum
 Sample pretreatment: Concentration $\approx 40\times$
 Sample load: A) 1.2 ml ($1\% \times V_t$)
 B) 3.2 ml ($1\% \times V_t$)
 Buffer: 50 mM NaH₂PO₄, 0.15 NaCl, pH 7.0
 Flow rate: A) 1.6 ml/min (50 cm/h)
 B) 4.4 ml/min (50 cm/h)
 (max. recommended flow rates)

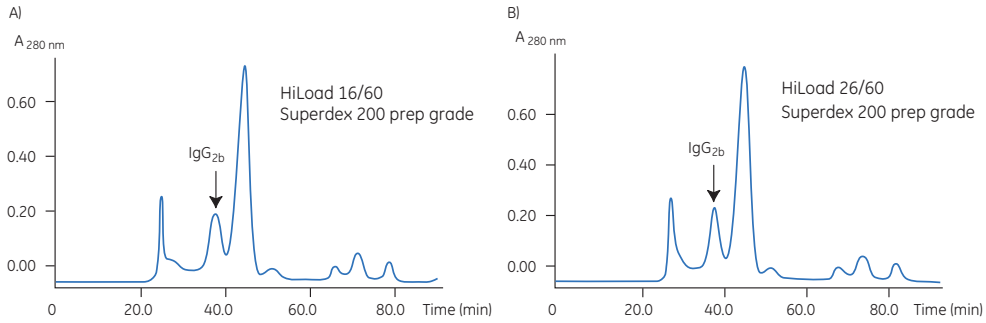


Fig 2.13. Purification of mouse monoclonal IgG_{2b} from cell supernatant using (A) HiLoad 16/60 Superdex 200 prep grade, column volume 120 ml and (B) HiLoad 26/60 Superdex 200 prep grade, column volume 320 ml. Almost identical separations are the result, even using prepacked columns of different sizes.

Column: Superdex 75 5/150 GL
 Sample: Trypsin (M_r 23 800) 11mg/ml
 Aprotinin (M_r 6 500) 3 mg/ml
 Trypsin 11 mg/ml and Aprotinin 3mg/ml
 Sample volume: 10 μ l
 Buffer: PBS, pH 7.4
 Flow rate: 0.3 ml/min

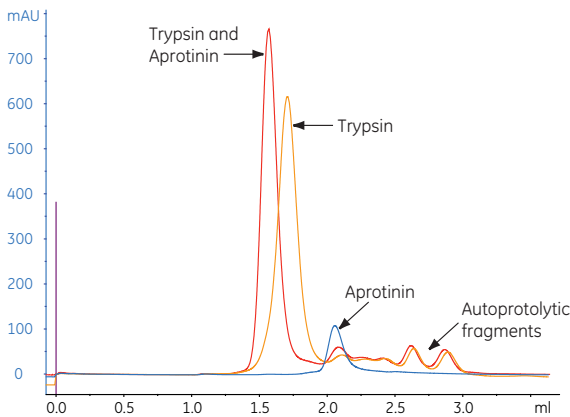


Fig 2.14. Monitoring of protein complex formation between trypsin and aprotinin.

Column:	Superdex 200 5/150 GL
Sample:	Integral membrane protein (M_r 60 000) from <i>E. coli</i>
Sample load:	10 μ l
Buffers (including 0.1 or 0.3 M NaCl):	0.02 M sodium acetate, 0.03% dodecyl maltoside, 0.5 mM TCEP, pH 5.2 0.02 M HEPES, 0.03% dodecyl maltoside, 0.5 mM TCEP, pH 7.5 0.02 M CAPSO, 0.03% dodecyl maltoside, 0.5 mM TCEP, pH 9.5
Flow rate:	0.35 ml/min
Detection:	280 nm

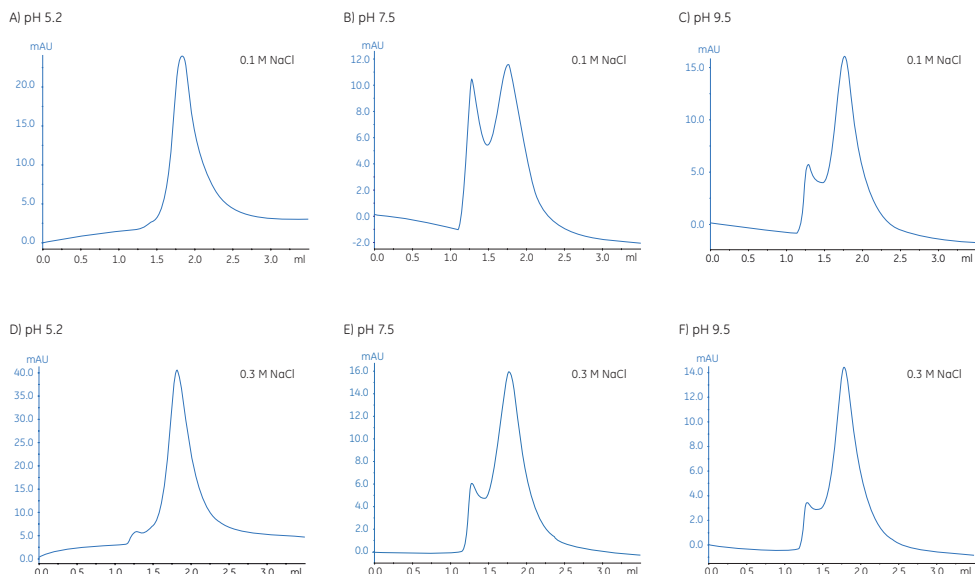


Fig 2.15. Screening of pH and ionic strength conditions for optimal homogeneity and stability of a detergent-protein complex. The chromatograms (A–F) represent the results from the different screening conditions.

Performing a separation

Buffer: 0.05 M sodium phosphate, 0.15 M NaCl pH 7, or select the buffer in which the sample should be stored or solubilized for the next step.

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



The sample should be fully dissolved. Centrifuge or filter to remove particulate material (see Appendix 3). Always use degassed buffers and maintain a constant temperature during the run to avoid introducing air into the column.



Set an appropriate pressure limit on the chromatography system to avoid damage to the column packing.

First time use or after long-term storage

1. Equilibrate the column with 1 column volume of buffer, but containing 0.05 M NaCl at 30 cm/h (0.4 ml/min for 10/300 GL, 1 ml/min for HiLoad 16/60 or 2.6 ml/min for HiLoad 26/60).
2. Equilibrate with 2 column volumes of buffer containing 0.15 M NaCl at 50 cm/h (0.65 ml/min for 10/300 GL, 1.6 ml/min for HiLoad 16/60 or 4.3 ml/min for HiLoad 26/60).

3. Reduce linear flow to 30 cm/h. Apply a sample volume equivalent to 0.5–4% of the column volume (up to 0.25 ml for 10/300 GL, 1.2 ml for HiLoad 16/60 or 3.2 ml for HiLoad 26/60). Note that the smaller the sample volume the better the resolution.
4. Elute with 1 column volume of buffer.
5. Before applying a new sample, re-equilibrate column with 1 column volume of buffer at 50 cm/h and until the baseline monitored at A_{280} is stable.

Column performance should be checked at regular intervals by determining the theoretical plate number per meter and peak symmetry. Prepacked columns are supplied with recommended values. See page 90 for how to check column efficiency.

See page 23 for advice on optimizing the separation.



Exposure to temperatures outside the range 4°C to 40°C will destroy the efficiency of a packed bed and the column will need to be repacked.

Cleaning

1. Wash with 1 column volume of 0.5 M NaOH at a flow of 25 cm/h (0.33 ml/min for 10/300 GL, 0.8 ml/min for HiLoad 16/60 or 2.2 ml/min for HiLoad 26/60) to remove most nonspecifically adsorbed proteins.
2. Wash with 1 column volume of distilled water at 25 cm/h.
3. Re-equilibrate with 2 column volumes of buffer at a flow of 50 cm/hr (0.4 ml/min for 10/300 GL, 1.6 ml/min HiLoad 16/60 or 4.3 ml/min for HiLoad 26/60) or until the baseline monitored at A_{280} and the pH of the eluent are stable.

Further equilibration may be necessary if the buffer contains detergent.



Routine cleaning after every 10–20 separations is recommended, but the frequency of cleaning will also depend on the nature of the samples being applied.

Removing severe contamination

1. Reverse the flow and wash at a linear flow of 25 cm/h at room temperature.
2. Wash with 4 column volumes of 1 M NaOH (to remove hydrophobic proteins or lipoproteins) followed by 4 column volumes of distilled water.
3. Wash with 0.5 column volume of 30% isopropanol to remove lipids and very hydrophobic proteins, followed by 2 column volumes of distilled water.
4. Equilibrate the column with at least 5 column volumes of buffer, or until the baseline monitored at A_{280} and the pH of the eluent are stable, before beginning a new separation.

For extreme cases of contamination, check the instructions supplied with the product.

Media characteristics

Matrix: Spherical composite of cross-linked agarose and dextran.

Table 2.3 Superdex media characteristics

Product	Efficiency*	pH stability†	Particle size	Mean particle size
Superdex Peptide	$\geq 30\,000\text{ m}^{-1}$	Long term: 1–14 Short term: 1–14	12–14 μm	13 μm
Superdex 75	$\geq 30\,000\text{ m}^{-1}$	Long term: 3–12 Short term: 1–14	12–14 μm	13 μm
Superdex 200	$\geq 30\,000\text{ m}^{-1}$	Long term: 3–12 Short term: 1–14	12–14 μm	13 μm
Superdex 30 prep grade	$\geq 13\,000\text{ m}^{-1}$	Long term: 3–12 Short term: 1–14	24–44 μm	34 μm
Superdex 75 prep grade	$\geq 13\,000\text{ m}^{-1}$	Long term: 3–12 Short term: 1–14	24–44 μm	34 μm
Superdex 200 prep grade	$\geq 13\,000\text{ m}^{-1}$	Long term: 3–12 Short term: 1–14	24–44 μm	34 μm

* Theoretical plates per meter (prepacked columns only).

† Long term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on its chromatography performance. Short term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges are estimates based on the experience and knowledge within GE Healthcare.

Chemical stability

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

The following solutions can be used for cleaning: up to 30% acetonitrile, up to 1 M sodium hydroxide, up to 70% ethanol (Superdex 30 prep grade), up to 24% ethanol (Superdex 75 prep grade and Superdex 200 prep grade), up to 1 M acetic acid, up to 30% isopropanol or up to 0.1 M HCl (Superdex 30 prep grade).

Storage

Store unused media 4°C to 30°C in 20% ethanol. Do not freeze.

Columns can be left connected to a chromatography system with a low flow rate (0.01 ml/min) of buffer passing through the column to prevent bacterial growth or the introduction of air into the column which destroys the packing.

For long term storage, wash with 4 column volumes of distilled water followed by 4 column volumes of 20% ethanol. Store at 4°C to 30°C.

Degas the ethanol/water mixture thoroughly and use a low flow rate, checking the back pressure as the column equilibrates.

Avoid changes in temperature which may cause air bubbles in the packing.

Chapter 3

Superose: broad fractionation range for laboratory scale

Superose are media with high physical and chemical stability based on highly cross-linked porous agarose particles. Typical fractionation ranges for Superose are shown in Figure 3.1. The mechanical rigidity of Superose allows even relatively viscous eluents, such as 8 M urea, to be run at practical flow rates. Under normal chromatography conditions nonspecific interactions between proteins and Superose are negligible when using buffers with ionic strengths in the range 0.15 M to 1.5 M.

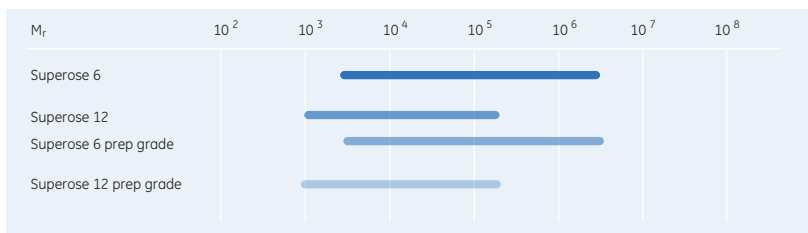


Fig 3.1. Fractionation ranges of Superose.

Some hydrophobic interactions have been noted, particularly for compounds such as smaller hydrophobic and/or aromatic peptides, membrane proteins and/or lipoproteins which may elute later than predicted. However, in some applications, these interactions can be an advantage for increasing the resolution of the separation.

Typical selectivity curves for Superose are shown in Figure 3.2.

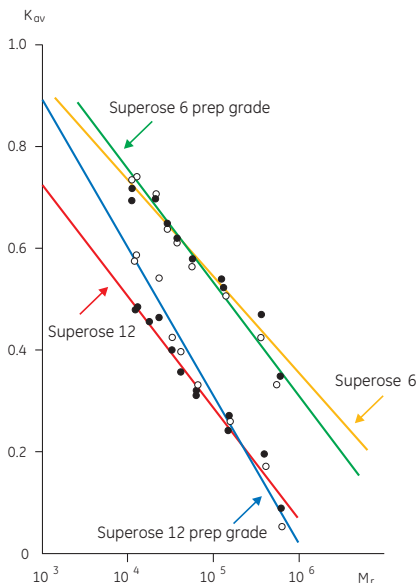
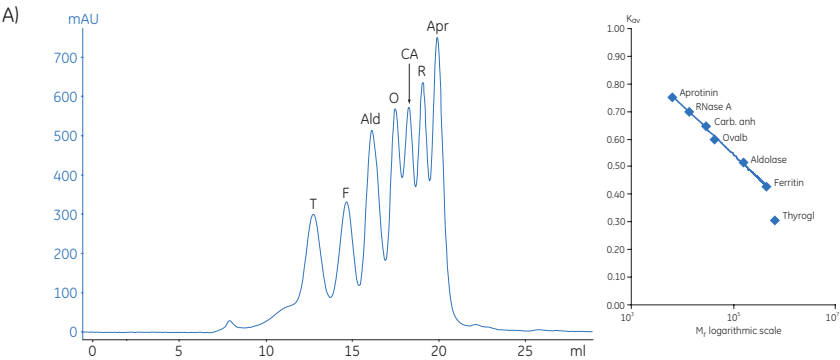


Fig 3.2. Selectivity curves of Superose for globular proteins.

Figure 3.3 gives a comparison of the different selectivities of Superose 6 and Superose 12 prepacked columns.

Column: Superose 6 10/300 GL
Sample: Proteins from Gel Filtration Calibration Kits LMW and HMW (GE Healthcare)
T = thyroglobulin (M_r 669 000)
F = ferritin (M_r 440 000)
Ald = aldolase (M_r 158 000)
O = ovalbumin (M_r 43 000)
CA = carbonic anhydrase (M_r 29 000)
R = RNase A (M_r 13 700)
Apr = aprotinin (M_r 6 500)
Sample load: 100 μ l
Eluent: 0.050 M Phosphate, 0.15 M NaCl, pH 7.2
Flow rate: 0.5 ml/min
System: ÄKTAexplorer™ 10
Detection: 280 nm



Column: Superose 12 10/300 GL
Sample: Proteins from Gel Filtration Calibration Kits LMW and HMW (GE Healthcare)
F = ferritin (M_r 440 000)
Ald = aldolase (M_r 158 000)
O = ovalbumin (M_r 43 000)
CA = carbonic anhydrase (M_r 29 000)
R = RNase A (M_r 13 700)
Apr = aprotinin (M_r 6 500)
Sample load: 100 μ l
Eluent: 0.050 M Phosphate, 0.15 M NaCl, pH 7.2
Flow rate: 0.5 ml/min
System: ÄKTAexplorer 10
Detection: 280 nm

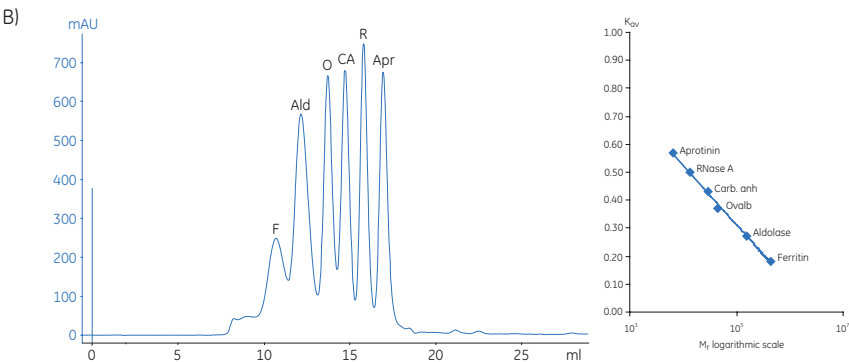


Fig 3.3. (A) Standard proteins separated on Superose 6 10/300 GL, M_r range: 5 000 to 5 000 000 and calibration curve. (B) Standard protein separated on Superose 12 10/300 GL, M_r range 1 000 to 300 000 and calibration curve.

Separation options

Superose is produced in different particle sizes (10 µm, 13 µm and 30 µm) and with two different selectivities, Superose 6 and Superose 12, and is available as prepacked columns and bulk packs (Table 3.1).

 Use 11 µm or 13 µm particles for analytical separations and 30 µm particles for preparative separations

Table 3.1. Separation options with Superose media

Product	Fractionation range, M_r (globular proteins)	Sample loading capacity*	Maximum operating back pressure	Recommended operation flow†
Superose 6 10/300 GL	5×10^3 – 5×10^6	25–250 µl	1.5 MPa, 15 bar, 217 psi	0.1–0.5 ml/min
Superose 6 PC 3.2/30	5×10^3 – 5×10^6	<50 µl	1.2 MPa, 12 bar, 175 psi	0.01–0.1 ml/min
Superose 6 prep grade (Bulk media)	5×10^3 – 5×10^6	0.5–4% of total column volume	Column-dependent	10–20 cm/h
Superose 12 10/300 GL	1×10^3 – 3×10^6	25–250 µl	3.0 MPa, 30 bar, 435 psi	0.5–1 ml/min
Superose 12 PC 3.2/30	1×10^3 – 3×10^6	<50 µl	2.4 MPa, 24 bar, 350 psi	0.04 ml/min
Superose 12 prep grade (Bulk media)	1×10^3 – 3×10^6	0.5–4% of total column volume	Column-dependent	10–20 cm/h

* For maximum resolution, apply as small sample volume as possible. Note that sample volumes less than 0.5% normally do not improve resolution.

† See Appendix 5 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa.

Separation examples

Figures 3.4 and 3.5 show examples of separations performed on Superose media.

Column: Superose 12 PC 3.2/30
Sample: 0.75 µl human tears
Buffer: 0.02 M sodium phosphate, 0.5 M NaCl, pH 5.3
Flow: 40 µl/min

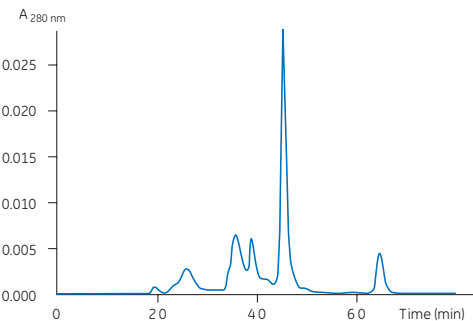


Fig 3.4. Microfractionation of 0.75 µl of human tears.

Column: 2× Superose 6 HR 10/30 in series
Sample: 10 µg *Hae* III cleaved pBR 322
Buffer: 0.05 M Tris-HCl, 1 mM EDTA, pH 8.0
Flow: 0.1 ml/min

Column: Superose 6 HR 10/30
Sample: φX-174 RF DNA-*Hae* III digest, 10 µg
Buffer: 0.05 M Tris-HCl, pH 8.0
Flow: 0.4 ml/min

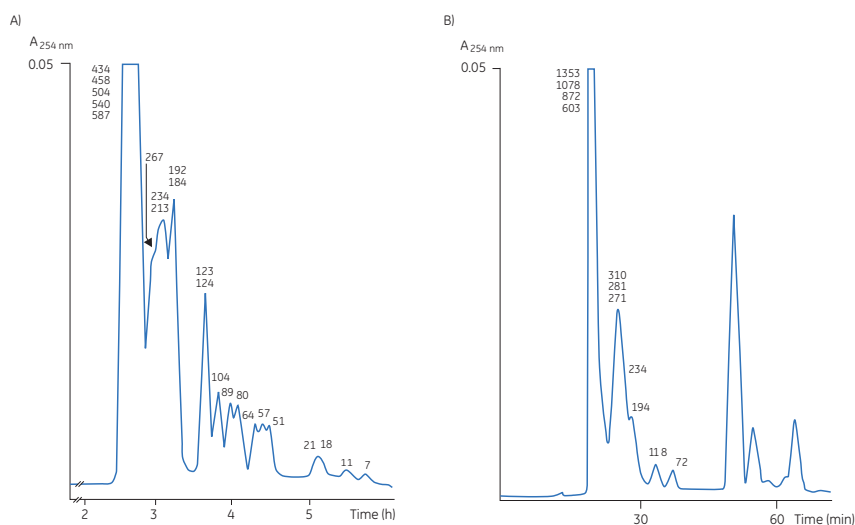


Fig 3.5. Separation of DNA fragments (Superose 6 HR 10/30 is replaced with Superose 6 10/300 GL). Peak figures correspond to number of base pairs.

Performing a separation

Buffer: 0.05 M sodium phosphate, 0.15 M NaCl pH 7, or select the buffer in which the sample should be stored or solubilized for the next step.

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



The sample should be fully dissolved. Centrifuge or filter to remove particulate material (see Appendix 3). Always use degassed buffers and maintain a constant temperature during the run to avoid introducing air into the column.



Set an appropriate pressure limit on the chromatography system to avoid damage to the column packing.

First time use or after long-term storage

Superose 6

Superose 6 swells slightly when transferred from ethanol to water. To avoid local high back pressure, take the following steps during initial equilibration:

- 12 ml distilled water at 0.2 ml/min at room temperature
- 38 ml distilled water at 0.5 ml/min at room temperature
- 50 ml eluent at 0.5 ml/min at room temperature

Ensure that the back pressure over the column does not exceed 1.2 MPa during equilibration.

Superose 12

- 50 ml distilled water at 0.2 to 0.5 ml/min at room temperature
- 50 ml eluent at 0.5 ml/min at room temperature

Ensure that the back pressure over the column does not exceed 2 MPa during equilibration.

Note: Precipitation of the sample may block the filter and cause compression of the gel. Therefore, the advise is to never set the pressure limit control to more than 0.2 MPa above the actual operating pressure.

Column performance should be checked at regular intervals by determining the theoretical plate number per meter and peak symmetry. Prepacked columns are supplied with recommended values. See page 90 for how to check column efficiency.

See page 23 for advice on optimizing the separation.



Exposure to temperatures outside the range 4°C to 40°C will destroy the efficiency of a packed bed and the column will need to be repacked.

Cleaning

1. Wash with 1 column volume 0.5 M NaOH at 40 cm/h (0.5 ml/min for 10/300 GL columns).
2. Rinse immediately with 1 column volume of distilled water or buffer at 40 cm/h.
3. Continue to re-equilibrate with 2 column volumes of buffer or until the baseline and the eluent pH are stable.

For extreme cases of contamination, check the instructions supplied with the product.

In special cases, it may be necessary to change the bottom filter or to remove and discard the top 2–3 mm of the gel. These operations must be done with extreme care to avoid serious loss of resolution. Note that Precision Columns should not be opened.

Superose prep grade may be autoclaved repeatedly at 121°C, pH 7 for 30 minutes without significantly affecting its chromatography properties. The medium must be removed from the column as autoclaving can damage column components (Note that Precision Columns cannot be repacked).

Media characteristics

Matrix: Cross-linked agarose.

Superose prep grade shows less tendency towards hydrophobic interactions than Superose in prepacked columns. Superose 6 shows less tendency towards hydrophobic interactions than Superose 12.

Table 3.2 Superose media characteristics

Product	Efficiency*	pH stability†	Particle size	Mean particle size
Superose 6	≥ 30 000 m ⁻¹	Long term: 3–12 Short term: 1–14	11–15 µm	13 µm
Superose 6 prep grade	†	Long term: 3–12 Short term: 1–14	20–40 µm	30 µm
Superose 12	≥ 40 000 m ⁻¹	Long term: 3–12 Short term: 1–14	8–12 µm	10 µm
Superose 12 prep grade	†	Long term: 3–12 Short term: 1–14	20–40 µm	30 µm

* Theoretical plates per meter (prepacked columns only).

† Long term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on its chromatography performance. Short term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges are estimates based on the experience and knowledge within GE Healthcare.

† A minimum column efficiency of 10 000 m⁻¹ should be expected for a well-packed column.

Chemical stability

Superose is stable in all commonly used aqueous buffers and additives such as detergents (1% SDS), denaturing agents (8 M urea or 6 M guanidine hydrochloride) and 30% acetonitrile.

Storage

Store unused media 4°C to 30°C in 20% ethanol. Do not freeze.

Columns can be left connected to a chromatography system with a low flow rate (0.01 ml/min) of buffer passing through the column to prevent bacterial growth or the introduction of air into the column which would destroy the packing.

For long term storage, wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Store at 4°C to 30°C.

Degas the ethanol/water mixture thoroughly and use a low flow rate, checking the back pressure as the column equilibrates.

Avoid changes in temperature which may cause air bubbles in the packing.

Chapter 4

Sephacryl: fast, high recovery separations at laboratory and industrial scale

Sephacryl High Resolution (HR) media provide a useful alternative to Superdex prep grade for applications that require a slightly broader fractionation range, as shown in Figure 4.1. High chemical stability and tolerance of high flow rates make Sephacryl well suited for industrial use.

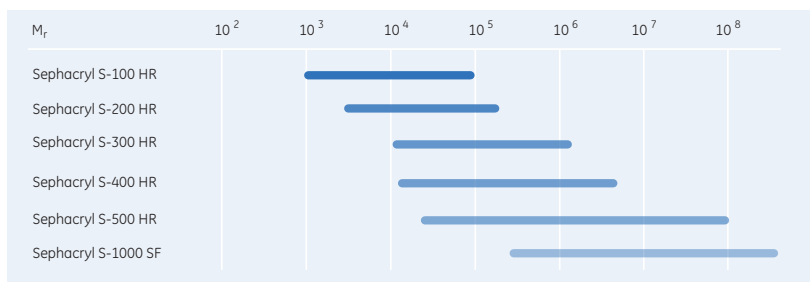


Fig 4.1. Fractionation ranges for Sephacryl HR.



Fig 4.2. Sephacryl is available as loose media and in prepacked columns.

Figure 4.3 shows comparisons of the different selectivities of Sephacryl HR.

Typical selectivity and pressure-flow relationship curves for Sephacryl are shown in Figures 4.4 and 4.5.

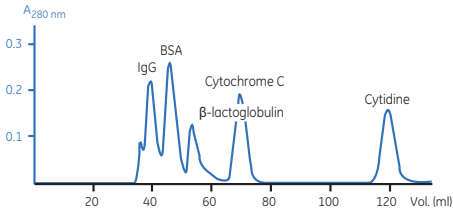
Column: A) HiPrep 16/60 Sephacryl S-100 HR
 B) HiPrep 16/60 Sephacryl S-200 HR
 C) HiPrep 16/60 Sephacryl S-300 HR

Sample: 500 μ l 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)

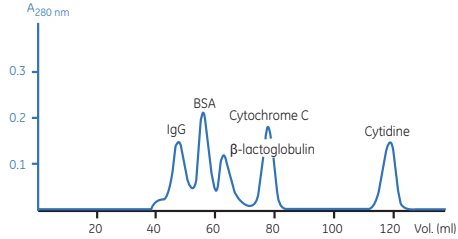
Buffer: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0

Flow rate: 0.8 ml/min (24 cm/h)

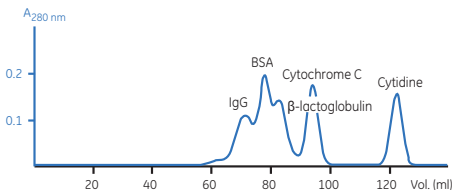
A) HiPrep 16/60 Sephacryl S-100 HR



B) HiPrep 16/60 Sephacryl S-200 HR



C) HiPrep 16/60 Sephacryl S-300 HR



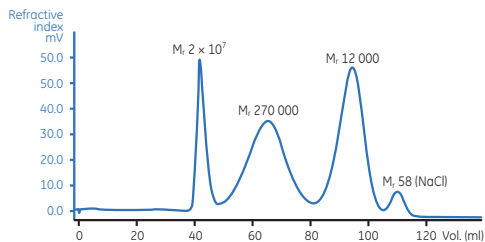
Column: D) HiPrep 16/60 Sephacryl S-400 HR
 E) HiPrep 16/60 Sephacryl S-500 HR

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: 0.25 M NaCl

Flow rate: 0.5 ml/min (15 cm/h)

D) HiPrep 16/60 Sephacryl S-400 HR



E) HiPrep 16/60 Sephacryl S-500 HR

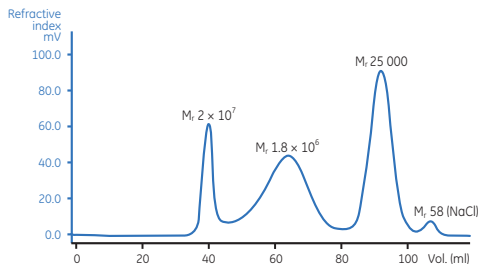


Fig 4.3. Comparison of the selectivity of the five different prepacked HiPrep Sephacryl HR columns.

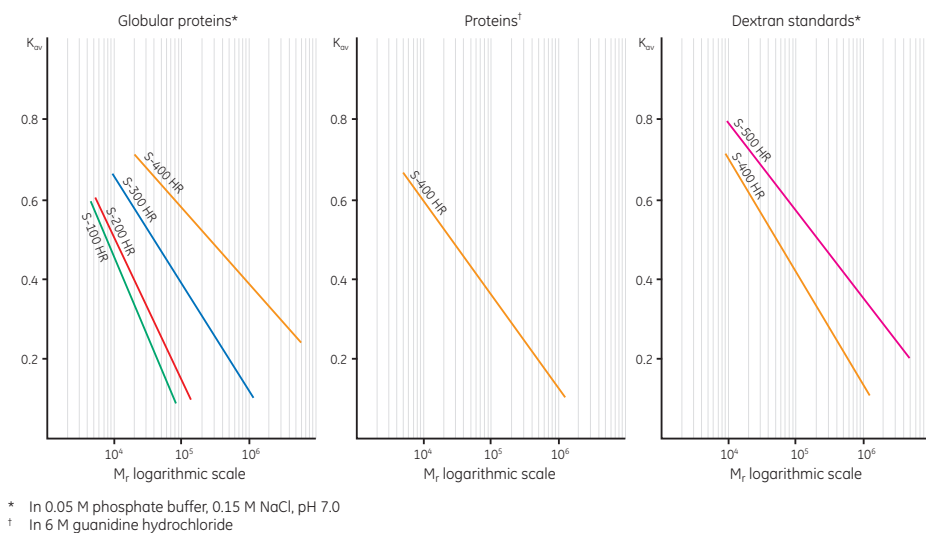


Fig 4.4. Selectivity curves for Sephacryl HR chromatography media.

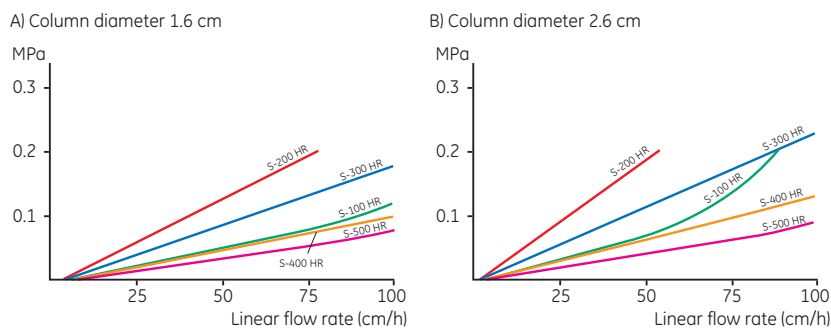


Fig 4.5. Pressure drop as a function of flow rate for Sephacryl HR. Bed height approximately 60 cm, distilled water, temperature 25°C. To calculate the volumetric flow rate, multiply the linear flow by the cross-sectional area of the column (2 cm² for XK 16 or 5.3 cm² for XK 26).

Sephacryl HR is a composite medium prepared by covalently cross-linking allyl dextran with N,N'-methylene bisacrylamide to form a hydrophilic matrix of high mechanical strength. The porosity of the medium, determined by the dextran component, has been controlled to yield five different selectivities. The mechanical rigidity of Sephacryl HR allows even relatively viscous eluents, such as 8 M urea, to be run at practical flow rates. Under normal chromatography conditions (A_{280} , 0.05 M phosphate, 0.15 M NaCl, pH 7.0) Sephacryl S-100 HR gave yields of at least 96% of the following substances: Blue Dextran 2000, ferritin, catalase, aldolase, BSA, ovalbumin, β -lactoglobulin A+B, chymotrypsinogen A, myoglobin, lysozyme, ribonuclease A, and cytochrome C. An ionic strength of at least 0.15 M is recommended for best results.

Separation options

Five Sephacryl HR chromatography media are available as prepacked columns and in bulk packs (Table 4.1).

Table 4.1. Separation options with Sephacryl media

Product	Fractionation range, M_r (globular proteins)	Fractionation range, M_r (dextrans)	Sample loading capacity*	Maximum operating back pressure	Recommended operation flow [†]
HiPrep 16/60 Sephacryl S-100 HR	$1 \times 10^3 - 1 \times 10^5$		≤ 5 ml	0.15 MPa, 5 bar, 21 psi	0.5 ml/min
HiPrep 26/60 Sephacryl S-100 HR	$1 \times 10^3 - 1 \times 10^5$		≤ 13 ml	0.15 MPa, 5 bar, 21 psi	1.3 ml/min
Sephacryl S-100 HR (Bulk media)	$1 \times 10^3 - 1 \times 10^5$		0.5–4% of total column volume	Column-dependent	10–35 cm/h
HiPrep 16/60 Sephacryl S-200 HR	$5 \times 10^3 - 2.5 \times 10^5$	$1 \times 10^3 - 8 \times 10^4$	≤ 5 ml	0.15 MPa, 5 bar, 21 psi	0.5 ml/min
HiPrep 26/60 Sephacryl S-200 HR	$5 \times 10^3 - 2.5 \times 10^5$	$1 \times 10^3 - 8 \times 10^4$	≤ 13 ml	0.15 MPa, 5 bar, 21 psi	1.3 ml/min
Sephacryl S-200 HR (Bulk media)	$5 \times 10^3 - 2.5 \times 10^5$	$1 \times 10^3 - 8 \times 10^4$	0.5–4% of total column volume	Column-dependent	10–35 cm/h
HiPrep 16/60 Sephacryl S-300 HR	$1 \times 10^4 - 1.5 \times 10^6$	$2 \times 10^3 - 4 \times 10^5$	≤ 5 ml	0.15 MPa, 5 bar, 21 psi	0.5 ml/min
HiPrep 26/60 Sephacryl S-300 HR	$1 \times 10^4 - 1.5 \times 10^6$	$2 \times 10^3 - 4 \times 10^5$	≤ 13 ml	0.15 MPa, 5 bar, 21 psi	1.3 ml/min
Sephacryl S-300 HR (Bulk media)	$1 \times 10^4 - 1.5 \times 10^6$	$2 \times 10^3 - 4 \times 10^5$	0.5–4% of total column volume	Column-dependent	10–35 cm/h
HiPrep 16/60 Sephacryl S-400 HR	$2 \times 10^4 - 8 \times 10^6$	$1 \times 10^4 - 2 \times 10^6$	≤ 5 ml	0.15 MPa, 5 bar, 21 psi	0.5 ml/min
HiPrep 26/60 Sephacryl S-400 HR	$2 \times 10^4 - 8 \times 10^6$	$1 \times 10^4 - 2 \times 10^6$	≤ 13 ml	0.15 MPa, 5 bar, 21 psi	1.3 ml/min
Sephacryl S-400 HR (Bulk media)	$2 \times 10^4 - 8 \times 10^6$	$1 \times 10^4 - 2 \times 10^6$	0.5–4% of total column volume	Column-dependent	10–35 cm/h
HiPrep 16/60 Sephacryl S-500 HR		$4 \times 10^4 - 2 \times 10^7$	≤ 5 ml	0.15 MPa, 5 bar, 21 psi	0.5 ml/min
HiPrep 26/60 Sephacryl S-500 HR		$4 \times 10^4 - 2 \times 10^7$	≤ 13 ml	0.15 MPa, 5 bar, 21 psi	1.3 ml/min
Sephacryl S-500 HR (Bulk media)		$4 \times 10^4 - 2 \times 10^7$	0.5–4% of total column volume	Column-dependent	10–35 cm/h
Sephacryl S-1000 SF (Superfine) (Bulk media)			0.5–4% of total column volume	not determined	2–30 cm/h

* For maximum resolution, apply as small sample volume as possible. Note that sample volumes less than 0.5% normally do not improve resolution.

[†] See Appendix 5 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa.

Separation examples

Figures 4.6 to 4.8 illustrate examples of separations performed on Sephacryl media.

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: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0
Flow rate: 2.0 ml/min (22 cm/h)

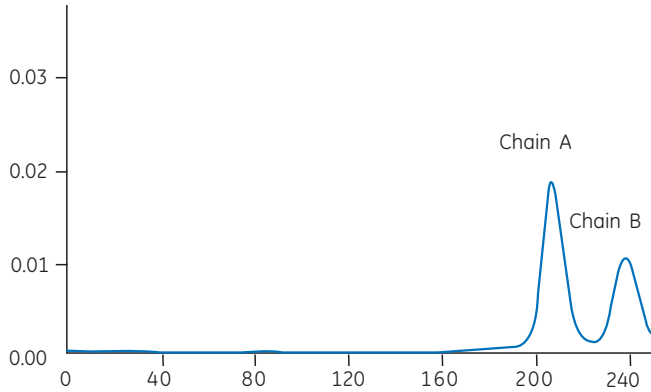


Fig 4.6. Separation of insulin chains on HiPrep 26/60 Sephacryl S-100 HR.

Column: BP 113/120 containing Sephacryl S-200 HR, bed height 100 cm
Sample: IgG fraction from previous ion exchange step (sample volume equivalent to 1% of V_t , (V_t = column volume))
Buffer: 0.05 M Tris-HCl, 0.15 M NaCl, pH 7.5
Flow rate: 7.5 cm/h

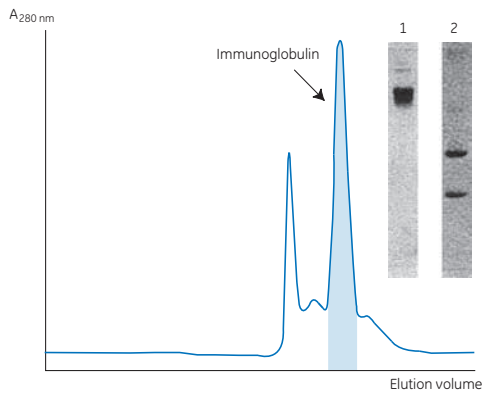


Fig 4.7. Purification of monoclonal antibodies on Sephacryl S-200 HR. Inset shows analysis by gradient the immunoglobulin pool. Lane 1, native sample; lane 2, sample reduced with 2-mercaptoethanol.

Medium: Sephacryl S-400 HR
Column: K 26/70, packed bed 2.6×61 cm
Sample: Integral membrane proteins prepared from human erythrocytes solubilized in 0.1 M phosphate, 100 mM SDS, 1 mM EDTA, 1 mM DTE, pH 7.4
Sample volume: 2 ml (2 mg/ml)
Buffer: 0.1 M phosphate, 50 mM SDS, 1 mM EDTA, 1 mM DTE, pH 7.4
Flow rate: 1 ml/min (11 cm/h)

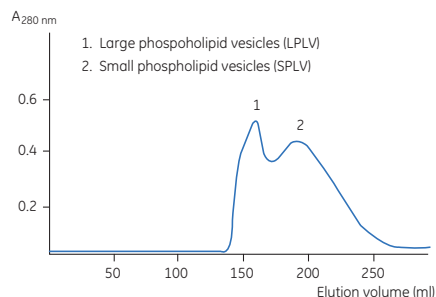


Fig 4.8. Gel filtration on Sephacryl S-400 HR quickly separates phospholipid vesicles (liposomes) into large (LPLV) and small (SPLV) phospholipid vesicles. Data provided by E. Greijer and P. Lundahl, Dept. of Biochemistry, Biomedical Centre, University of Uppsala, Sweden.

Performing a separation

Buffer: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.2 or select the buffer in which the sample should be stored or solubilized for the next step.

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



The sample should be fully dissolved. Centrifuge or filter to remove particulate material (see Appendix 3). Always use degassed buffers and maintain a constant temperature during the run to avoid introducing air into the column.



Set an appropriate pressure limit on the chromatography system to avoid damage to the column packing.

First time use or after long-term storage

1. Equilibrate the column with at least 0.5 column volume of distilled water at 15 cm/h (0.5 ml/min for 16/60 column or 1.3 ml/min for 26/60).
2. Equilibrate with 2 column volumes of buffer at 30 cm/h (1.0 ml/min for 16/60 column or 2.6 ml/min for 26/60).
3. Reduce flow to 15 cm/h and, for best resolution, apply a sample volume equivalent to 1% of the column volume (1.2 ml for 16/60 column or 3.2 ml for 26/60). Sample volumes between 0.5% and 4% can be applied.
4. Elute with 1 column volume of buffer.
5. Before applying a new sample re-equilibrate column with 1 column volume of buffer at 30 cm/h until the baseline monitored at A_{280} is stable.



Column performance should be checked at regular intervals by determining the theoretical plate number per meter and peak symmetry. Prepacked columns are supplied with recommended values. See page 90 on how to check column efficiency.

See page 23 for advice on optimizing the separation.



Exposure to temperatures outside the range 4°C to 40°C will destroy the efficiency of a packed bed and the column will need to be repacked.

Cleaning

1. Wash with 0.5 column volume of 0.2 M NaOH at a flow of 15 cm/h (0.5 ml/min for column 16/60 or 1.3 ml/min for 26/60) to remove most nonspecifically adsorbed proteins.
2. Re-equilibrate immediately with 2 column volumes of buffer or until the baseline monitored at A_{280} and the pH of the eluent are stable.

Further equilibration may be necessary if the buffer contains detergent.



Routine cleaning after every 10–20 separations is recommended, but the frequency of cleaning will also depend on the nature of the samples being applied.

If required Sephacryl HR may be autoclaved repeatedly at 121°C, pH 7, for 30 minutes without significantly affecting its chromatography properties. The medium must be removed from the column as autoclaving can damage column components (Note that HiPrep columns cannot be repacked).

To remove severe contamination

Reverse the flow and wash at a flow rate of 10 cm/h (0.3 ml/min for column 16/60 or 0.8 ml/min for 26/60) at room temperature using the following solutions:

1. Wash with 0.25 column volumes of 0.5 M NaOH (to remove hydrophobic proteins or lipoproteins) followed by 4 column volumes of distilled water.
2. Wash with 0.5 column volume of 30% isopropanol (to remove lipids and very hydrophobic proteins), followed by 2 column volumes of distilled water.

For extreme cases of contamination, check the instructions supplied with the product.



Reversing flow through a column packed with Sephacryl media should only be considered under cases of severe contamination. There is a risk that reversing the flow may cause channeling through the packed bed leading to poor resolution, loss of efficiency and the need to repack the column. Professionally packed columns are less likely to be affected, but extreme care must be taken.

Media characteristics

Matrix: Cross-linked copolymer of allyl dextran and N,N'-methylene bisacrylamide.

Table 4.2 Sephacryl media characteristics

Product	Efficiency: theoretical plates per meter (prepacked columns only)	pH stability*	Particle size	Mean particle size
Sephacryl S-100 HR	$\geq 5\,000\text{ m}^{-1}$	Long term: 3–11 Short term: 2–13	25–75 μm	47 μm
Sephacryl S-200 HR	$\geq 5\,000\text{ m}^{-1}$	Long term: 3–11 Short term: 2–13	25–75 μm	47 μm
Sephacryl S-300 HR	$\geq 5\,000\text{ m}^{-1}$	Long term: 3–11 Short term: 2–13	25–75 μm	47 μm
Sephacryl S-400 HR	$9\,000\text{ m}^{-1}$	Long term: 3–11 Short term: 2–13	25–75 μm	47 μm
Sephacryl S-500 HR	$9\,000\text{ m}^{-1}$	Long term: 3–11 Short term: 2–13	25–75 μm	47 μm
Sephacryl S-1000 SF (Superfine)	[†]	Long term: 3–11 Short term: 2–13	40–105 μm	65 μm

* Long term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on its chromatography performance. Short term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges are estimates based on the experience and knowledge within GE Healthcare.

[†] Not determined.

Chemical stability

Sephacryl HR is stable in all commonly used aqueous buffers and additives such as detergents (1% SDS), denaturing agents (8 M urea or 6 M guanidine hydrochloride). The medium is also stable in 30% acetonitrile, 0.5 M sodium hydroxide, up to 24% ethanol, up to 1 M acetic acid and up to 30% isopropanol.

Storage

Store unused media 4°C to 30°C in 20% ethanol. Do not freeze.

Columns can be left connected to a chromatography system with a low flow rate (0.01 ml/min) of buffer passing through the column to prevent bacterial growth or the introduction of air into the column which would destroy the packing.

For long-term storage, wash with 4 column volumes of distilled water followed by 4 column volumes of 20% ethanol. Store at 4°C to 30°C.

Degas the ethanol/water mixture thoroughly and use a low flow rate, checking the back pressure as the column equilibrates.

Avoid changes in temperature which may cause air bubbles in the packing.

Chapter 5

Sephadex: desalting, buffer exchange and sample clean up

Gel filtration based on Sephadex enables group separation of biomolecules that are above the exclusion limit of the medium, from contaminants such as salts, dyes, and radioactive labels. Sephadex is prepared by cross-linking dextran with epichlorohydrin.

The different types of Sephadex vary in their degree of cross-linking and hence in their degree of swelling and selectivity for specific molecular sizes (see Media characteristics on page 70).

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

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

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

Table 5.1. Sephadex G-25 media

Sephadex G-25	Application
Superfine	For highest column efficiency (highest resolution), but operating pressures increase
Fine	For laboratory-scale separations
Coarse and Medium	Use when a high flowrate at a low operating pressure is essential, e.g., in large-scale applications
Coarse	For batch procedures



Fig 5.1. Prepacked Sephadex G-25 columns: (A) HiPrep 26/10 Desalting, (B) HiTrap Desalting 5 ml.



Use desalting/buffer exchange when needed, before purification, between purification steps, and/or after purification. These are very fast methods compared to dialysis, but remember that each extra step can reduce yield and that desalting often dilutes the sample (centrifugation protocols do not dilute samples).



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

Desalting provides several advantages over dialysis. Dialysis is generally a slow technique that requires large volumes of buffer and carries the risk that material and target protein activity will be lost during handling. When desalting, sample volumes of up to 30% of the total volume of the desalting column can be processed. The high speed and capacity of the separation allows even relatively large sample volumes to be processed rapidly and efficiently in the laboratory, as illustrated in Figure 5.2. Sample concentration does not influence the separation as long as the concentration of proteins does not exceed approximately 70 mg/ml when using normal aqueous buffers, and provided that the target protein is stable and soluble at the concentration used. Use 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate if volatile buffers are required.



When desalting is the first chromatography step, the sample should first be clarified; centrifugation and/or filtration is recommended.

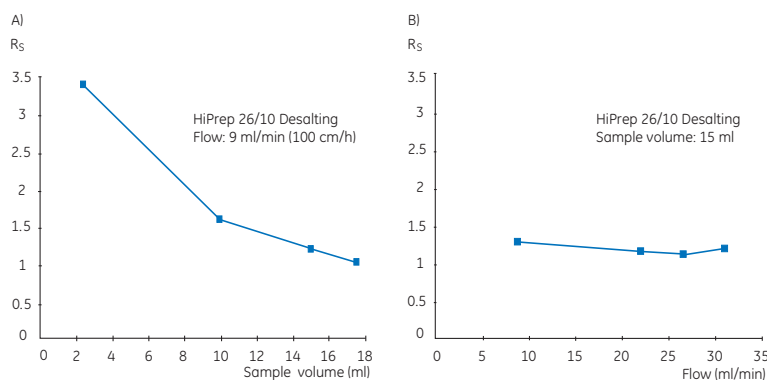


Fig 5.2. (A) Influence of sample volume on resolution. (B) Influence of flow rate on resolution.

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

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

Separation options

For group separations the medium should be selected so that the high molecular weight molecules are eluted at the void volume with minimum peak broadening or dilution and minimum time on the column. The lowest molecular weight substances should appear by the time one column volume of buffer has passed through the column.

Sephadex media are available in prepacked columns (also spin columns), microplates, and as bulk medium (Table 5.2).

Table 5.2. Group separation options with Sephadex media

	Columns and 96-well plates	Chromatography medium	Loaded volume (ml)	Eluted volume (ml)	Dilution factor	Operation
	PD SpinTrap™ G-25	Sephadex G-25 Medium	0.07–0.13	0.07–0.13*	No dilution	Centrifuge
	PD MultiTrap™ G-25	Sephadex G-25 Medium	0.07–0.13	0.07–0.13*	No dilution	Centrifuge
	PD MiniTrap™ G-25	Sephadex G-25 Medium	0.2–0.5	0.1–0.5	No dilution	Centrifuge
			0.1–0.5	1.0	2–10	Gravity flow
	PD MidiTrap™ G-25	Sephadex G-25 Medium	0.75–1.0	0.5–1.0	No dilution	Centrifuge
			0.5–1.0	1.5	1.5–3	Gravity flow
	PD-10 Desalting columns	Sephadex G-25 Medium	1.75–2.5	1.0–2.5	No dilution	Centrifuge
			1.0–2.5	3.5	1.5–3.5	Gravity flow
	PD MiniTrap G-10	Sephadex G-10 Medium	0.1–0.3	0.5	1.7–5	Gravity flow
	PD MidiTrap G-10	Sephadex G-10 Medium	0.4–1.0	1.2	1.2–3.0	Gravity flow
	HiTrap Desalting	Sephadex G-25 Superfine	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
	2× HiTrap Desalting	Sephadex G-25 Superfine	3.0 (max.)	4.0–5.0	1.3–1.7	Syringe/pump/system
	3× HiTrap Desalting	Sephadex G-25 Superfine	4.5 (max.)	6.0–7.0	1.3–1.7	Syringe/pump/system
	HiPrep 26/10	Sephadex G-25 Fine	10	10–15	1.0–1.5	Pump/system
			15 (max.)	15–20	1.0–1.3	Pump/system
	2× HiPrep 26/10	Sephadex G-25 Fine	30 (max.)	30–40	1.0–1.3	Pump/system
	3× HiPrep 26/10	Sephadex G-25 Fine	45 (max.)	45–55	1.0–1.2	Pump/system
	4× HiPrep 26/10	Sephadex G-25 Fine	60 (max.)	60–70	1.0–1.2	Pump/system

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

* Applied volume = eluted volume; For sample volumes less than 100 µl it is recommended to apply a stacker volume of 30 µl equilibration buffer after the sample has fully absorbed.

For convenience and reliable performance, use prepacked Sephadex columns such as HiTrap Desalting 5 ml and HiPrep 26/10 Desalting.



Always use disposable columns if there is a risk of biological or radioactive contamination or when any possibility of carryover between samples is unacceptable.

The type of equipment available and the sample volume to be processed also govern the choice of prepacked column, as shown in Figure 5.3.

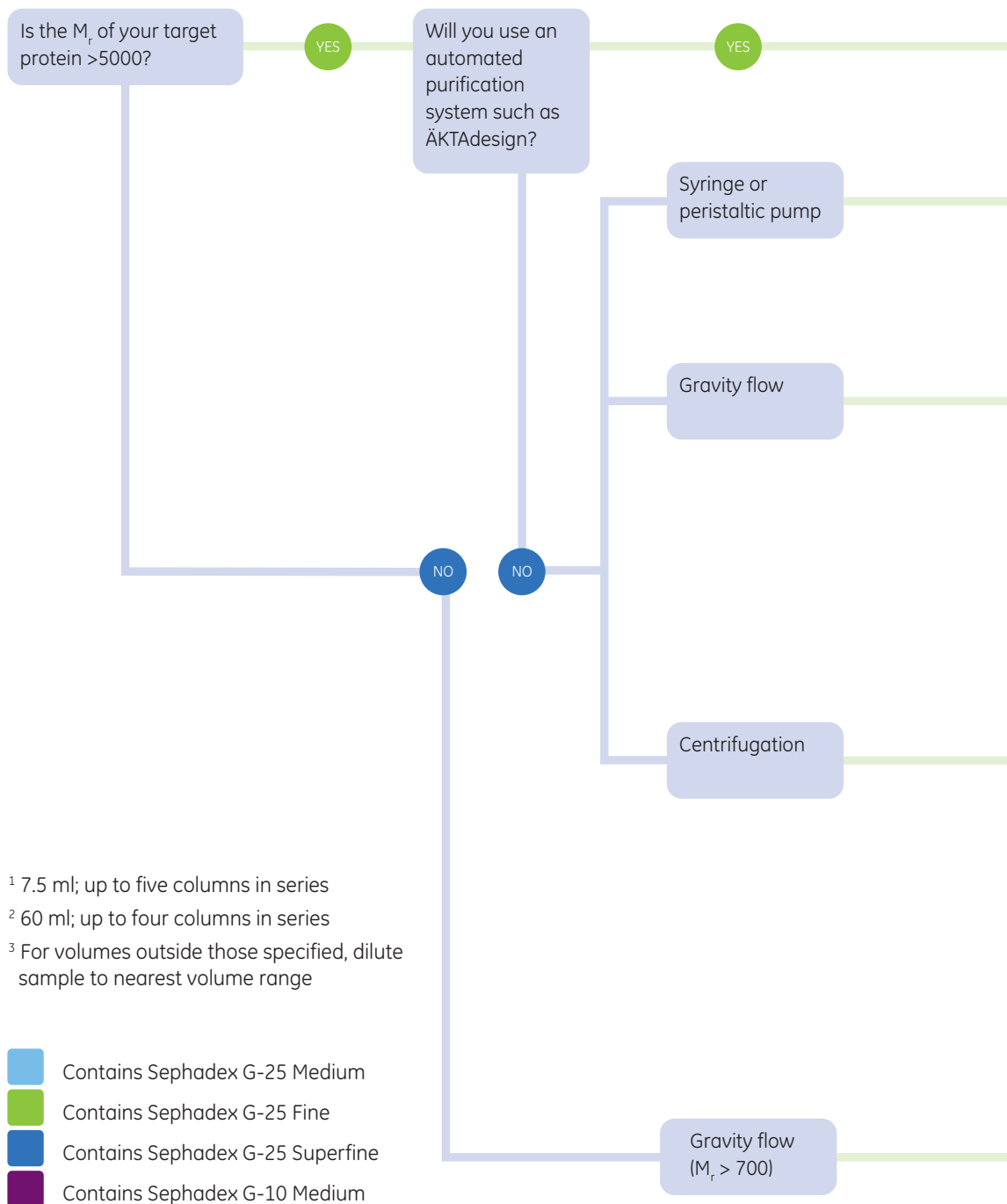
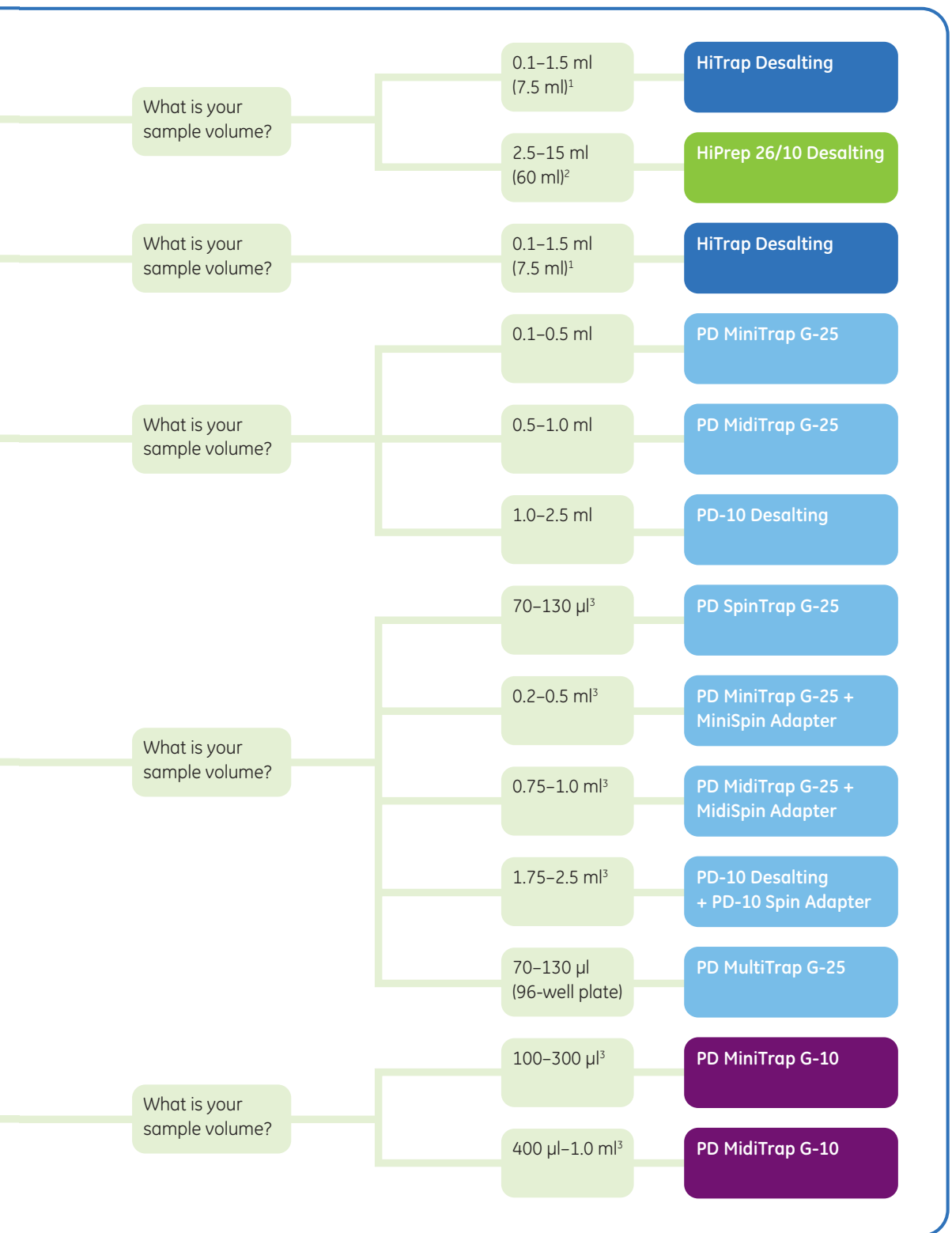


Fig 5.3. Selecting prepacked columns for desalting and buffer exchange.



Separation examples

Figures 5.4 and 5.5 show examples of separations performed on Sephadex media.

Sample: (Histidine)₆-protein eluted from HiTrap Chelating HP with sodium phosphate 20 mM, sodium chloride 0.5 M, imidazole 0.5 M, pH 7.4
Column: HiTrap Desalting 5 ml
Buffer: Sodium phosphate 20 mM, sodium chloride 0.15 M, pH 7.0

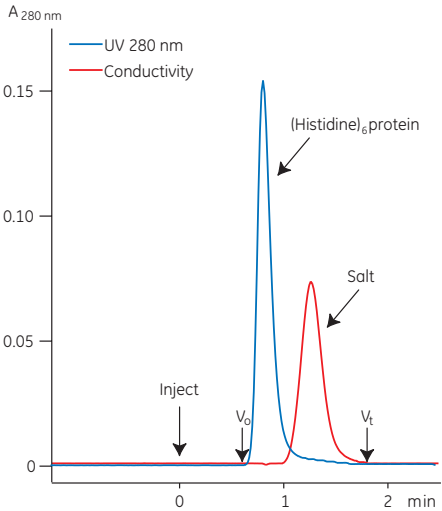


Fig 5.4. Desalting a (Histidine)₆ fusion protein using HiTrap Desalting 5 ml on ÄKTApri™. The UV (protein) and conductivity (salt) traces enable pooling of the desalted fractions and facilitate optimization of the separation.

Column: HiPrep 26/10 Desalting
Sample: 2 mg/ml BSA, 0.07 mg/ml N-Hydroxysuccinimide (NHS) in 50 mM sodium phosphate, 0.15 M NaCl, pH 7.0. Filtered through a 0.45 µm filter
Sample volume: 13 ml
Buffer: 50 mM sodium phosphate, 0.15 M NaCl, pH 7.0
Flow rate: 31 ml/min (350 cm/h)

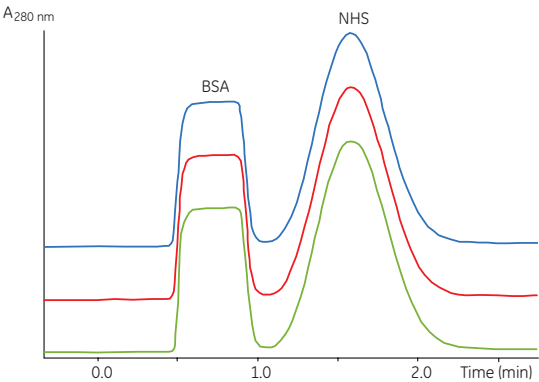


Fig 5.5. Reproducible removal of N-hydroxysuccinimide from bovine serum albumin.

Performing a separation

Desalting and buffer exchange can take less than 5 minutes per sample with greater than 95% recovery for most proteins.



To prevent possible ionic interactions the presence of a low salt concentration (25 mM NaCl) is recommended during desalting and in the final sample buffer. 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 NaCl.



The sample should be fully dissolved. Centrifuge or filter to remove particulate material (see Appendix 3). Always use degassed buffers to avoid introducing air into the column.



Sample concentration up to 70 mg/ml protein should not influence the separation when using normal aqueous buffers.

If possible use a chromatography system with a UV and a conductivity monitor to facilitate optimization of the sample loading. The elution of the protein peak at A_{280} and the appearance of the salt peak can be followed exactly and different separations can be easily compared, as shown in Figure 5.6.

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 column volume.

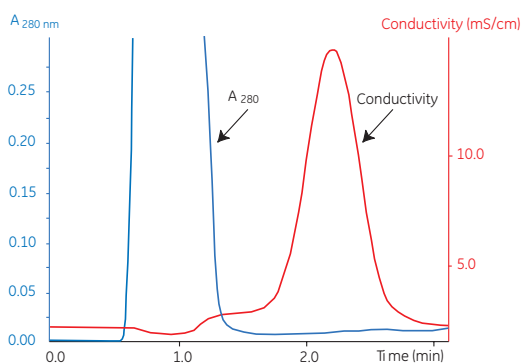


Fig 5.6. Buffer exchange of mouse plasma on HiPrep 26/10 Desalting.

General considerations

Small-scale desalting of samples

For sample volumes ranging from 0.2 to 2.5 ml, it is possible to run multiple samples in parallel with PD-10 Desalting, PD MidiTrap G-25, and PD MiniTrap G-25 columns. Two different protocols are available for these columns: one for manual use on the laboratory bench and one for use together with a standard centrifuge in combination with a Spin Adapter. For smaller proteins ($M_r > 700$), PD MiniTrap G-10 and PD MidiTrap G-10 columns may be used.

For smaller sample volumes in the range of 70 to 130 μ l, multiple samples can be run on PD SpinTrap G-25 spin columns together with a microcentrifuge or PD MultiTrap G-25 96-well plate using centrifugation for extraction. Although possible to perform, using PD MultiTrap G-25 with vacuum is not recommended due to reduced reproducibility compared with operation using centrifugation.

Desalting larger sample volumes using HiTrap and HiPrep columns

Connect up to three HiTrap Desalting columns in series to increase the sample volume capacity. For example, two columns allow a sample volume of 3 ml, and three columns allow a sample volume of 4.5 ml (Table 5.2).

Connect up to four HiPrep 26/10 Desalting columns in series to increase the sample volume capacity. For example, two columns allow a sample volume of 30 ml, and four columns allow a sample volume of 60 ml. Even with four columns in series, the sample can be processed in 20 to 30 min without back pressure problems.

Buffer preparation

For substances carrying charged groups, an eluent containing a buffer salt is recommended. A salt concentration of at least 150 mM is recommended to prevent possible ionic interactions with the chromatography medium. Sodium chloride is often used for this purpose. Often a buffer with 25 to 50 mM concentration of the buffering substance is sufficient. At salt concentrations above 1 M, hydrophobic substances may be retarded or may bind to the chromatography medium.

At even higher salt concentrations, > 1.5 M ammonium sulfate, the column packing shrinks.

Sample preparation

Sample concentration does not influence the separation as long as the viscosity does not differ by more than a factor of 1.5 from that of the buffer used. This corresponds to a maximum concentration of 70 mg/ml for proteins, when normal, aqueous buffers are used. The sample should be fully solubilized. Centrifuge or filter (0.45 µm filter) immediately before loading to remove particulate material if necessary.

Buffer exchange

Protein solubility often depends on pH and/or ionic strength (salt concentration), and the exchange of buffer may therefore result in precipitation of the protein. Also, protein activity can be lost if the change of pH takes it outside of the range where the protein is active. Samples that have been obtained after purification will usually be free from particles, unless the purified protein or a contaminant has been aggregated.

The protocols in the following sections describe desalting and buffer exchange using different formats of prepacked columns.

HiTrap Desalting columns



Fig 5.7. HiTrap Desalting column allows easy and efficient group separations with a syringe, pump, or chromatography system.

HiTrap Desalting is a 5 ml column (Fig 5.7) packed with the gel filtration medium Sephadex G-25 Superfine, which is based on cross-linked dextran beads. The fractionation range for globular proteins is between M_r 1000 and 5000, with an exclusion limit of approximately M_r 5000. This ensures group separations of proteins/peptides larger than M_r 5000 from molecules with a molecular weight less than M_r 1000.

HiTrap Desalting can be used with aqueous solutions in the pH range 2 to 13. The prepacked medium is stable in all commonly used buffers, solutions of urea (8 M), guanidine hydrochloride (6 M), and all nonionic and ionic detergents. Lower alcohols (methanol, ethanol, propanol) can be used in the buffer or the sample, but we recommend that the concentration be kept below 25% v/v. Prolonged exposure (hours) to pH below 2 or above 13, or to oxidizing agents, should be avoided.

The recommended range of sample volumes is 0.1 to 1.5 ml when complete removal of low molecular weight components is desired. The separation is not affected by the flow rate, in the range of 1 to 10 ml/min. The maximum recommended flow rate is 15 ml/min. Separations are easily performed with a syringe, pump, or chromatography system. Up to three columns can be connected in series, allowing larger sample volumes to be handled. To avoid cross-contamination, use the column only with the same type of sample.

Manual purification with a syringe



Fig 5.8. Using HiTrap columns with a syringe. (A) Prepare buffers and sample. Remove the column's top cap and twist off the end. (B) Equilibrate the column, load the sample and begin collecting fractions. (C) Wash and elute, continuing to collect fractions.

1. Fill the syringe with binding buffer. Remove the stopper and connect the column to the syringe (use the connector supplied) "drop to drop" to avoid introducing air into the column.
2. Remove the snap-off end at the column outlet.
3. Equilibrate the column with 5 column volumes of binding buffer.
4. Apply the pretreated sample using a syringe fitted to the Luer connector on the column. For best results, use a flow rate of 0.2 to 1 ml/min (1 ml column) and 0.5 to 5 ml/min (5 ml column) during sample application*.
5. Wash with 5 to 10 column volumes of binding buffer or until no material appears in the effluent. Maintain a flow rate of 1 to 2 ml/min (1 ml column) and 5 to 10 ml/min (5 ml column) for washing. Optional: collect the flowthrough (in 1 ml fractions for the 1 ml column and 2 ml fractions for the 5 ml column) and reserve until the procedure has been successfully completed. Retain a sample for analysis by SDS-PAGE to measure the efficiency of protein binding to the medium.
6. Elute with 5 to 10 column volumes of elution buffer. Maintain a flow rate of 0.2 to 1 ml/min (1 ml column) and 0.5 to 5 ml/min (5 ml column) for elution.
7. After elution, regenerate the column by washing it with 3 to 5 column volumes of binding buffer. The column is now ready for a new purification.

* 1 ml/min corresponds to approximately 30 drops/min when using a syringe with a HiTrap 1 ml column; 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column



For large sample volumes, a simple peristaltic pump can be used to apply sample and buffers.

Simple desalting with ÄKTAprime plus

ÄKTAprime plus contains pre-programmed templates for individual HiTrap Desalting 5 ml and HiPrep 26/10 Desalting columns.



Fig 5.9. ÄKTAprime plus.



Prepare at least 500 ml of each buffer.

1. Follow instructions supplied on the ÄKTAprime plus cue card to connect the column and load the system with binding buffer.
2. Select the Application Template.
3. Start the method.
4. Enter the sample volume and press OK to start.

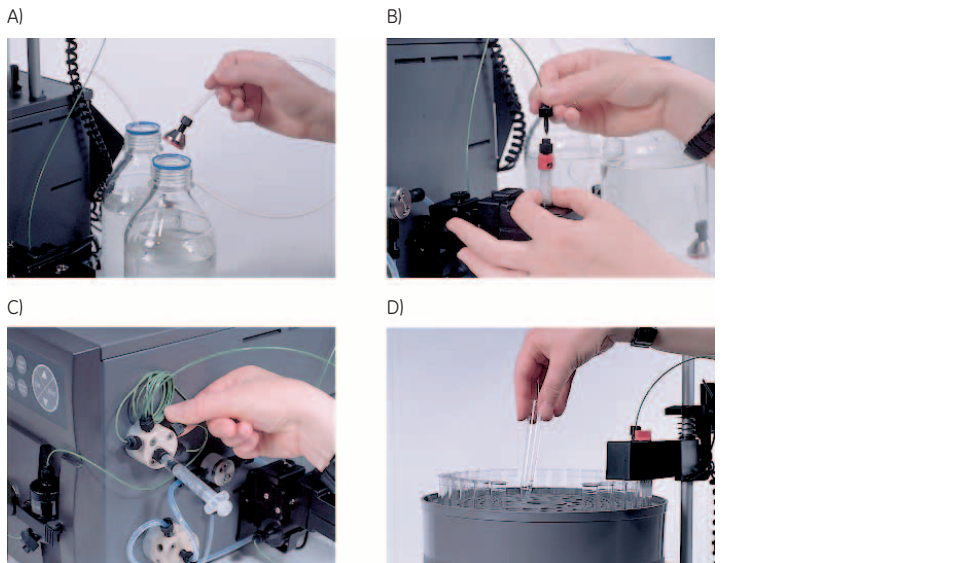


Fig 5.10. Typical procedures using ÄKTAprime plus. (A) Prepare the buffers. (B) Connect the column. (C) Prepare the fraction collector. (D) Load the sample.

Desalting on a gravity-feed PD-10 column

Buffer Preparation

1. Remove top cap and pour off the excess liquid.
2. Cut off the bottom tip.
3. Place column in the Desalting Workmate supplied onto the plastic tray and equilibrate with 25 ml buffer. Discard the eluent.
4. Add a total sample volume of 2.5 ml. If the sample volume is less than 2.5 ml, add buffer to reach a final volume of 2.5 ml. Discard the eluent.
5. Add 3.5 ml buffer to elute high molecular weight components and collect the eluent.



Using the standard procedure described above protein yield is typically greater than 95% with less than 4% salt (low molecular weight) contamination. The dilution factor is 1:4.



Sephadex G-10 can be packed into empty PD-10 columns and run in the same manner as PD-10 Desalting columns.

Optimization of desalting

1. When possible select a prepacked column that is best suited to the volume of sample that needs to be desalted (see Separation Options). For the majority of separations the instructions supplied ensure satisfactory results and very little optimization should be necessary.
2. Ensure that buffer conditions are optimal for the separation.
3. Select the highest flow rate recommended. Figure 5.11 shows an example of the influence of flow rate on group separation.
4. Determine the maximum sample volume that can be loaded. Figure 5.12 shows an example of the influence of sample volume on group separation.

Column: HiTrap Desalting 5 ml
Sample: Bovine serum albumin, 2 mg/ml in 0.5 M NaCl, 0.05 M sodium phosphate, pH 7.0
Buffer: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0
Sample volume: 0.8 ml
Flow rate: 1.7, 3.3, 6.7, 10.0, 13.3, 16.7, 20.0 ml/min

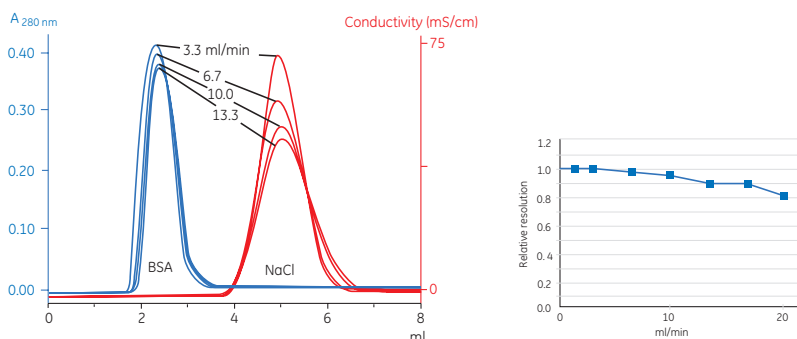


Fig 5.11. Influence of flow rate on separation using a HiTrap Desalting column.

Column: HiTrap Desalting 5 ml
Sample: Bovine serum albumin, 2 mg/ml in 0.5 M NaCl, 0.05 M sodium phosphate, pH 7.0
Buffer: 0.05 M sodium phosphate, 0.15 M NaCl, pH 7.0
Sample volume: 0.8, 1.3, 1.7, 2.2 ml
Flow rate: 5 ml/min

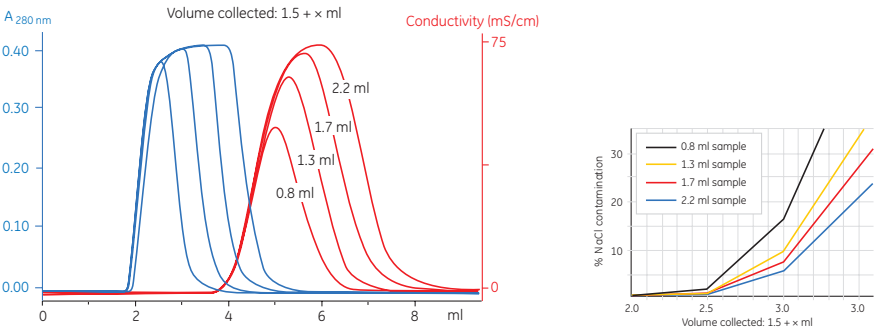


Fig 5.12. Influence of sample volume on separation using a HiTrap Desalting column.

As the sample volume increases (up to a maximum of 30% of the total column volume) the dilution factor decreases and there may be a slight increase in the amount of salt remaining in the sample after elution.

 Sample volumes up to 30% of the total column volume give a separation with minimal sample dilution. Larger sample volumes can be applied, but resolution will be reduced.

Scale-up and processing larger sample volumes

Connecting columns in series increases the effective column volume and so increases sample loading capacity. Table 5.3 shows the sample loading capacities and dilution factors when using prepacked desalting columns alone or in series, see also Figure 5.13 for HiTrap application examples.

Table 5.3. Selection guide for desalting/buffer exchange columns

Column	Loaded volume (ml)	Eluted volume (ml)	Dilution factor	Operation
HiPrep 26/10 Desalting	10	10–15	1–1.3	pump
	15 (max)	15–20	1–1.5	pump
2 × HiPrep 26/10 Desalting	30 (max)	30–40	1–1.3	pump
3 × HiPrep 26/10 Desalting	45 (max)	40–55	1–1.2	pump
4 × HiPrep 26/10 Desalting	60 (max)	60–70	1–1.2	pump
HiTrap Desalting	0.25	1.0	4	syringe/pump
	0.5	1.5	3	syringe/pump
	1.0	2.0	2	syringe/pump
	1.5 (max)	2.0	1.3	syringe/pump
2 × HiTrap Desalting	3.0	4–5	1.3–1.7	syringe/pump
3 × HiTrap Desalting	4.5 (max)	6–7	1.3–1.7	syringe/pump
PD-10 Desalting columns	1.5	3.5	2.3	gravity
	2.0	3.5	1.7	gravity
	2.5	3.5	1.4	gravity

Increasing sample loading capacity from 1.5 ml up to 7.5 ml

Column: HiTrap Desalting, 1 × 5 ml, 3 × 5 ml, 5 × 5 ml
Sample: 2 mg/ml BSA in 50 mM sodium phosphate, 0.5 M sodium chloride, pH 7.0
Sample volume: $28\% \times V_t$ (1.4, 4.3 and 7.1 ml respectively)
Buffer: 50 mM sodium phosphate, 0.15 M sodium chloride, pH 7.0
Flow rate: 5 ml/min

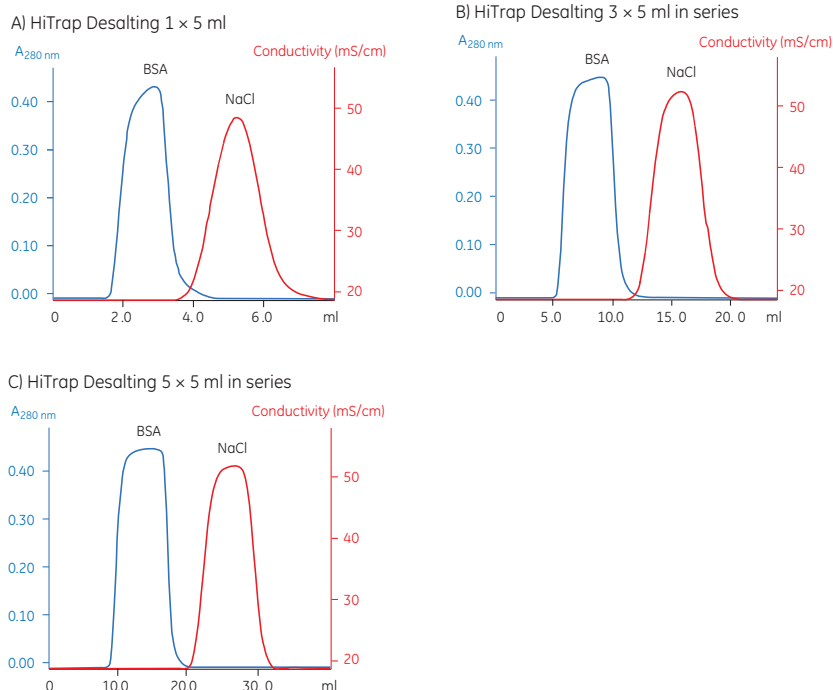


Fig 5.13. Scale-up using HiTrap columns connected in series.

Increasing sample loading capacity from 15 ml up to 60 ml

Connect HiPrep 26/10 Desalting columns in series. With 2 columns, a sample volume of 30 ml can be applied, and 4 columns allow a sample volume of 60 ml (Fig 5.14). Even with four columns in series, high flow rates can be maintained without causing back pressure difficulties so that up to 60 ml of sample can be processed in 20–30 minutes.

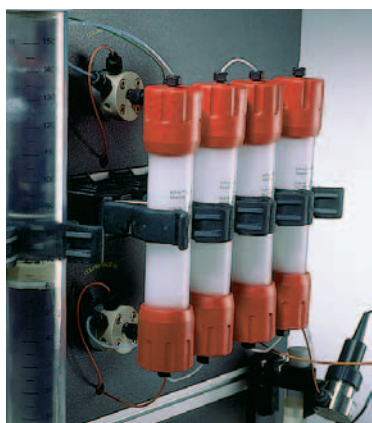


Fig 5.14. Four HiPrep 26/10 Desalting columns connected in series.

For sample volumes greater than 60 ml

Select a suitable particle size of Sephadex G-25, rehydrate 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 particle size determines the flow rates and sample volumes that can be applied, as shown in Figure 5.15.

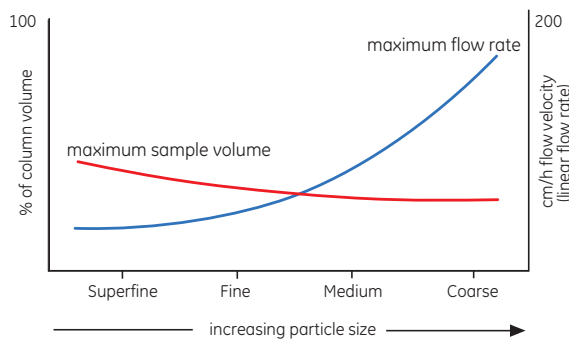


Fig 5.15 Sephadex G-25: recommended sample volumes and flow rates vary with particle size.

- Use Superfine grade with a bed height of approximately 15 cm when requiring the highest efficiencies.
- Use Fine grade with an approximate bed height of 15 cm for laboratory scale separations.
- Use Medium and Coarse grades for preparative processes where a high flow rate at a low operating pressure is essential. Pack in a column less than 50 cm in bed height. The Coarse grade is suitable for batch procedures.

Media characteristics

Sephadex is prepared by cross-linking dextran with epichlorohydrin. Variations in the degree of cross-linking create the different Sephadex media and influence their degree of swelling and their selectivity for specific molecular sizes.

Table 5.4. Sephadex media characteristics

Product	Fractionation range, M_r (globular proteins)	pH stability*	Bed volume ml/g dry Sephadex	Maximum operating flow	Particle size in 0.15 M sodium chloride
Sephadex G-10	$< 7 \times 10^2$	Long term: 2–13 Short term: 2–13	2–3	Darcy's law†	55–165 μm
Sephadex G-25 Coarse	1×10^3 – 5×10^3	Long term: 2–13 Short term: 2–13	4–6	Darcy's law†	87–510 μm
Sephadex G-25 Medium	1×10^3 – 5×10^3	Long term: 2–13 Short term: 2–13	4–6	Darcy's law†	38–235 μm
Sephadex G-25 Fine	1×10^3 – 5×10^3	Long term: 2–13 Short term: 2–13	4–6	Darcy's law†	17–132 μm
Sephadex G-25 Superfine	1×10^3 – 5×10^3	Long term: 2–13 Short term: 2–13	4–6	Darcy's law†	15–88 μm
Sephadex G-50 Fine	1×10^3 – 3×10^4	Long term: 2–10 Short term: 2–13	9–11	Darcy's law†	34–208 μm

* Long term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on its chromatography performance. Short term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges are estimated based on the experience and knowledge within GE Healthcare.

† In practice this means that the pressure/flow considerations that must be made when using other gel filtration media do not apply to Sephadex. Doubling flow rate will double column pressure. See Appendix 2 for an explanation of Darcy's law.

Column Packing

See Appendix 1.

Cleaning

PD-10, SpinTrap, MultiTrap, MiniTrap, MidiTrap, and HiTrap Desalting columns are disposable, but, depending on the type of sample and if cross-contamination is not a concern, they can be re-used a few times.

For HiPrep 26/10 Desalting columns proceed as follows:

1. Wash the column with 2 column volumes of 0.2 M sodium hydroxide or a solution of a non ionic detergent (typically 0.1–0.5% Triton X-100 dissolved in distilled water or 0.1 M acetic acid) at a flow rate of 10 ml/min. Ensure that the pressure drop does not exceed 0.15 MPa (1.5 bar, 22 psi).
2. Wash the column with 5 column volumes of distilled water at a flow rate of 15 ml/min.
3. Before use, re-equilibrate the column with at least 5 column volumes of buffer until the UV base line and pH are stable.

To remove precipitated proteins and peptides, fill the column with 1 mg pepsin/ml in 0.1 M acetic acid, 0.5 M NaCl and leave at room temperature overnight or 1 hour at 37°C. Repeat the normal cleaning procedure above.

Chemical stability

Sephadex is stable in all commonly used aqueous buffers and additives such as ionic and non-ionic detergents, denaturing agents (8 M urea or 6 M guanidine hydrochloride). The media are stable in short chain alcohols such as ethanol, methanol and propanol, but concentrations above 25% should not normally be used. Note that Sephadex shrinks in alcohol solutions.

Storage

Store unused media 4°C to 30°C in 20% ethanol. Do not freeze.

Wash used media with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Store at 4°C to 30°C.

Alternatively, wash with 2 column volumes of distilled water followed by 2 column volumes 0.01 M NaOH. Sodium hydroxide solution is bacteriostatic, easily disposed of and does not shrink the medium.

Degas the ethanol/water mixture thoroughly and use a low flow rate, checking the back pressure as the column equilibrates.

Avoid changes in temperature which may cause air bubbles in the packing.

Chapter 6

Sephadex LH-20 – gel filtration in presence of organic solvents

Sephadex LH-20 is specifically designed for the separation and purification of natural products that require the presence of organic solvents to maintain their solubility, including molecules such as steroids, terpenoids, lipids and low molecular weight peptides (up to 35 amino acid residues). Compounds are usually separated by a form of liquid/liquid partitioning or absorption chromatography. Sephadex LH-20 can have a very high selectivity for aromatic compounds in certain solvents and can be used at analytical or industrial scale for the preparation of closely related species.

Sephadex LH-20 is made from hydroxypropylated dextran beads that have been cross-linked to yield a polysaccharide network. The medium can be swollen in water and organic solvents.

Sephadex LH-20 is suitable for an initial purification before polishing by ion exchange or reversed phase chromatography, or for a final polishing step, for example during the preparation of diastereoisomers.

Depending upon the chosen solvents, Sephadex LH-20 can also separate components by partitioning between the matrix and the organic solvent. Sephadex LH-20 exhibits both hydrophilic and hydrophobic properties, the combination of which can offer unique chromatography selectivity for certain applications.



Sephadex has been used for gel filtration in organic solvents, for example dimethylformamide may be used with Sephadex G-10 and mixtures of water with the shorter chain alcohols may be used with Sephadex G-10, G-25 and G-50.

Media characteristics

Table 6.1

Product	Fractionation range (globular proteins)	Sample loading capacity*	Maximum operating back pressure	Maximum operating flow
Sephadex LH-20	$< 5 \times 10^3$ (exclusion limit will depend on the solvent)	2% of column volume	Solvent-dependent	12 cm/min (720 cm/h, bed height 14 cm, 15 MPa back pressure)

* If Sephadex LH-20 is used in adsorption mode then the sample volume is unlimited until reaching the point of column saturation.

Separation examples

An HIV-1 reverse transcriptase inhibitor has been isolated from *Phyllanthus niruri*, a natural medicine that has been used for many years to combat edema and jaundice. The active component that inhibits HIV-1 reverse transcriptase has been identified as repandusinic acid A monosodium salt, a small tannin-like molecule. The structure of the free acid is shown in Figure 6.1.

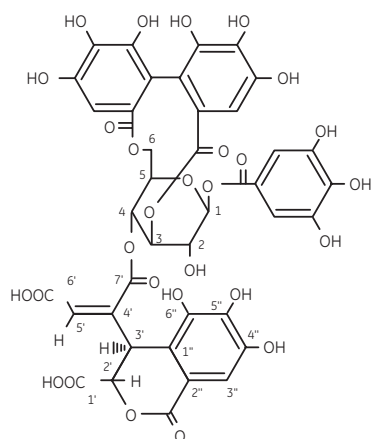


Fig 6.1. Structure of free acid form of repandusinic acid A.

Table 6.2 shows the recovery of active inhibitor from an analytical separation on Sephadex LH-20.

Table 6.2 Summary of data for the isolation of repandusinic acid A from *P. niruri*

Purification step	Yield (mg)	ID50* (µg/ml)	Specific activity (× 10 ² IU/mg)	Total activity (× 10 ³ IU) [†]
H ₂ O extract	6600	50	4	2640
MeOH insoluble	22 500	20	10	22 500
Sephadex LH-20 fr. 4–11 [‡]	247	3.0–3.6	56–67	11 616
Cellulose				
Fr 1	189	7.8	26	484
Fr 2	24	5.0	40	96
Fr 3	18	2.4	83	150
Fr 4	9	3.4	58	52
Fr 5	14	1.8	111	156
RA (pure substance)	5.9	0.3	668	394

* ID50 indicates the effectiveness of inhibitors expressed as concentrations which cause 50% inhibition of HIV-1-RT. Crude HIV-1-RT was used in this experiment

[†] IU are arbitrary inhibitory activity units obtained by dividing the total weight of the fraction at each step by the weight of each fraction required to achieve 50% inhibition of [³H]dTTP incorporation into the polymer in the HIV-1-RT assay.

[‡] Fractions 4–10 and fraction 11 were combined because both fractions had the inhibitory activity

Figure 6.2 shows Sephadex LH-20 used at a preparative scale for the separation of 2-acetamidobenzoic acid and 4-acetamidobenzoic acid. In this separation the hydrophilicity and hydrophobicity of the medium provide a unique chromatography selectivity resulting in high resolution of closely related species. The molecules differ only by the position of the acetamide moiety on the benzene ring.

Column: Sephadex LH-20, 2.5 × 200 cm
Sample: Mixture of 2- and 4-acetamidobenzoic acid
Eluent: Acetone
Flow rate: 8 ml/min
Detection: Refractive Index
Yield: 250 mg 2-acetamidobenzoic acid
 254 mg 4-acetamidobenzoic acid

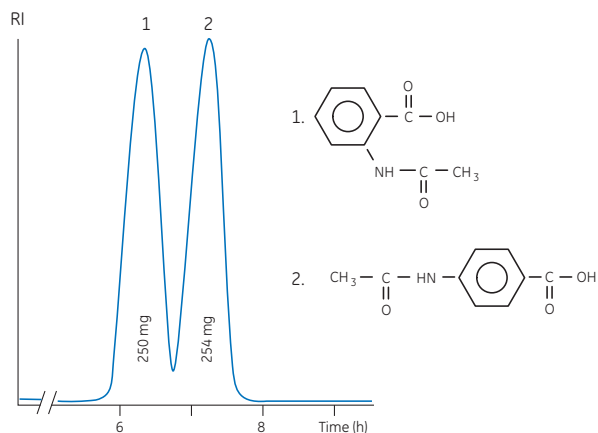


Fig 6.2. Separation of 2- and 4-acetamidobenzoic acid on Sephadex LH-20.

Packing a column

Sephadex LH-20 should be packed in a solvent resistant column selected from Table 6.3 according to the column volume required for the separation.

Table 6.3. Solvent resistant columns

Column	Volume (ml)	Bed height (cm)
SR 25/454	73–220	15–45
SR 25/100	343–490	70–100



Simple steps to clarify a sample before applying it to a column will avoid the risk of blockage, reduce the need for stringent washing procedures and extend the life of the chromatography medium. Filter or centrifuge all solvents and samples before use.

1. Refer to Table 6.4, page 77, to calculate the amount of dry medium required as the extent of swelling depends upon the solvent system. Swell Sephadex LH-20 for at least 3 hours at room temperature in the solvent to be used for the separation.
2. Prepare a slurry 75:25 settled medium:solvent and decant any fine particles of medium.
3. Equilibrate all materials to room temperature.
4. Resuspend and pour the slurry into the column in one continuous step (using a glass rod will help to eliminate air bubbles).
5. Fill the column reservoir to the top with solvent. Seal, attach to a pump and open the column outlet.
6. Pack at 300 cm/h until the bed has reached a constant height. Stop the flow, empty and remove the packing reservoir.
7. Carefully fill the column with solvent and insert a wetted adaptor into the column. Ensure no air bubbles are trapped under the net and adjust the adaptor O-ring to give a sliding seal against the column wall.

8. Connect all tubings, ensuring that there are no air bubbles in the flow path.
9. Slowly slide down the adaptor so that any air in the tubings is displaced by solvent and lock the adaptor into position on the surface of the medium.
10. Open the column outlet and continue packing until the packed bed is stable and a final adjustment of the top adaptor can be made.

Note: In solvents such as chloroform Sephadex LH-20 is less dense than the solvent and the medium will float. Pour the medium into the column and drain until the second adaptor can be inserted. Lock the adaptor in position at the surface of the medium and direct the flow of chloroform upwards. The bed will be packed against the top adaptor and the lower adaptor can be pushed slowly upwards towards the lower surface of the medium. Close the column outlet when moving the adaptor to avoid compressing the bed.

Performing a separation

Start at a linear flow of 1 cm/h to check resolution. Low flow rate gives better the resolution.

1. Equilibrate the column with at least 2 column volumes of the solvent until a stable baseline is achieved.
2. Apply a sample volume equivalent to 1–2% of the total column volume.
3. Elute in 1 column volume. Re-equilibration is not needed between runs with the same solvent.

Cleaning

Wash the column with 2–3 column volumes of the solvent, followed by re-equilibration in a new solvent if changing the separation conditions.

Table 6.4. 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	4.4–4.6
Pyridine	4.2–4.4
Water	4.0–4.4
Dimethylformamide	4.0–4.4
Methanol	3.9–4.3
Saline	3.8–4.2
Ethylene dichloride	3.8–4.1
Chloroform*	3.8–4.1
Propanol	3.7–4.0
Ethanol†	3.6–3.9
Isobutanol	3.6–3.9
Formamide	3.6–3.9
Methylene dichloride	3.6–3.9
Butanol	3.5–3.8
Isopropanol	3.3–3.6
Tetrahydrofuran	3.3–3.6
Dioxane	3.2–3.5
Acetone	2.4–2.6
Acetonitrile‡	2.2–2.4
Carbon tetrachloride‡	1.8–2.2
Benzene‡	1.6–2.0
Ethyl acetate‡	1.6–1.8
Toluene‡	1.5–1.6

* Containing 1% ethanol.

† Containing 1% benzene.

‡ Solvents that give a bed volume of less than 2.5 mg/ml dry Sephadex LH-20 are not generally useful.

Chemical stability

Sephadex LH-20 is stable in most aqueous and organic solvent systems. The medium is not stable below pH 2.0 or in strong oxidizing agents.

Storage

Store dry at 4°C to 30°C. Store packed columns and used medium at 4°C to 8°C in the presence of a bacteriostatic agent.

Transferring Sephadex LH-20 from aqueous solution to organic solvents

Transfer Sephadex LH-20 from an aqueous solution to the organic solvent by moving through a graded series of solvent mixtures. 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: transfer to 70% A:30% B then to 30% A:70% B and finally 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 6.3.

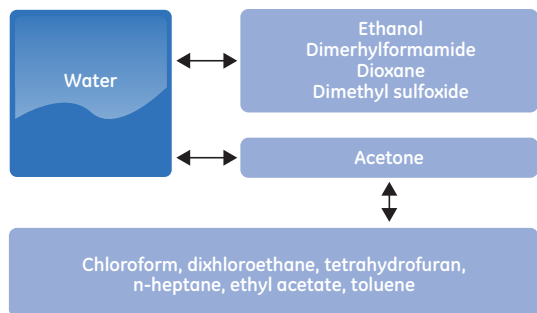


Fig 6.3. Suggested routes for changing to organic solvents.

1. Transfer the required amount of medium to a sintered glass Buchner funnel and remove the excess aqueous solution by gentle suction.
2. Add the next solvent and resuspend the medium by stirring gently.
3. Suck off the 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 medium into solvent resistant SR 25/45 or SR 25/100 columns.

Chapter 7

Gel filtration in theory

Defining the process

Results from gel filtration are usually expressed as an elution profile or chromatogram that shows the variation in concentration (typically in terms of UV absorbance, for proteins usually at 280 nm) of sample components as they elute from the column in order of their molecular size. Figure 7.1 shows a theoretical chromatogram of a high-resolution fractionation. Molecules that do not enter the matrix are eluted together in the void volume, V_0 , as they pass directly through the column at the same speed as the flow of buffer. 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 elute from the column in order of decreasing size. Small molecules such as salts that have full access to the pores move down the column, but do not separate from each other. These molecules usually elute just before one total column volume, V_t , of buffer has passed through the column.

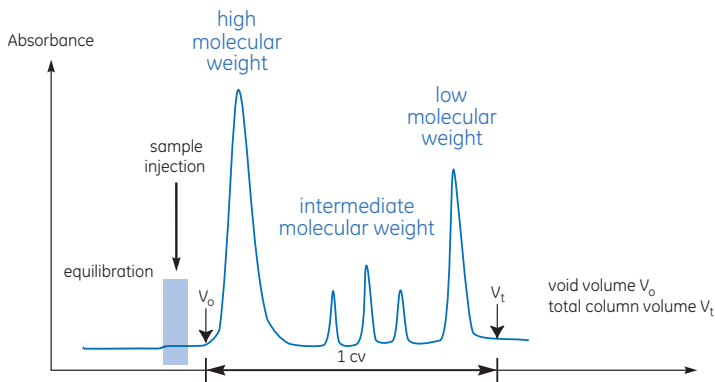


Fig 7.1. Theoretical chromatogram of a high resolution fractionation.

The behavior of each component can be expressed in terms of its elution volume, V_e from the chromatogram. As shown in Figure 7.2, there are three different ways of measuring V_e , dependent on the volume of sample applied to the column. V_e is the direct observation of the elution properties of a certain component.

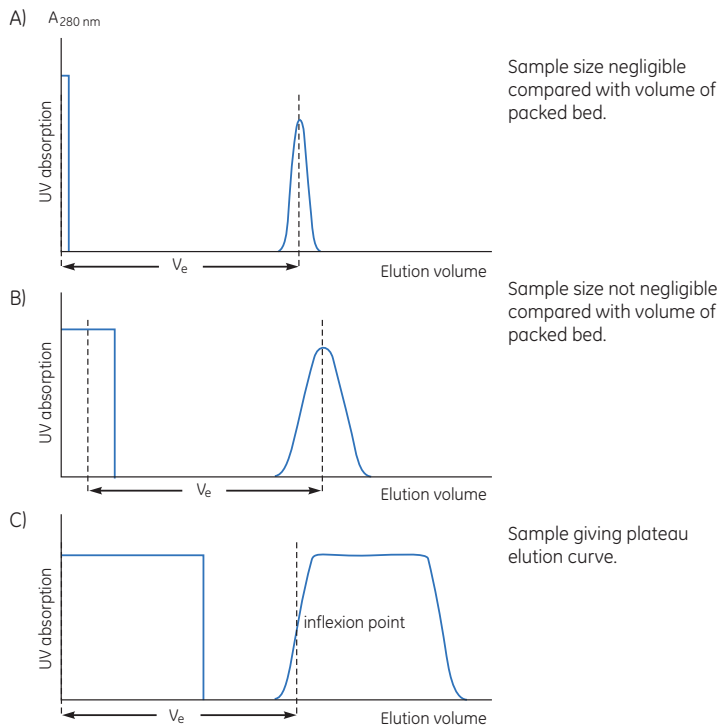


Fig 7.2. Measurement of elution volume, V_e . (A) Sample size negligible compared with volume of packed bed. (B) Sample size not negligible compared with volume of packed bed. (C) Sample giving plateau elution curve

Since symmetrical peaks are common in gel filtration, elution volumes are easily determined. However, V_e will vary with the total volume of the packed bed (V_t) and the way in which the column has been packed. The elution of a sample is best characterized by a distribution coefficient (K_d). K_d is used for column comparison since it is independent of column dimensions and thus allows comparison and prediction between columns with different sizes if the same medium and sample is used.

K_d is derived as follows:

The volume of the mobile phase (buffer) is equal to the void volume, V_o , which is the elution volume of molecules that remain in the buffer because they are larger than the largest pores in the matrix and therefore pass straight through the packed bed (Fig 7.3). In a well-packed column, the void volume is approximately 30% of the total physical volume.

The volume of the stationary phase, V_s , is equal to $V_t - V_o$, the volume of buffer inside the matrix. This volume is available only to very small molecules. V_i is the elution volume of molecules that distribute freely between the mobile and stationary phases minus the void volume. K_d represents the fraction of the stationary phase that is available for diffusion of a given molecular species:

$$K_d = \frac{V_e - V_o}{V_t - V_o - V_{\text{gel matrix}}} = \frac{V_e - V_o}{V_i}$$

Since, in practice, V_s or V_i are difficult to determine, it is more convenient to employ the term $(V_t - V_o)$. The estimated volume of the stationary phase will therefore include the volume of solid material which forms the matrix.

The stationary phase volume V_s can be substituted by the term $(V_t - V_o)$ in order to obtain a value K_{av} .

$$K_{av} = (V_e - V_o) / (V_t - V_o)$$

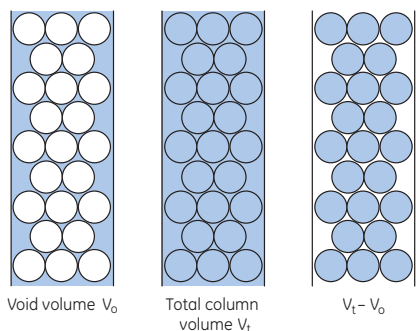


Fig 7.3. Diagrammatic representation of V_t and V_0 . Note that $V_t - V_0$ will include the volume of the solid material which forms the matrix (Fischer, L. Laboratory Techniques in Biochemistry and Molecular Biology. Vol. 1 part II. An Introduction to Gel Chromatography. North Holland Publishing Company, Amsterdam. Reproduced by kind permission of the author and publisher).

Since $(V_t - V_0)$ includes the volume of the matrix that is inaccessible to all solute molecules, K_{av} is not a true partition coefficient. However, for a given medium there is a constant ratio of $K_{av}:K_d$ 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. Other methods of normalizing data give values which vary depending upon how well the column is packed. The approximate relationships between some of these terms are shown in Figure 7.4.

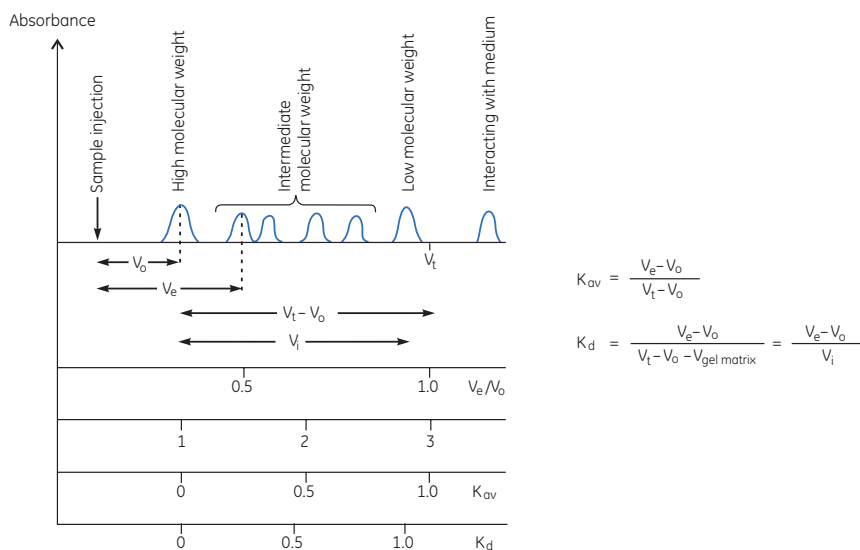
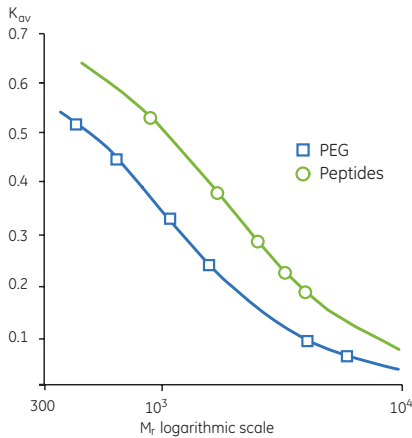


Fig 7.4. Relationship between several expressions used for normalizing elution behavior.

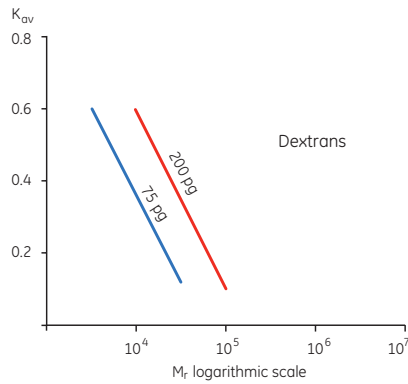
Selectivity curves and media selection

The partition coefficient K_{av} is related to the size of a molecule. Molecules of similar shape and density demonstrate a sigmoidal relationship between their K_{av} values and the logarithms of their molecular weights (M_r). Over a considerable range there is a virtually linear relationship between K_{av} and $\log M_r$. The selectivity of a gel filtration medium depends solely on its pore size distribution and is described by a selectivity curve. By plotting K_{av} against the log of the molecular weight for a set of standard proteins, selectivity curves are created for each gel filtration medium, as shown in Figure 7.5.

Superdex 30 prep grade



Superdex 75 prep grade and Superdex 200 prep grade



Superdex 75 prep grade and Superdex 200 prep grade

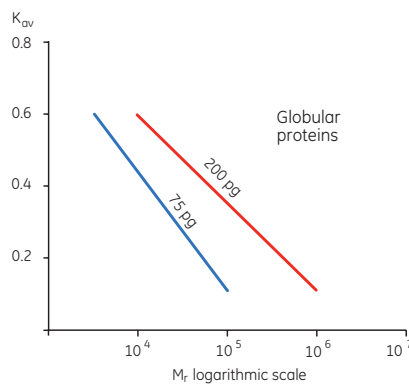


Fig 7.5. Selectivity curves for Superdex 30 prep grade, Superdex 75 prep grade and Superdex 200 prep grade.

Gel filtration media 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 ($K_{av} = 1$).



Under ideal conditions, no molecules can be eluted with a K_{av} greater than 1 or less than 0.



If the K_{av} is greater than 1, molecules have bound nonspecifically to the chromatographic medium.



If K_{av} is less than 0 after calibration then there is channeling in the chromatography bed and the column must be repacked.



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

Resolution

Resolution (R_s) is defined by the following expression:

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

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_{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 $1/2 (W_1 + W_2)$ the mean peak width of the two peaks as shown in Figure 7.6.

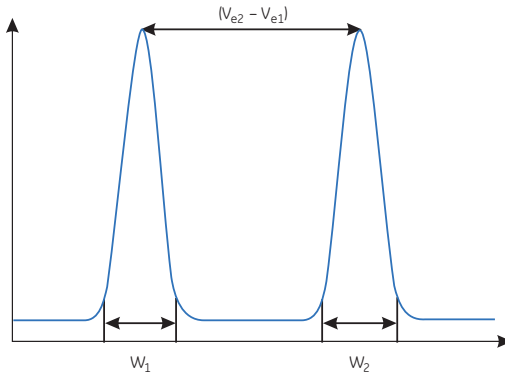


Fig 7.6. Parameters used to define resolution (R_s).

Final resolution, the degree of separation between peaks, is influenced by many factors: the ratio of sample volume to column volume, flow rate, column dimensions, particle size, particle size distribution, packing density, porosity of the particle, and viscosity of the mobile phase. The success of gel filtration depends primarily on choosing conditions that give sufficient selectivity and counteract peak broadening effects during the separation.

Resolution is a function of the selectivity of the medium and the efficiency of that medium to produce narrow peaks (minimal peak broadening) as illustrated in Figure 7.7.

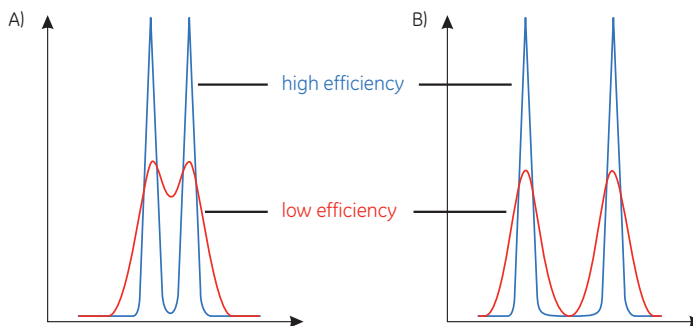


Fig 7.7. Resolution depends on the selectivity of the media and the counteraction of peak broadening (lower efficiency). (A) Good resolution (blue curve) and poor resolution (red curve). (B) Excellent resolution (blue curve) and good resolution (red curve).

The homogeneity of the packed bed and the particles influences the uniformity of the flow through the column and hence affects the shape and eventual peak width. Gel filtration media with high uniformity and narrow particle size distribution facilitate the elution of molecules in sharp peaks.

Gel filtration media 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 and so improve resolution by reducing peak width.

Sample dilution is inevitable because diffusion occurs. In order 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 7.8 shows how, if no zone broadening occurs, the maximum sample volume could be as great as the separation volume (V_{sep}):

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

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

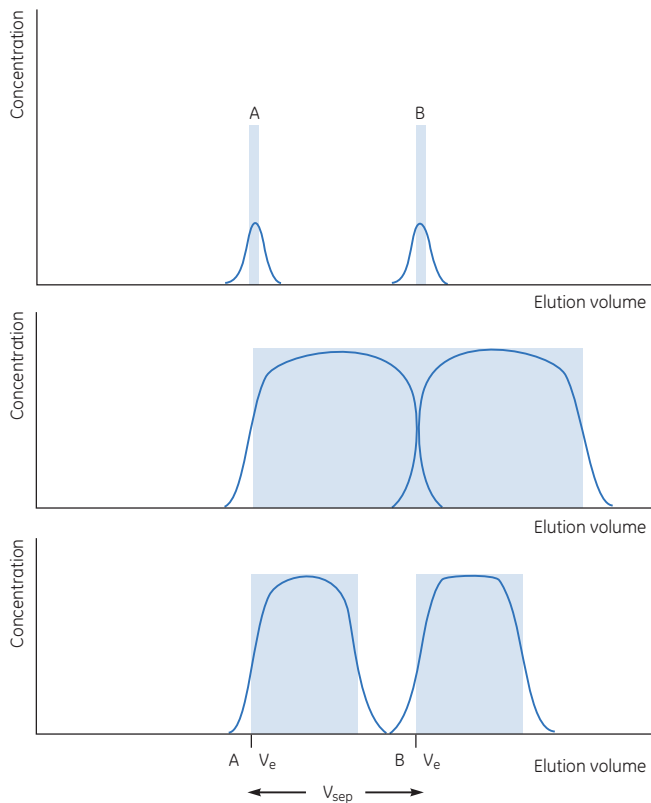


Fig 7.8. Elution curves for different sample sizes. The top diagram corresponds to the application of a small sample. The center diagram corresponds to the maximum sample volume that gives complete separation if there is no zone broadening. The bottom diagram corresponds to the maximum sample volume 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 8

Gel filtration in a purification strategy

The three phase purification strategy of Capture, Intermediate Purification and Polishing (CIPP) is used in both the pharmaceutical industry and in the research laboratory to ensure faster method development, a shorter time to pure product and good economy. This chapter gives a brief overview of this approach, which is recommended for any multistep protein purification. The Protein Purification Handbook from GE Healthcare is recommended as a guide to planning efficient and effective protein purification strategies.

As shown in Figure 8.1, an important first step for any purification is correct sample preparation and this is covered in more detail in Appendix 3. Gel filtration is often used for desalting and buffer exchange during sample preparation using Sephadex G-25, and samples volumes up to 30% of the total column volume can be applied.

In high-resolution mode, gel filtration is ideal for the final polishing steps in a purification when sample volumes have been reduced (sample volume significantly influences speed and resolution in gel filtration). Samples are eluted isocratically (single buffer, no gradient) and buffer conditions can be varied to suit the sample type or the requirements for subsequent purification, analysis or storage, since buffer composition does not directly affect resolution.

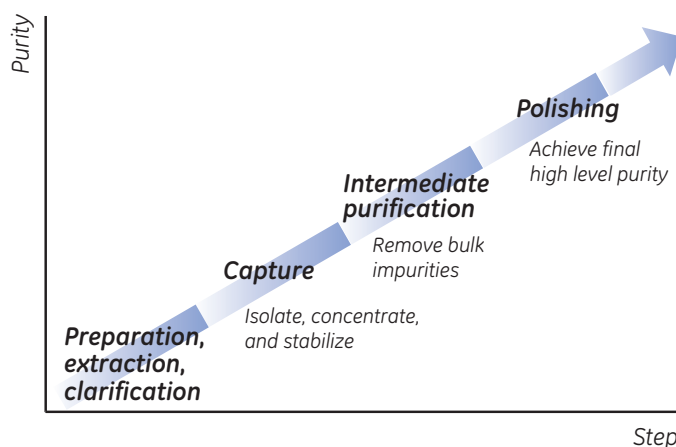


Fig 8.1. Sample preparation and CIPP purification strategy.

The purification strategy according to CIPP



Imagine the purification has three phases: Capture, Intermediate Purification and Polishing. Each phase may include one or more purification steps.



Assign a specific objective to each step within the purification process.

The problem associated with a particular purification step will depend greatly upon the properties of the starting material. Thus, the objective of a purification step will vary according to its position in the process.

In the capture phase the objectives are to isolate, concentrate and stabilize the target product. The product should be concentrated and transferred to an environment that will conserve potency/activity.

During the intermediate purification phase the objective is to remove most of the bulk impurities, such as other proteins and nucleic acids, endotoxins and viruses.

In the polishing phase most impurities have already been removed except for trace amounts or closely related substances. The objective is to achieve final purity by removing any remaining trace impurities or closely related substances.

 The optimal selection and combination of purification techniques for Capture, Intermediate Purification and Polishing is crucial for an efficient purification.

A guide describing the main features of the most common purification techniques and their use in the different phases in CIPP is shown in Table 8.1.

Table 8.1. Properties of different purification techniques and strategies for their use in CIPP

Method	Typical characteristics		Purification phase			Sample start conditions	Sample end conditions
	Resolution	Capacity	Capture	Intermediate	Polishing		
AC	+++ or ++	+++ or ++	+++	++	+	Various binding conditions	Specific elution conditions
IMAC	+++	++	+++	++	+	For purifying histidine-tagged proteins using Ni Sepharose columns:: 20-40 mM imidazole, pH > 7, 500 mM NaCl; no chelators Other proteins: low concentration of imidazole	High concentration of imidazole, pH > 7, 500 mM NaCl
GF	++	+	+		+++	Most conditions acceptable, limited sample volume	Buffer exchange possible Diluted sample
IEX	+++	+++	+++	+++	+++	Low ionic strength. pH depending on protein and IEX type	High ionic strength or pH changed
HIC	+++	++	++	+++	+++	High ionic strength Addition of salt required	Low ionic strength
Chromato-focusing	+++	+			++	Low ionic strength	Polybuffer Low ionic strength
RPC	+++	++		+	++	Ion-pair reagents and organic modifiers may be required	Organic solvents (risk for loss of biological activity)

Gel filtration as a polishing step

Most commonly, separations by charge, hydrophobicity or affinity will have been used in earlier stages of a purification strategy so that high resolution gel filtration is ideal for the final polishing step. The product can be purified and transferred into the required buffer in one step and dimers and aggregates can be removed, as shown in Figure 8.2.

Gel filtration is also the slowest of the chromatography techniques and the size of the column determines the volume of sample that can be applied. It is therefore most logical to use gel filtration after techniques that reduce sample volume so that smaller columns can be used.

Column: XK 16/60 packed with Superdex 75 prep grade
 Sample: partly purified ZZ-brain IGF
 Sample load: 1.0 ml
 Buffer: 0.3 M ammonium 0.5 ml/min
 Flow: 0.5 ml/min (15 cm/h)

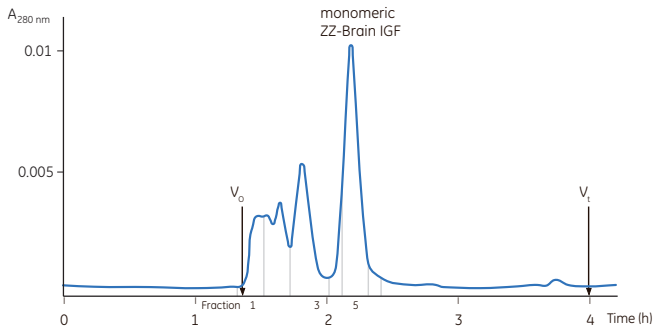


Fig 8.2. Final polishing step: separation of dimers and multimers on Superdex 75 prep grade.



Media for polishing steps should offer highest possible resolution. Superdex is the first choice at laboratory scale and Superdex prep grade for large-scale applications.

CIPP does not mean that there must always be three purification steps. For example, capture and intermediate purification may be achievable in a single step, as may intermediate purification and polishing. Similarly, purity demands may be so low that a rapid capture step is sufficient to achieve the desired result. For purification of therapeutic proteins, a fourth or fifth purification step may be required to fulfill the highest purity and safety demands. The number of steps used will always depend upon the purity requirements and intended use for the protein.

Purification of humanized IgG₄ monoclonal antibody

A humanized IgG₄ monoclonal antibody was expressed in a myeloma cell culture and purified by a combination of affinity chromatography and gel filtration (Fig 8.3). The antibody was captured by affinity chromatography using MabSelect™. Gel filtration on HiLoad Superdex 200 pg column was then used to separate the monomer from the dimer and larger polymers.

Capture by affinity chromatography

Sample: 1282 ml myeloma cell culture containing humanized IgG₄ (~0.33 mg/ml)

Column: MabSelect (18 ml), XK 16/20 column

Binding buffer: 20 mM sodium phosphate, 0.15 M NaCl, pH 7.4

Elution buffer: 100 mM sodium citrate, pH 3.0

Flow: 220 cm/h (7.4 ml/min)

Operation: Equilibration: 5 column volumes (CV) binding buffer
Sample application: 1 282 ml.
Wash: 10 CV binding buffer.
Elution: step gradient 100% 5 CV elution buffer

Purification of the monomer (polishing step)

Sample: 7.5 ml of the pooled fractions from MabSelect column

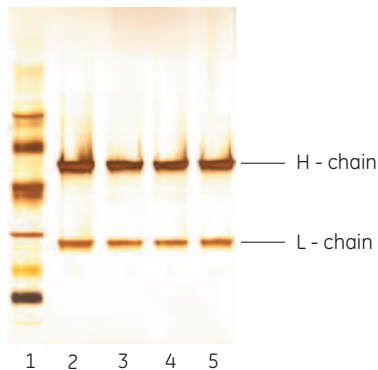
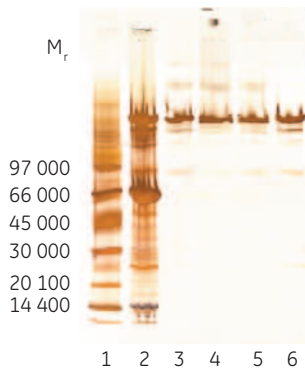
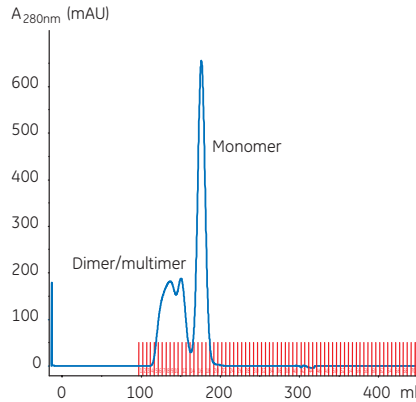
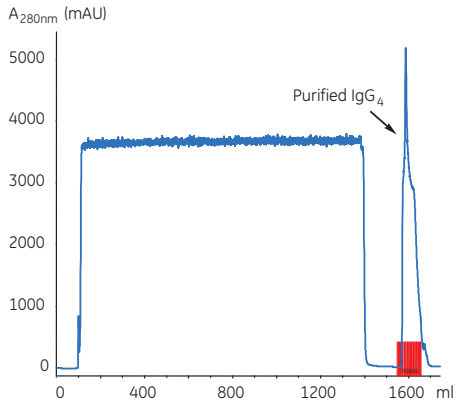
Column: HiLoad 26/60 Superdex 200 prep grade

Buffer: 50 mM sodium phosphate, 0.15 M NaCl, pH 7.0

Flow: 22.6 cm/h (2 ml/min)

Operation: Equilibration 2 CV, Sample application, Isocratic elution 1 CV

CV = total column volume = V_t



Analysis: SDS-PAGE, silver staining

Unreduced sample

Lanes

1. LMW Marker
2. Crude sample from myeloma cell culture
3. Pooled eluted sample from capture step
4. Fraction 4-9 from polishing step
5. Fraction 10-12 from polishing step
6. Fraction 14-18 from polishing step

Reduced sample

1. LMW Marker
2. Pooled eluted sample from capture step
3. Fraction 4-9 from polishing step
4. Fraction 10-12 from polishing step
5. Fraction 14-18 from polishing step

Fig 8.3. Two step purification of humanized IgG₄.

Appendix 1

Column packing and preparation

A well-packed column is essential for a high resolution fractionation on any gel filtration medium. Prepacked columns from GE Healthcare will ensure reproducible results and the highest performance. If the column volume or medium you require is not available as a prepacked column, contact your local GE Healthcare sales representative to inquire about our column packing services.

Packing a column is a very critical stage in any gel filtration experiment. A poorly packed column will give rise to uneven flow, peak broadening, and loss of resolution, it can also affect achievable flow rates. If you decide to pack a gel filtration column yourself then the guidelines in this appendix will apply at any scale of operation.

An instructive video on a CD, is available to demonstrate how to produce a well-packed column (see Ordering information for "Column Packing – The Movie"). It focuses particularly on the importance of column packing for gel filtration. Gel filtration is simple to perform once a well-packed column has been obtained. Providing that a column is used and maintained carefully it can be expected to give reproducible, high resolution results for a long time.



Ensure that there is sufficient buffer for long, unattended runs or that the pump is programmed to stop the flow after a suitable time. Gel filtration columns that run dry must be repacked.

Columns for packing gel filtration media

Empty columns from GE Healthcare are fully compatible with the high flow rates achievable with modern media and a broad range of column dimensions is available. Ordering information for empty columns and main accessories can be found at the back of this handbook. For a complete listing of all spare parts refer to the www.gelifesciences.com/protein-purification

Table A1.1. Maximum bed heights (cm) and bed volumes (ml) using one adaptor or two adaptors in the various XK columns.

Column	With one adaptor		With two adaptors	
	Volume (ml)	Bed height (cm)	Volume (ml)	Bed height (cm)
XK 16/20	5-31	2.5-15.5	0-31	0-15.5
XK 16/40	45-70	22.5-35	16-70	8-35
XK 16/70	105-130	52.5-65	76-130	38-65
XK 16/100	165-190	82.5-95	136-190	68-95
XK 26/20	5-66	1-12.5	0-66	0-12.5
XK 26/40	122-186	23-35	45-186	8.5-35
XK 26/70	281-344	53-65	204-344	38.5-65
XK 26/100	440-504	83-95	365-504	68.5-95
XK 50/20	0-274	0-14	0-274	0-14
XK 50/30	265-559	14-28	0-559	0-28
XK 50/60	794-1088	40-56	500-1088	26-56
XK 50/100	1588-1862	81-95	1274-1862	65-95

Adaptors are adjustable column end pieces that help to eliminate any disturbances to the surface of the packed medium as sample is applied and to prevent insoluble particles from entering and blocking the column.

Tricorn™ and XK empty columns are delivered with one adaptor, but a second adaptor can be used instead of a column end piece if a shorter bed height is required. HiScale™ columns are equipped with dual adaptors. A range of accessories are available for all empty columns.

Table A1.2. Bed volumes and heights for the various Tricorn columns

Bed volumes and heights		With one adaptor		With two adaptors	
Tricorn Column	Column Size i.d. (mm)	Volume (ml)	Bed Height (mm)	Volume (ml)	Bed height (mm)
Tricorn 10/20	10	0.00–2.29	0–29	0.00–2.07	0–26
Tricorn 10/100	10	6.21–8.57	79–109	3.64–8.36	46–106
Tricorn 10/150	10	10.14–12.50	129–159	7.57–12.28	96–156
Tricorn 10/200	10	14.07–16.42	179–209	11.50–16.21	146–206
Tricorn 10/300	10	21.92–24.28	279–309	19.35–24.06	246–306
Tricorn 10/600	10	45.48–47.84	579–609	42.91–47.63	546–606

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

Column	Column volume (ml) with one adaptor
HiScale 16/20	Max bed height 20 cm, max volume 40 ml
HiScale 26/20	Max bed height 20 cm, max volume 106 ml
HiScale 16/40	Max bed height 40 cm, max volume 80 ml
HiScale 26/40	Max bed height 20 cm, max volume 212 ml
HiScale 50/20	Max bed height 20 cm, max volume 393 ml
HiScale 50/40	Max bed height 20 cm, max volume 785 ml

 Longer columns (50 cm and more) can be difficult to pack under normal laboratory conditions. As alternatives, 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.

Checking column efficiency

Column performance should be checked at regular intervals by determining the theoretical plate number and peak symmetry. Prepacked columns are supplied with recommended values.

Typical values for column performance:

Superdex: Efficiency N>10 000, Peak symmetry A_s = 0.70–1.30

Sephacryl HR: Efficiency N>9 000, Peak symmetry A_s = 0.80–1.50

1. Equilibrate the packed column in distilled water at a linear flow of 60 cm/h.
2. Inject acetone (10 mg/ml in water) in a volume equivalent to 0.2% of the total packed column volume.
3. Monitor UV absorbance 280 nm from the time of injection until the acetone peak has eluted and the signal has returned to baseline.
4. Calculate column efficiency i.e. the number of theoretical plates (N):

$$N = 5.54 (V_e / W_{1/2})^2 \times 1000/L$$

where

V_e = peak elution (retention) volume

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

L = bed height (mm)

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

Calculate the symmetry factor (A_s):

$$A_s = b/a$$

where

a = first half peak width at 10% peak height

b = second half peak width at 10% peak height

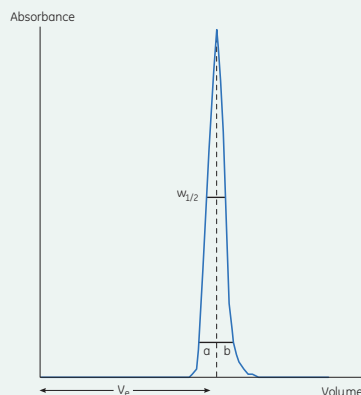


Fig A1.1. Determination of column efficiency by number of theoretical plates and peak symmetry.

Column packing for high resolution fractionation using Superdex prep grade and Sephacryl High Resolution

Superdex prep grade and Sephacryl High Resolution should be packed and equilibrated at a high flow rate using a column from the XK-series. XK columns are optimally designed for gel filtration with a bed design that ensures a uniform liquid flow and a dead space at the column outlet of less than 0.1% of the column volume in order to minimize dilution and to prevent remixing of separated peaks. XK columns are manufactured from materials which do not interfere with labile biological substances. They are easy to dismantle and reassemble for thorough cleaning, particularly important when handling biological samples.



Ensure that the column and all components are clean and in good condition. It is particularly importance that the nets, net fasteners and glass tube are not damaged. Use well degassed buffers and equilibrate all materials to the temperature at which the separation will be performed. Avoid columns with large dead volumes as this will affect resolution.



For high resolution fractionation, use bed heights between 30–60 cm. Apply sample volumes equivalent to 1–2% of the column volume. The sample volume can be increased up to 4% if resolution in the particular application still is good enough.



The settled medium should have a volume of 1.15 times that of the required packed column volume, see Table A1.1 to A1.3 for examples.

1. Sephacryl HR and Superdex prep grade are supplied swollen in a suspension containing 20% ethanol as a preservative. Suspend the medium by shaking gently and pour a sufficient quantity into a graduated glass cylinder or beaker.

Avoid using magnetic stirrers, spatulas or glass rods since they may damage the matrix.

2. Wash the medium with 5–10 column volumes of distilled water on a glass filter and resuspend in distilled water to a final concentration of 50% settled medium. The medium must be thoroughly washed to remove the 20% ethanol storage solution. Residual ethanol may interfere with subsequent procedures.



To produce a more evenly dispersed slurry of Superdex prep grade, Tween™ 20 (250 ml per 500 ml washed slurry) can be added in order to reduce surface tension.

3. Wet the bottom filter by injecting distilled water through the effluent tubing. Close the end piece outlet. Mount filter and bottom end piece onto the column.

4. Attach the packing reservoir tightly to the column.



For XK 16 and XK 26 columns using a second column instead of a packing reservoir makes it easier to obtain a well-packed column. The second column is used with Packing Connector XK 16 or XK 26 as appropriate.

5. Mount the column and packing reservoir vertically on a laboratory stand.
6. Fill the column with distilled water to a height of 2 cm above the column end piece. Avoid air bubbles.
7. Degas the suspended medium under vacuum and carefully pour the suspended medium down the wall of the column using a glass rod. Avoid introducing air bubbles. Pour everything in a single operation and fill the reservoir to the top with distilled water.
8. Connect the pump outlet to the inlet on the packing reservoir. Open the column outlet and start the flow of buffer, see Table A1.4 for flow recommendations.



To achieve satisfactory column efficiency, Superdex prep grade must be packed in two steps: Step 1 for 2 hours or until the bed has reached a constant height and Step 2 for 60 min. Table A1.4 shows the flow rates for each step.



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 was unsatisfactory after the first attempt.

9. Stop the pump and remove the packing reservoir. Carefully fill the column with distilled water to form an upward meniscus at the top and insert the adaptor. Adjust the adaptor to the surface of the packed bed.
10. Continue packing the column at the flow rate used in Step 2 for approximately 10 minutes. If the recommended flow rate cannot be obtained, use the maximum flow rate the pump can deliver. Mark the position of the top of the packed medium, stop the pump, close the column outlet, move the adaptor down onto to the surface of the medium and then push the adaptor a further 3 mm into the medium. The column is now ready to use. See Table A1.4 for maximum recommended flow rate and operating pressure for Sephacryl HR and Superdex prep grade media.



Maximum pressures (Sephacryl HR 0.3 MPa, 0.3 bar and Superdex prep grade 5 MPa, 5 bar) should not be exceeded during packing.



Always check the specific storage instructions supplied with the product.

Table A1.4. Recommended flow rates during column packing

Column	Bed height cm	Step 1 Sephacryl HR ml/min	Step 2 Sephacryl HR ml/min	Step 1 Superdex prep grade ml/min	Step 2 Superdex prep grade ml/min
XK 16/40	35	2	12–14	2	10–12
XK 16/70	65	2	12–14	2	10–12
XK16/100	95	2	12–14	2	10–12
XK 26/40	35	4	6–8	4	12
XK 26/70	65	4	6–8	4	12
XK 26/100	95	4	6–8	4	12
XK 50/20	10–15	9	12	10	20
XK 50/30	20–25	9	12	10	20
XK 50/60	55	9	12	10	20
XK 50/100	95	9	12	10	20

Column packing for group separations using Sephadex

Sephadex is supplied as a dry powder and must be allowed to swell in excess buffer before use. After swelling, adjust with buffer to form a thick slurry from which air bubbles are removed under vacuum. Approximately 75% settled medium is suitable. Fine particles can be decanted.



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

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



Ensure that the column and all components are clean and in good condition. It is particularly important that the nets, net fasteners and glass tube are not 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 the formation of bubbles.



For group separations, use up to 10 cm bed height. Sample volumes can be up to 30% of the column volume. Pack a quantity of medium up to 5 times the volume of the sample to be desalted.

Note: These instructions assume that a column with two adaptors is used for packing.

1. Weigh out the correct amount of dry Sephadex and allow the medium to swell according to the instructions above. Avoid using magnetic stirrers, spatulas or glass rods since they may damage the medium.
2. Wet the bottom filter by injecting distilled water through the effluent tubing. Close the end piece outlet. Mount filter and bottom end piece onto the column.

3. If the slurry volume is greater than the volume of the column, attach a packing reservoir to the column.
4. Mount the column and packing reservoir vertically on a laboratory stand.
5. Fill the column with distilled water or buffer to a height of approximately 2 cm above the column end piece. Avoid air bubbles.
6. Pour the well-mixed and well-degassed suspension in a single operation down the inside wall using a glass rod. Avoid introducing air bubbles.
7. Connect the pump outlet to the inlet of the packing reservoir. Open the column outlet and start the flow of buffer. Pass 2–3 column volumes of buffer through the column in order to stabilize the bed and equilibrate completely. Use a slightly higher flow rate than the flow rate to be used during separations.
8. Maintain the packing flow rate for at least 3 column volumes after a constant bed height is obtained.
9. Mark the bed height on the column and close the column outlet. Remove the packing reservoir.
10. Add buffer carefully to fill the column and form an upward meniscus.
11. Connect all tubings. Slacken the adaptor tightening mechanism and insert the adaptor at an angle into the column so that no air is trapped under the net. Slide the adaptor slowly down the column until the mark is reached. Note that the outlet of the adaptor should be open and the column outlet should be closed.
12. Adjust the tightening mechanism to give a sliding seal between the column wall and O-ring. Screw the adaptor onto the column.
13. Continue packing the column for approximately 10 minutes. Stop the pump, close the column outlet and move the top adaptor down onto the surface of the medium. Push the adaptor a further 3 mm into the medium. The column is now ready for equilibration.

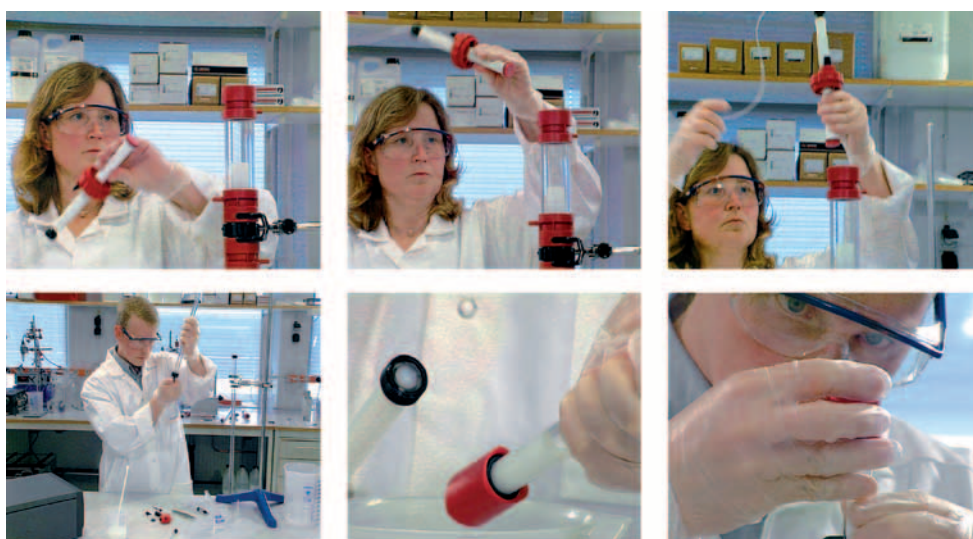


Fig A1.2. "Column Packing — The Movie" provides a step-by-step demonstration of column packing.



Sephadex G-10, G-25 and G-50 obey Darcy's law, for example if the flow rate is doubled then the column pressure will double, hence maximum values for flow or operating pressures do not need to be considered (see Appendix 2 for an explanation of Darcy's law).

Controlling flow

The safest and easiest way in which to control flow during column packing and chromatography separation is to use a pump controlled within an ÄKTA design chromatography system. Accurate and reproducible flow control is particularly important for efficient column packing and when repeating experiments or performing routine preparative work. A peristaltic pump can be used with Sephadex packed in smaller columns.



The maximum flow rate achievable will depend on column diameter and buffer viscosity. Narrow columns allow a higher pressure and higher linear flow (cm/h) than wide columns.



Always connect a pump so that buffer is pumped onto the column (rather than connecting the pump after the column and drawing buffer through the column). This reduces the risk of bubble formation due to suction effects.



Always use a flow rate for column packing that is higher than the flow rate used for separation.



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



Do not exceed 75% of the packing flow rate during any separation.



Do not use a peristaltic pump when packing Superdex or Sephacryl media in larger columns since they cannot achieve high enough flowrate needed to get high resolution fractionation.

Appendix 2

Sephadex and Darcy’s law

Sephadex G-10, G-25 and G-50 may be assumed to behave as rigid spheres in gel filtration and therefore obey Darcy’s Law. This Law describes a general relationship for flow in porous media:

$$U = K \times \Delta P \times L^{-1}$$

Equation (1)

U = linear flow rate expressed in cm/h (see Appendix 5)
ΔP = pressure drop over bed expressed in cm H₂O
L = bed height expressed in cm
K = constant of proportionality depending on the properties of the bed material and the buffer

Assuming a buffer with viscosity of 1 cP: $U = K_o \times \Delta P \times L^{-1}$

Equation (2)

K_o = the “specific permeability” depending on the particle size of the medium and the water regain

-  Note that flow is proportional to the pressure drop over the bed and, assuming a constant pressure head, inversely proportional to the bed height. In practice this means that the pressure/flow considerations that must be made when using other gel filtration media do not apply to Sephadex and that a doubling of flow rate leads to a doubling in column pressure. To a good approximation, flow rate is independent of the column diameter.
-  Flow at viscosities greater than 1 cP can be obtained by using the relationship: flow rate is inversely proportional to viscosity. High buffer viscosities can be compensated for by increasing the operating pressure to maintain a high flow rate.

Theoretical flow (not maximum) can be calculated from equation (2) by inserting values for ΔP and L. Specific permeabilities (K) are given in Table A2.1.

Table A2.1. Specific permeabilities of Sephadex

Sephadex type	Permeability K
Sephadex G-10	19
Sephadex G-25 Superfine	9
Sephadex G-25 Fine	30
Sephadex G-25 Medium	80
Sephadex G-25 Coarse	290
Sephadex G-50 Fine	36

Appendix 3

Sample preparation

Samples for chromatographic purification should be clear and free from particulate matter. Simple steps to clarify a sample before beginning purification will avoid clogging the column, may reduce the need for stringent washing procedures, and can extend the life of the chromatographic medium.

Sample extraction procedures and the selection of buffers, additives, and detergents are determined largely by the source of the material, the stability of the target molecule, the chromatographic techniques that will be employed, and the intended use of the product. These subjects are dealt with in general terms in the *Protein Purification Handbook* and more specifically according to target molecule in the *Recombinant Protein Handbook*, and *Antibody Purification Handbook*, available from GE Healthcare.

Sample clarification

Centrifugation and filtration are standard laboratory techniques for sample clarification and are used routinely when handling small samples.



It is highly recommended to centrifuge and filter any sample immediately before chromatographic purification.

Centrifugation

Centrifugation removes lipids and particulate matter, such as cell debris. If the sample is still not clear after centrifugation, use filter paper or a 5 µm filter as a first step and one of the filters below as a second step filter.



For small sample volumes or proteins that adsorb to filters, centrifuge at 10 000 g for 15 minutes.



For cell lysates, centrifuge at 40 000 to 50 000 g for 30 minutes.



Serum samples can be filtered through glass wool after centrifugation to remove any remaining lipids.

Filtration

Filtration removes particulate matter. Membrane filters that give the least amount of nonspecific binding of proteins are composed of cellulose acetate or PVDF.

For sample preparation before chromatography, select a filter pore size in relation to the bead size of the chromatographic medium (Table A3.1).

Table A3.1. Filter pore size recommendations

Nominal pore size of filter	Particle size of chromatography medium
1 µm	90 µm and upwards
0.45 µm	34 µm
0.22 µm	3, 10, 15 µm or when extra clean samples or sterile filtration is required



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

Desalting

Detailed procedures for buffer exchange and desalting are given in Chapter 5.

Denaturation

Table A3.2. Common denaturing agents

Denaturing agent	Typical conditions for use	Removal/comment
Urea	2 M–8 M	Remove using maintain solubility. Sephadex G-25.
Guanidine hydrochloride	3 M–6 M	Remove using Sephadex G-25 or during IEX.
Triton X-100	2%	Remove using Sephadex G-25 or during IEX.
Sarcosyl	1.5%	Remove using Sephadex G-25 or during IEX.
N-octyl glucoside	2%	Remove using Sephadex G-25 or during IEX.
Sodium dodecyl sulfate	0.1%–0.5%	Exchange for non-ionic detergent during first chromatographic step, avoid anion exchange chromatography.
Alkaline pH	>pH 9, NaOH	May need to adjust pH during chromatography to maintain solubility.

Details taken from:
Scopes R.K., *Protein Purification, Principles and Practice*, Springer, (1994), J.C. Janson and L. Rydén, *Protein Purification, Principles, High Resolution Methods and Applications*, 2nd ed. Wiley Inc, (1998) and other sources.

See Chapter 5, page 57.

Precipitation and resolubilization

Specific sample preparation steps may be required if the crude sample is known to contain contaminants such as lipids, lipoproteins, or phenol red that may build up on a column or if certain gross impurities, such as bulk protein, should be removed before any chromatographic step.

Fractional precipitation Fractional precipitation is occasionally used at laboratory scale to remove gross impurities but is generally not required in purification of affinity-tagged proteins. In some cases, though, precipitation can be useful as a combined protein concentration and purification step.

Precipitation techniques separate fractions by the principle of differential solubility. For example, because protein species differ in their degree of hydrophobicity, increased salt concentrations can enhance hydrophobic interactions between the proteins and cause precipitation. Fractional precipitation can be applied to remove gross impurities in three different ways, as shown in Figure A3.1.

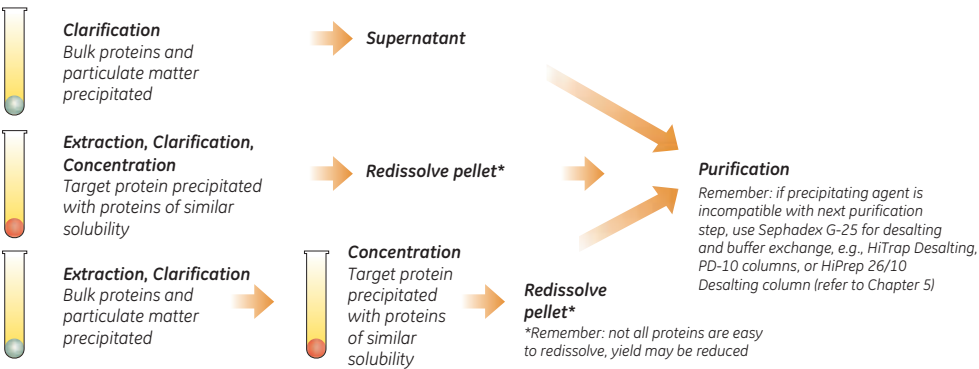


Fig A3.1. Three ways to use precipitation.



Precipitation techniques may be affected by temperature, pH, and sample concentration. These parameters must be controlled to ensure reproducible results.



Most precipitation techniques are not suitable for large-scale preparation.

Examples of precipitation agents are reviewed in Table A3.3. The most common precipitation method using ammonium sulfate is described in more detail on below.

Table A3.3. Examples of precipitation techniques

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulfate	As described below.	> 1 mg/ml proteins, especially immunoglobulins.	Stabilizes proteins, no denaturation; supernatant can go directly to HIC. Helps to reduce lipid content.
Dextran sulfate	Add 0.04 ml of 10% dextran sulfate and 1 ml of 1 M CaCl ₂ per ml of sample, mix 15 min, centrifuge at 10 000 × g, discard pellet.	Samples with high levels of lipoprotein, e.g., ascites.	Precipitates lipoprotein.
Polyvinylpyrrolidone	Add 3% (w/v), stir 4 h, centrifuge at 17 000 × g, discard pellet.	Samples with high levels of lipoprotein, e.g., ascites.	Alternative to dextran sulfate.
Polyethylene glycol (PEG, M _w > 4000)	Up to 20% (w/v)	Plasma proteins.	No denaturation, supernatant goes directly to IEX or AC, complete removal may be difficult. Stabilizes proteins.
Acetone (cold)	Up to 80% (v/v) at 0°C. Collect pellet after centrifugation at full speed in an Eppendorf™ centrifuge.		May denature protein irreversibly. Useful for peptide precipitation or concentration of sample for electrophoresis.
Polyethyleneimine	0.1% (w/v)		Precipitates aggregated nucleoproteins.
Protamine sulfate	1% (w/v)		Precipitates aggregated nucleoproteins.
Streptomycin sulfate	1% (w/v)		Precipitates nucleic acids.
Caprylic acid	(X/15) g where X = volume of sample.	Antibody concentration should be > 1 mg/ml.	Precipitates bulk of proteins from sera or ascites, leaving immunoglobulins in solution.

Details taken from: Scopes R.K., Protein Purification, Principles and Practice, Springer, (1994), J.C. Janson and L. Rydén, Protein Purification, Principles, High Resolution Methods and Applications, 2nd ed. Wiley Inc, (1998).

Ammonium sulfate precipitation

Ammonium sulfate precipitation is frequently used for initial sample concentration and cleanup. As the concentration of the salt is increased, proteins will begin to “salt out.” Different proteins salt out at different concentrations, a process that can be taken advantage of to remove contaminating proteins from the crude extract. The salt concentration needs to be optimized to remove contaminants and not the desired protein. An additional step with increased salt concentration should then precipitate the target protein. If the target protein cannot be safely precipitated and redissolved, only the first step should be employed. HIC is often an excellent next purification step, as the sample already contains a high salt concentration and can be applied directly to the HIC column with little or no additional preparation. The elevated salt level enhances the interaction between the hydrophobic components of the sample and the chromatography medium.

Solutions needed for precipitation:

Saturated ammonium sulfate solution (add 100 g ammonium sulfate to 100 ml distilled water, stir to dissolve).

1 M Tris-HCl, pH 8.0.

Buffer for first purification step.



Some proteins may be damaged by ammonium sulfate. Take care when adding crystalline ammonium sulfate: high local concentrations may cause contamination of the precipitate with unwanted proteins.



For routine, reproducible purification, precipitation with ammonium sulfate should be avoided in favor of chromatography.



In general, precipitation is rarely effective for protein concentrations below 1 mg/ml.

1. Filter (0.45 μ m) or centrifuge the sample (10 000 $\times$ g at 4°C).
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add ammonium sulfate solution, drop by drop. Add up to 50% saturation*. Stir for 1 h.
4. Centrifuge 20 min at 10 000 $\times$ g.
5. Remove supernatant. Wash the pellet twice by resuspension in an equal volume of ammonium sulfate solution of the same concentration (i.e., a solution that will not redissolve the precipitated protein or cause further precipitation). Centrifuge again.
6. Dissolve pellet in a small volume of the buffer to be used for the next step.
- 7 Ammonium sulfate is removed during clarification/buffer exchange steps with Sephadex G-25, using desalting columns (see Chapter 5).

* The % saturation can be adjusted either to precipitate a target molecule or to precipitate contaminants.

The quantity of ammonium sulfate required to reach a given degree of saturation varies according to temperature. Table A3.4 shows the quantities required at 20°C.

Table A3.4. Quantities of ammonium sulfate required to reach given degrees of saturation at 20°C

	Final percent saturation to be obtained																		
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100		
Starting percent saturation	Amount of ammonium sulfate to add (grams) per liter of solution at 20°C																		
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761		
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723		
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685		
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647		
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609		
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571		
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533		
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495		
40					0	31	63	96	130	166	202	241	281	322	365	410	457		
45						0	31	64	98	132	169	206	245	286	329	373	419		
50							0	32	65	99	135	172	210	250	292	335	381		
55								0	33	66	101	138	175	215	256	298	343		
60									0	33	67	103	140	179	219	261	305		
65										0	34	69	105	143	183	224	267		
70											0	34	70	107	146	186	228		
75												0	35	72	110	149	190		
80													0	36	73	112	152		
85														0	37	75	114		
90															0	37	76		
95																0	38		

Removal of lipoproteins

Lipoproteins and other lipid material can rapidly clog chromatography columns and it is advisable to remove them before beginning purification. Precipitation agents such as dextran sulfate and polyvinylpyrrolidone, described under Fractional precipitation, are recommended to remove high levels of lipoproteins from samples such as ascites fluid.



Centrifuge samples to avoid the risk of nonspecific binding of the target molecule to a filter.



Samples such as serum can be filtered through glass wool to remove remaining lipids.

Appendix 4

Selection of purification equipment

Simple buffer exchange and desalting steps can be performed using a syringe or peristaltic pump together with prepacked HiTrap columns. A chromatography system is needed to deliver accurately controlled flow rates for high resolution separations.

Way of working	Standard ÄKTA design configurations					Syringe or peristaltic pump + HiTrap Desalting column	Gravity-fed columns
	ÄKTAmicro™	ÄKTAprime plus	ÄKTExpress™	ÄKTApurifier™	ÄKTA avant		
Simple, one step desalting, buffer exchange	x	x	x	x	x	x	x
Reproducible performance for routine separation	x	x	x	x	x		
Micropreparative and analysis	x						
System control and data handling for regulatory requirements, e.g., GLP	x		x	x	x		
Automatic method development and optimization				(x)	x		
Automatic buffer preparation				(x)	x		
Automatic pH scouting				(x)	x		
Automatic media or column scouting				(x)	x		
Automatic multi-step purification		(x)	x				
Scale-up, process development					x		



Appendix 5

Converting from linear flow (cm/h) to volumetric flow rates (ml/min) and *vice versa*

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

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

$$\begin{aligned}\text{Volumetric flow rate (ml/min)} &= \frac{\text{Linear flow (cm/h)}}{60} \times \text{column cross-sectional area (cm}^2\text{)} \\ &= \frac{Y}{60} \times \frac{\pi \times d^2}{4}\end{aligned}$$

where

Y = linear flow 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 is 150 cm/h?

Y = linear flow = 150 cm/h

d = inner diameter of the column = 1.6 cm

$$\begin{aligned}\text{Volumetric flow rate} &= \frac{150 \times \pi \times 1.6 \times 1.6}{60 \times 4} \text{ ml/min} \\ &= 5.03 \text{ ml/min}\end{aligned}$$

From volumetric flow rate (ml/min) to linear flow (cm/hour)

$$\begin{aligned}\text{Linear flow (cm/h)} &= \frac{\text{Volumetric flow rate (ml/min)} \times 60}{\text{column cross-sectional area (cm}^2\text{)}} \\ &= Z \times 60 \times \frac{4}{\pi \times d^2}\end{aligned}$$

where

Z = volumetric flow rate in ml/min

d = column inner diameter in cm

Example:

What is the linear flow 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

$$\begin{aligned}\text{Linear flow} &= 1 \times 60 \times \frac{4}{\pi \times 0.5 \times 0.5} \text{ cm/h} \\ &= 305.6 \text{ cm/h}\end{aligned}$$

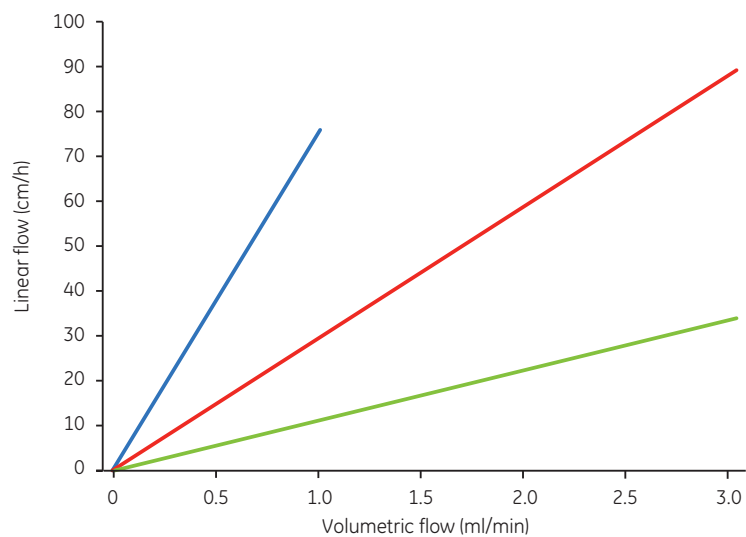


Fig A5.1. Linear flow as a function of volumetric flow: Blue curve 10 mm, red curve 16 mm, and green curve 26 mm column diameter.

From ml/min to using a syringe

1 ml/min = approximately 30 drops/min on a HiTrap 1 ml column

5 ml/min = approximately 120 drops/min on a HiTrap 5 ml column

Appendix 6

Conversion data

Proteins

Protein size and amount conversion

Mass (g/mol)	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

Absorbance coefficient for proteins

Protein	A_{280} for 1 mg/ml
IgG	1.35
IgM	1.20
IgA	1.30
Protein A	0.17
Avidin	1.50
Streptavidin	3.40
Bovine Serum Albumin	0.70

Nucleic Acids

Approximate molecular weights of nucleic acids

M.W. of ssRNA = (# nucleotides $\times$ 320.5) + 159.0

M.W. of ssDNA = (# nucleotides $\times$ 303.7) + 79.0

M.W. of dsDNA = (# nucleotides $\times$ 607.4) + 157.9

Absorbance units to nucleic acid concentration conversion

1 A_{280} dsDNA = 50 µg/ml

1 A_{280} ssDNA = 37 µg/ml

1 A_{280} ssRNA = 40 µg/ml

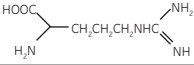
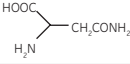
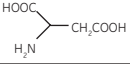
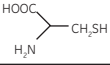
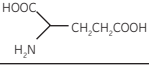
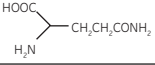
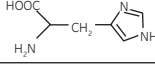
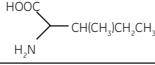
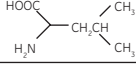
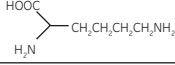
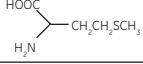
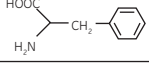
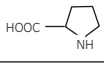
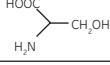
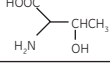
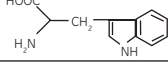
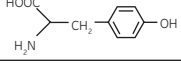
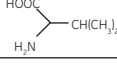
Column pressures

The maximum operating back pressure refers to the pressure above which the column contents may begin to compress.

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 7

Amino acids table

Amino acid	Three-letter code	Single-letter code	Structure
Alanine	Ala	A	
Arginine	Arg	R	
Asparagine	Asn	N	
Aspartic Acid	Asp	D	
Cysteine	Cys	C	
Glutamic Acid	Glu	E	
Glutamine	Gln	Q	
Glycine	Gly	G	
Histidine	His	H	
Isoleucine	Ile	I	
Leucine	Leu	L	
Lysine	Lys	K	
Methionine	Met	M	
Phenylalanine	Phe	F	
Proline	Pro	P	
Serine	Ser	S	
Threonine	Thr	T	
Tryptophan	Trp	W	
Tyrosine	Tyr	Y	
Valine	Val	V	

Formula	M _r	Middle unit residue (-H ₂ O)		Side-chain charge at neutral pH	Hydrophilic (nonpolar)	Uncharged (polar)	Hydrophilic (polar)
		Formula	M _r				
C ₃ H ₇ NO ₂	89.1	C ₃ H ₅ NO	71.1	Neutral	●		
C ₆ H ₁₄ N ₄ O ₂	174.2	C ₆ H ₁₂ N ₄ O	156.2	Basic (+ve)			●
C ₄ H ₈ N ₂ O ₃	132.1	C ₄ H ₆ N ₂ O	114.1	Neutral		●	
C ₄ H ₇ NO ₄	133.1	C ₄ H ₅ NO ₃	115.1	Acidic (+ve)			●
C ₃ H ₇ NO ₂ S	121.2	C ₃ H ₅ NOS	103.2	Neutral		●	
C ₅ H ₉ NO ₄	147.1	C ₅ H ₇ NO ₃	129.1	Acidic (+ve)			●
C ₅ H ₁₀ N ₂ O ₃	146.1	C ₅ H ₈ N ₂ O ₂	128.1	Neutral		●	
C ₂ H ₅ NO ₂	75.1	C ₂ H ₃ NO	57.1	Neutral		●	
C ₆ H ₉ N ₃ O ₂	155.2	C ₆ H ₇ N ₃ O	137.2	Basic (+ve)			●
C ₆ H ₁₃ NO ₂	131.2	C ₆ H ₁₁ NO	113.2	Neutral	●		
C ₆ H ₁₃ NO ₂	131.2	C ₆ H ₁₁ NO	113.2	Neutral	●		
C ₆ H ₁₄ N ₂ O ₂	146.2	C ₆ H ₁₂ N ₂ O	128.2	Basic (+ve)			●
C ₅ H ₁₁ NO ₂ S	149.2	C ₅ H ₉ NOS	131.2	Neutral	●		
C ₉ H ₁₁ NO ₂	165.2	C ₉ H ₉ NO	147.2	Neutral	●		
C ₅ H ₉ NO ₂	115.1	C ₅ H ₇ NO	97.1	Neutral	●		
C ₃ H ₇ NO ₃	105.1	C ₃ H ₅ NO ₂	87.1	Neutral		●	
C ₄ H ₉ NO ₃	119.1	C ₄ H ₇ NO ₂	101.1	Neutral		●	
C ₁₁ H ₁₂ N ₂ O ₂	204.2	C ₁₁ H ₁₀ N ₂ O	186.2	Neutral	●		
C ₉ H ₁₁ NO ₃	181.2	C ₉ H ₉ NO ₂	163.2	Neutral		●	
C ₅ H ₁₁ NO ₂	117.1	C ₅ H ₉ NO	99.1	Neutral	●		

Appendix 8

Analysis and characterization

Analytical assays are essential to follow the progress of purification. They are used to assess the effectiveness of the purification in terms of yield, biological activity, recovery and degree of purification. The importance of a reliable assay for the target molecule cannot be overemphasized.

Protein detection and quantification

Detection and quantification of the target protein are needed when optimizing purification protocols. For over-expressed proteins, the high concentration in itself can be used for detection of the target protein fraction in a chromatogram, but in such a case verification of the identity of the protein in the final preparation is needed. Specific detection of tagged proteins can often be accomplished by analyzing the presence of the tag by activity or immunoassay, or simply by the spectral properties of the tag. Specific detection of the target protein can be obtained by functional assays, immunodetection, and mass spectrometry. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is the key method for checking purity of proteins. The target protein band can often be identified using the apparent M_r obtained by including standard molecular weight markers in the analysis. Subsequent verification of protein identity should always be obtained. Optimizing purification protocols may require functional assays to assess the intactness of the target protein. In general:

- The relative yield of tagged protein can often be determined by measuring the absorbance at 280 nm because the purity after a single purification step is high, that is, most of the eluted material may be considered to be the target protein. The extinction coefficient of the target protein will be needed. A good estimation may be obtained by theoretical calculation from the amino acid composition of the protein.
- The yield of protein may also be determined by standard chromogenic methods (e.g., Lowry, BCA™ protein assay, Bradford, etc.).
- Immunoassays (Western blot, ELISA, immunoprecipitation, etc) can be used for quantitation if a suitable standard curve can be produced. In this case, it is not necessary to purify the tagged protein so long as a purified standard is available. Therefore, these techniques may be used for quantitation during protocol development. The immunoassay technique is also particularly suitable for screening large numbers of samples when a simple yes/no answer is required (e.g., when testing fractions from a chromatographic run).

Purity check and protein characterization

Purity

Purity of the target protein is most often estimated by SDS-PAGE, capillary electrophoresis, RPC or mass spectrometry. The absorbance at 280 nm gives a rough but fast and non-destructive estimate of total protein content in crude samples. Lowry or Bradford assays are also frequently used to determine the total protein. The Bradford assay is particularly suited to samples where there is a high lipid content which may interfere with the Lowry assay. Absorbance data can be calibrated to high accuracy for pure proteins.

Obtaining a single band in electrophoresis is indicative of a pure protein, although potentially other proteins may co-migrate. At this stage, additional methods based on orthogonal separation principles, such as chromatography in analytical scale should be used to confirm homogeneity/purity.

Characterization

Characterization of purified components can be divided into physical, chemical and biological/functional characterization.

Physical parameters

Physical parameters important for identification, further experimental work, and documentation include mass, isoelectric point and UV/Vis spectral properties.

Mass is conveniently determined with mass spectrometry (MS) also for proteins, with accuracy better than 0.1%, providing a good fingerprint. Differences in single amino acids or attached monosaccharides are clearly revealed.

Isoelectric point is determined by analytical isoelectric focusing (IEF) with accuracy better than 0.1 pH units and may thus sometimes even reveal variations among non-charged amino acids in the vicinity of charged ones.

The UV/Vis spectrum can sometimes be unique for a specific protein, in particular if light-absorbing cofactors (flavins, heme groups, other metal ions etc) are present. The specific extinction coefficient can also be calibrated, allowing very accurate concentration determination once the protein is free from contaminants.

Chemical characterization

Chemical characterization includes total amino acid composition, complete or partial amino acid sequence, MS fragment pattern (peptide maps) and determination of posttranslational modifications including glycosylation. The total amino acid composition is still carried out in specialized laboratories, while most other data can be obtained by different applications of MS.

Functional characterization

Biological/functional characterization comprises enzymatic specificity and kinetic parameters including the action of inhibitors. Examples of important non-enzymatic properties are binding to signal substances, toxins, lectins, and antibodies. The interaction of a component with detecting antibodies may also be found in this category.

Enzymatic properties are determined by a multitude of specific protocols. Readily reversible protein-ligand interactions are typically determined by electrophoretic or chromatographic methods, whereas slow (strong) protein-ligand interactions are determined by filter binding assays. Protein-protein interactions and many protein-ligand interactions conveniently studied with Biacore™ assays that also allow the study of binding kinetics.

	Sensitivity limit	Quantitative	Living cells	Linear dynamic range
Coomassie blue staining	20 ng	+++	no	7
Negative staining	15 ng	+	no	3
Silver staining	200 pg	++	no	3
Fluorescent staining	400 pg	++++	no	10 ⁴
Fluorescent labeling	a few pg	+++++	no	10 ⁴
<i>Radioactive labelling:</i>				
X-ray film	1 pg	+++	yes	20
Phosphor-imager plates	0.2 pg	++++	yes	10 ⁵
Stable isotope labelling	< 1 pg	++++ (with MS)	yes	?

Appendix 9

Storage of biological samples

The advice given here is of a general nature and cannot be applied to every biological sample. Always consider the properties of the specific sample and its intended use before following any of these recommendations.

General recommendations

-  Add stabilizing agents when necessary. Stabilizing agents are often required for storage of purified proteins.
-  Serum, culture supernatants, and ascites should be kept frozen at -20°C or -70°C, in small aliquots.
-  Avoid repeated freeze/thawing or freeze drying/redissolving that may reduce biological activity.
-  Avoid conditions close to stability limits, in terms of for example pH or salt concentrations, reducing or chelating agents.
-  Keep refrigerated at 4°C in a closed vessel to minimize bacterial growth and protease activity. For prolonged storage at 4°C (more than 24 h), add a preserving agent (e.g., merthiolate 0.01%).
-  Sodium azide can interfere with coupling methods, and some biological assays, and can be a health hazard. It can be removed by using a desalting column (see Chapter 5).

Common storage conditions for purified proteins

-  Store as a precipitate in a high concentration of ammonium sulfate, for example 4.0 M.
-  Freeze in 50% glycerol, especially suitable for enzymes.
-  Avoid using preserving agents if the product is to be used for a biological assay. Preserving agents should not be added if *in vivo* experiments are to be performed. Store samples in small aliquots and keep frozen.
-  Sterile filter to prolong storage time.
-  Add stabilizing agents, for example, glycerol (5% to 20%), serum albumin (10 mg/ml), ligand (concentration is selected based on concentration of active protein) to maintain biological activity. Remember that any additive will reduce the purity of the protein and may need to be removed at a later stage.
-  Avoid repeated freeze/thawing or freeze drying/redissolving that may reduce biological activity.
-  Certain proteins, including some mouse antibodies of the IgG₃ subclass, should not be stored at 4°C as they precipitate at this temperature (cryoproteins). Store at room temperature in the presence of a preserving agent

Appendix 10

Molecular weight estimation and molecular weight distribution analysis

Unlike electrophoretic techniques, gel filtration 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, free from the constraints imposed by the charge state of the molecules. In order to understand and follow the procedures outlined, it is important to have read Chapter 7, Gel filtration in theory.

For molecular weight determination, several theoretical models have been proposed to describe the behavior of solutes during gel filtration. Most models assume that the partition of solute molecules between the particles and surrounding liquid is an entirely steric effect. However, 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 gel filtration can be made by comparing an elution volume parameter, such as 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 versus 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 measured elution volume. Various elution parameters, such as V_e , V_e/V_o , K_d , and K_{av} have been used in the literature for the preparation of calibration curves but the use of K_{av} is recommended since: 1) it is less sensitive to errors which may be introduced as a result of variations in column preparation and column dimensions, 2) it does not require the unreliable determination of the internal volume (V_i) as is required with K_d .

For good estimation of molecular weight, the calibration standards must have the same relationship between molecular weight and molecular size as the substance of interest. Calibration Kits from GE Healthcare provide well-characterized, globular protein standards for protein molecular weight estimation. The Low Molecular Weight (LMW) Gel Filtration Calibration Kit contains five individually lyophilized proteins with molecular weights in the range 6500 to 75 000 and Blue Dextran 2000 (see Table A10.1). The High Molecular Weight (HMW) Gel Filtration Calibration Kit contains five individually lyophilized proteins with molecular weights in the range 43 000 to 669 000 and Blue Dextran 2000 (see Table A10.2). Blue Dextran 2000 determines the void fraction in the column.

These well-defined protein standards show excellent behavior in gel filtration and enable simple, reliable calibration of gel filtration columns.

Table A10.1. Characteristics of Gel Filtration Calibration Kit LMW

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 A10.2. Characteristics of Gel Filtration Calibration HMW

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 calibration results from chromatographic runs and calculated calibration curves using prepacked Superdex columns are shown in Figures A10.1 and A10.2.

The method used for Figures A10.1 and A10.2:

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.: 100 µl

Buffer: 50 mM phosphate buffer, 150 mM NaCl, pH 7.2

Flow rate: Figure A10.1 0.5 ml/min
Figure A10.2 0.6 ml/min

Detection: 280 nm

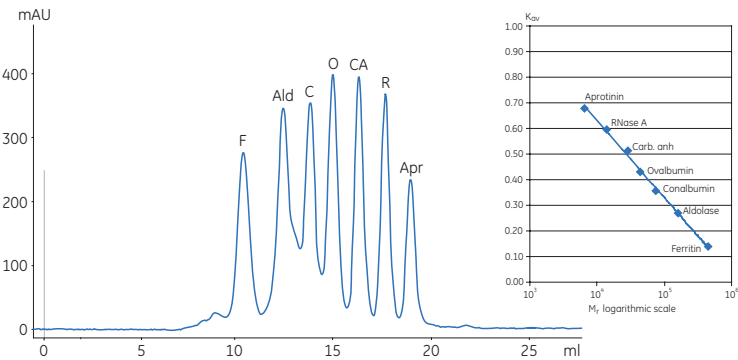


Fig A10.1. Chromatographic separation and calibration curve for the standard proteins on Superdex 200 10/300 GL column.

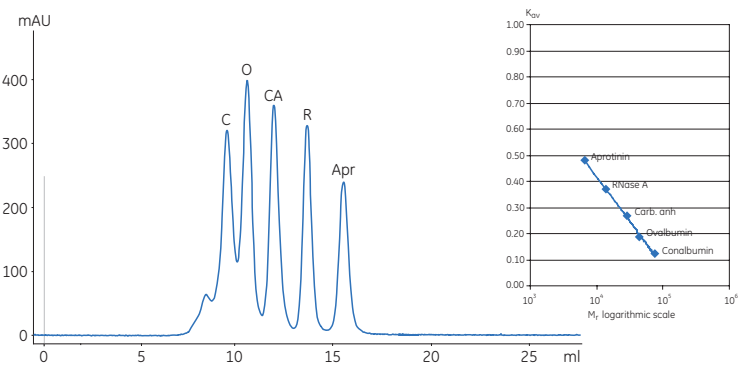


Fig A10.2. Chromatographic separation and calibration curve for the standard proteins on Superdex 75 10/300 GL column.

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



Use a medium 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 (see gel filtration media fractionation guide Chapter 1 page 16).



Use a prepacked column whenever possible. Homemade columns must be packed very carefully (see Appendix 1).



Use freshly prepared calibration standards, selected so that the expected molecular weight values are covered by the entire calibration range. Always filter Blue Dextran before use. Apply samples in a volume less than 2% of the total column volume.



Use the same buffer for the separation of calibrants and sample, for example 50 mM sodium phosphate, 0.15 M NaCl at pH 7. Use the recommended flow rate for the prepacked column or medium selected.



If the molecular weight is unknown, use a medium with a wide fractionation range such as Sephacryl HR. This is also recommended for molecular weight distribution analysis and for polymeric materials such as dextrans and polyethylene glycols.



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

Deviation from a K_{av} :log M_r calibration curve may occur if the molecule of interest does not have the same molecular shape as the standards.

Performing a molecular weight determination

1. If using a self-packed column, prepare a fresh, filtered solution of Blue Dextran 2000 (1.0 mg/ml) in the running buffer. Apply Blue Dextran to the column, using a volume <2% of the total column volume (V_t) to determine the void volume (V_o), and to check the column packing.
2. Dissolve the selected calibration references in the running buffer (at concentrations recommended by the manufacturer). Allow a few minutes for dissolution, stirring gently. Do not heat or mix vigorously. If necessary, filter the calibration solution.
3. Apply the calibration solution to the column, in a volume <2% of the total column volume (V_t).
4. Determine the elution volumes (V_e) for the standards by measuring the volume of the eluent from the point of application to the centre of the elution peak.
5. Calculate the K_{av} values for the standards and prepare a calibration curve of K_{av} versus the logarithm of their molecular weights, as follows:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where V_e = elution volume for the protein

V_o = column void volume = elution volume for Blue Dextran 2000

V_t = total bed volume

Use a computer to plot the K_{av} value for each protein standard against the corresponding logarithmic molecular weight and use Excel to calculate the regression line.

6. Apply the sample in a volume $<2\%$ of the total column volume (V_t) and determine the elution volume (V_e) of the molecule of interest.
7. Calculate the corresponding K_{av} for the component of interest and determine its molecular weight from the calibration curve.



A calibrated column can be used for extended periods as long as the column is kept in good condition and not allowed to dry out, eliminating the need to set up a separate experiment for each determination.

Related literature

Purification	Code no.
Antibody Purification Handbook	18-1037-46
Strategies for Protein Purification	28-9833-31
Recombinant Protein Handbook, Principles and Methods	18-1142-75
Purifying Challenging Proteins, Principles and Methods	28-9095-31
Affinity Chromatography Handbook: Principles and Methods	18-1022-29
Ion Exchange Chromatography Handbook: Principles and Methods	18-1114-21
Hydrophobic Interaction and Reversed Phase Chromatography Handbook, Principles and Methods	11-0012-69
Sample preparation for analysis of proteins, peptides and carbohydrates, Selection guide	18-1128-62
Gel Filtration columns and media, Selection guide and product profile	18-1124-19
Solutions for antibody purification, Selection Guide	28-9351-97
Ion Exchange columns and media, Selection Guide	18-1127-31
Affinity chromatography columns and media, Selection Guide	18-1121-86
HiTrap-convenient protein purification, Column Guide	18-1129-81
ÄKTA Protein purification by design	28-4026-97
Prepacked chromatography columns for ÄKTA design systems, Selection guide	28-9317-78
Column Packing - The Movie, CD	18-1165-33
Pure simplicity for tagged proteins, Brochure	28-9353-64
Years of experience in every column, Brochure	28-9090-94
Protein Analysis	
www.gelifesciences.com/proteinanalysis_techsupport	

Ordering information

High-resolution chromatography

Column	Quantity	Code no.
High-resolution fractionation		
Superdex		
Superdex Peptide PC 3.2/30	1 × 2.4 ml column	17-1458-01
Superdex 75 PC 3.2/30	1 × 2.4 ml column	17-0771-01
Superdex 200 PC 3.2/30	1 × 2.4 ml column	17-1089-01
Superdex Peptide 10/300 GL	1 × 24 ml column	17-5176-01
Superdex 75 10/300 GL	1 × 24 ml column	17-5174-01
Superdex 200 10/300 GL	1 × 24 ml column	17-5175-01
Superdex 200 5/150 GL	1 × 3 ml column	28-9065-61
Superdex 75 5/150 GL	1 × 3 ml column	28-9205-04
HiLoad 16/60 Superdex 30 prep grade	1 × 120 ml column	17-1139-01
HiLoad 26/60 Superdex 30 prep grade	1 × 320 ml column	17-1140-01
HiLoad 16/60 Superdex 75 prep grade	1 × 120 ml column	17-1068-01
HiLoad 26/60 Superdex 75 prep grade	1 × 320 ml column	17-1070-01
HiLoad 16/60 Superdex 200 prep grade	1 × 120 ml column	17-1069-01
HiLoad 26/60 Superdex 200 prep grade	1 × 320 ml column	17-1071-01
Superdex 30 prep grade	150 ml	17-0905-01
Superdex 75 prep grade	150 ml	17-1044-01
Superdex 200 prep grade	150 ml	17-1043-01
Superose		
Superose 6 PC 3.2/30	1 × 2.4 ml column	17-0673-01
Superose 12 PC 3.2/30	1 × 2.4 ml column	17-0674-01
Superose 6 10/300 GL	1 × 24 ml column	17-5172-01
Superose 12 10/300 GL	1 × 24 ml column	17-5173-01
Superose 6 prep grade	125 ml	17-0489-01
Superose 12 prep grade	125 ml	17-0536-01
Sephacryl		
HiPrep 16/60 Sephacryl S-100 HR	1 × 120 ml column	17-1165-01
HiPrep 26/60 Sephacryl S-100 HR	1 × 320 ml column	17-1194-01
HiPrep 16/60 Sephacryl S-200 HR	1 × 120 ml column	17-1166-01
HiPrep 26/60 Sephacryl S-200 HR	1 × 320 ml column	17-1195-01
HiPrep 16/60 Sephacryl S-300 HR	1 × 120 ml column	17-1167-01
HiPrep 26/60 Sephacryl S-300 HR	1 × 320 ml column	17-1196-01
HiPrep 16/60 Sephacryl S-400 HR	1 × 120 ml column	28-9356-04
HiPrep 26/60 Sephacryl S-400 HR	1 × 320 ml column	28-9356-05
HiPrep 16/60 Sephacryl S-500 HR	1 × 120 ml column	28-9356-06
HiPrep 26/60 Sephacryl S-500 HR	1 × 320 ml column	28-9356-07
Sephacryl S-100 HR	150 ml	17-0612-10
Sephacryl S-100 HR	750 ml	17-0612-01
Sephacryl S-200 HR	150 ml	17-0584-10

Column	Quantity	Code no.
Sephacryl S-200 HR	750 ml	17-0584-01
Sephacryl S-300 HR	150 ml	17-0599-10
Sephacryl S-300 HR	750 ml	17-0599-01
Sephacryl S-400 HR	150 ml	17-0609-10
Sephacryl S-400 HR	750 ml	17-0609-01
Sephacryl S-500 HR	150 ml	17-0613-10
Sephacryl S-500 HR	750 ml	17-0613-01
Sephacryl S-1000 SF	750 ml	17-0476-01

Desalting and group separations

HiTrap Desalting	5 × 5 ml columns	17-1408-01
HiPrep 26/10 Desalting	1 × 53 ml column	17-5087-01
HiPrep 26/10 Desalting	4 × 53 ml column	17-5087-02
PD-10 Desalting Column	30 gravity-fed columns	17-0851-01
Empty PD-10 Desalting Column	50 gravity-fed empty columns	17-0435-01
PD Spin Trap G-25	50 columns	28-9180-04
PD MultiTrap G-25	4 × 96-well plates	28-9180-06
PD MiniTrap G-25	50 columns	28-9180-07
PD MidiTrap G-25	50 columns	28-9180-08
PD MiniTrap G-10	50 columns	28-9180-10
MiniSpin Adapter	10	28-9232-43
MidiSpin Adapter	10	28-9232-44
PD-10 Spin Adapter	10	28-9232-45
Collection plate 500 µl V-bottom	5 × 96-well plates	28-4039-43
LabMate PD-10 Buffer Reservoir	10	18-3216-03
Sephadex G-10	100 g	17-0010-01
Sephadex G-10	500 g	17-0010-02
Sephadex G-25 Coarse	100 g	17-0034-01
Sephadex G-25 Coarse	500 g	17-0034-02
Sephadex G-25 Fine	100 g	17-0032-01
Sephadex G-25 Fine	500 g	17-0032-02
Sephadex G-25 Medium	100 g	17-0033-01
Sephadex G-25 Medium	500 g	17-0033-02
Sephadex G-25 Superfine	100 g	17-0031-01
Sephadex G-50 Fine	100 g	17-0042-01
Sephadex G-50 Fine	500 g	17-0042-02

Separation in organic solvents

Sephadex LH-20	25 g	17-0090-10
Sephadex LH-20	100 g	17-0090-01
Sephadex LH-20	500 g	17-0090-02

Calibration Kits

Gel Filtration Calibration Kit LMW	1	28-4038-41
Gel Filtration Calibration Kit HMW	1	28-4038-42

Column	Quantity	Code no.
Empty columns		
XK columns		
XK 16/20 column	1	18-8773-01
XK 16/40 column	1	18-8774-01
XK 16/70 column	1	18-8775-01
XK 16/100 column	1	18-8776-01
XK 26/20 column	1	18-1000-72
XK 26/40 column	1	18-8768-01
XK 26/70 column	1	18-8769-01
XK 26/100 column	1	18-8770-01
XK 50/20 column	1	18-1000-71
XK 50/30 column	1	18-8751-01
XK 50/60 column	1	18-8752-01
XK 50/100 column	1	18-8753-01
Tricorn columns		
Tricorn 10/100	1	28-4065-15
Tricorn 10/150	1	28-4064-16
Tricorn 10/200	1	28-4064-17
Tricorn 10/300	1	28-4064-18
Tricorn 10/600	1	28-4064-19
HiScale columns		
HiScale 16/20	1	28-9644-41
HiScale 16/40	1	28-9644-24
HiScale 26/20	1	28-9645-14
HiScale 26/40	1	28-9645-13
HiScale 50/20	1	28-9644-45
HiScale 50/40	1	28-9644-44
Solvent resistant columns		
SR 25/45 column	1	19-0879-01
SR 25/100 column	1	19-0880-01
Accessories and spare parts		
Packing Connector XK 16	1	18-1153-44
Packing Connector XK 26	1	18-1153-45
Packing equipment 10/100 (Tricorn)	1	18-1153-25
Packing Connector 10-10	1	18-1153-23

More details and products can be found on www.gelifesciences.com/protein-purification

For local office contact information,
please visit www.gelifesciences.com/contact

www.gelifesciences.com/protein-purification

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden



imagination at work

GE, imagination at work, and GE monogram are trademarks of General Electric Company.

ÅKTA, ÅKTAexplorer, ÅKTAmicro, ÅKTAprime, ÅKTApurifier, ÅKTAexpress, Biacore, BioProcess, HiLoad, HiPrep, HiScale, HiTrap, MabSelect, MidiTrap, MiniTrap, MultiTrap, Sephacryl, Sephadex, Sepharose, SpinTrap, Superdex, Superose, and Tricorn are trademarks of GE Healthcare companies.

US patent numbers 5,284,933 and 5,310,663, and equivalent patents and patent applications in other countries (assignee: Hoffman La Roche, Inc) relate to the purification and preparation of fusion proteins and affinity peptides comprising at least two adjacent histidine residues (commonly known as the histidine-tag technology).

Any customer that wishes to use Chelating Sepharose Fast Flow, Ni Sepharose 6 Fast Flow or IMAC Sepharose 6 Fast Flow for non-research/commercial applications under these patents is requested to contact Hoffman-La Roche AG, Corporate licensing, attention Dr Andreas Maurer, CH-4070 Basel, Switzerland, telephone +41 61 687 2548, fax +41 61 687 2113, for the purpose of obtaining a license.

All third party trademarks are the property of their respective owners.

© 2010 General Electric Company—All rights reserved.
First published Dec. 2000.

All goods and services are sold subject to the terms and conditions of sale of the company within GE Healthcare which supplies them. A copy of these terms and conditions is available on request. Contact your local GE Healthcare representative for the most current information.

GE Healthcare UK Limited Amersham Place
Little Chalfont
Buckinghamshire, HP7 9NA
UK

GE Healthcare Europe, GmbH
Munzinger Strasse 5
D-79111 Freiburg
Germany

GE Healthcare Bio-Sciences Corp.
800 Centennial Avenue, P.O. Box 1327
Piscataway, NJ 08855-1327
USA

GE Healthcare Bio-Sciences KK
Sanken Bldg., 3-25-1, Hyakunincho
Shinjuku-ku, Tokyo 169-0073
Japan