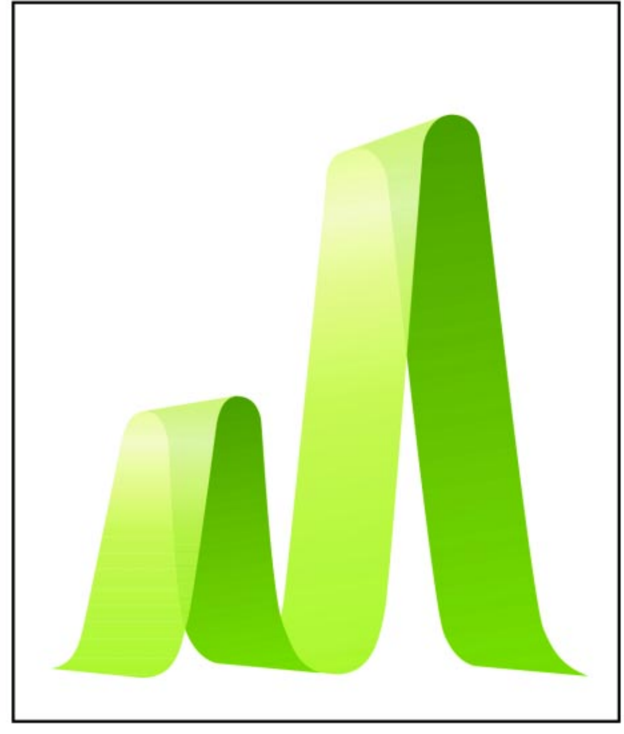
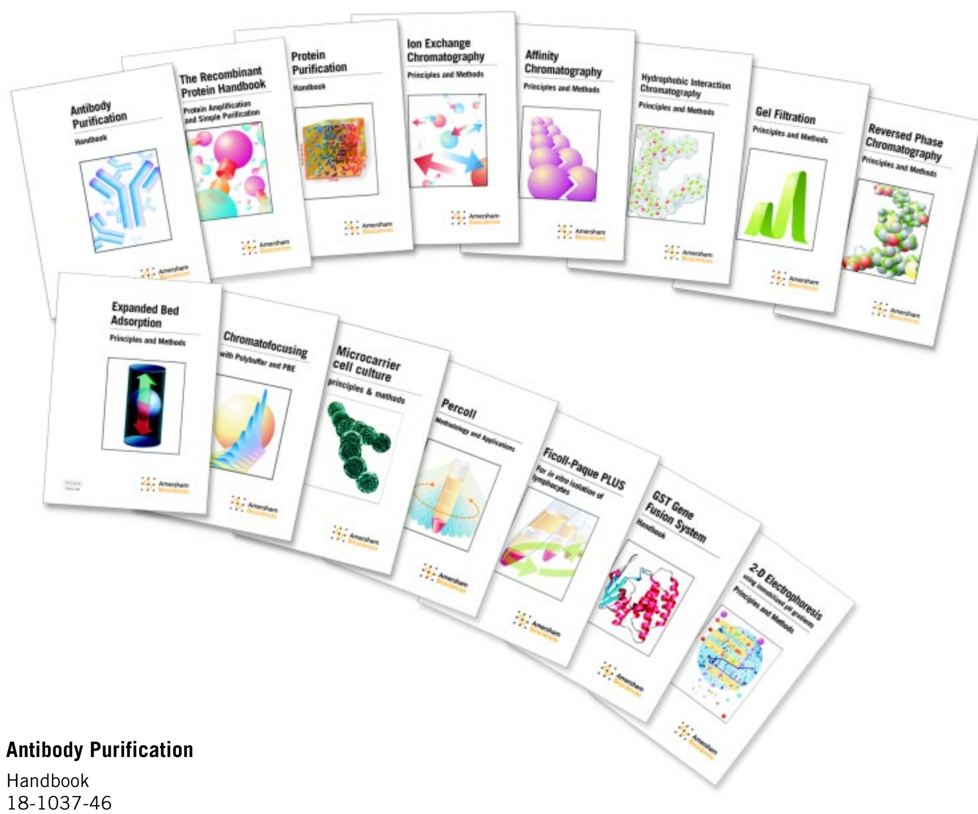
Gel Filtration

Principles and Methods



18-1022-18
Edition AI

Handbooks
from Amersham Biosciences



Antibody Purification

Handbook
18-1037-46

The Recombinant Protein Handbook

Protein Amplification and Simple Purification
18-1142-75

Protein Purification

Handbook
18-1132-29

Ion Exchange Chromatography

Principles and Methods
18-1114-21

Affinity Chromatography

Principles and Methods
18-1022-29

Hydrophobic Interaction Chromatography

Principles and Methods
18-1020-90

Gel Filtration

Principles and Methods
18-1022-18

Reversed Phase Chromatography

Principles and Methods
18-1134-16

Expanded Bed Adsorption

Principles and Methods
18-1124-26

Chromatofocusing

with Polybuffer and PBE
18-1009-07

Microcarrier cell culture

Principles and Methods
18-1140-62

Percoll

Methodology and Applications
18-1115-69

Ficoll-Paque Plus

For *in vitro* isolation of lymphocytes
18-1152-69

GST Gene Fusion System

Handbook
18-1157-58

2-D Electrophoresis
using immobilized pH gradients

Principles and Methods
80-6429-60

Gel filtration

Principles and Methods

Contents

Introduction 5
Symbols 6
Common abbreviations 7

Chapter 1
Gel filtration in practice 9
Introduction 9
Separation by gel filtration 9
Resolution in gel filtration 13
Media selection 16
Sample preparation 20
Buffer composition and preparation 21
Denaturing (chaotropic) agents and detergents 22
Column and media preparation 23
Sample application 23
Elution and flow rates 24
Method development for high resolution fractionation 26
Care of gel filtration media 27
Equipment selection 27
Scaling up 27
BioProcess Media for large-scale production 29
Troubleshooting 29

Chapter 2
Gel filtration media 35
Components of gel filtration media 35
Superdex: first choice for high resolution, short run times and high recovery 36
Separation options 38
Separation examples 39
Performing a separation 42
Cleaning 43
Media characteristics 44
Chemical stability 44
Storage 44
Sephacryl: fast, high recovery separations at laboratory and industrial scale 45
Separation options 48
Separation examples 48
Performing a separation 49
Cleaning 50
Media characteristics 51
Chemical stability 51
Storage 51
Superose: broad fractionation range, but not suitable for industrial scale separations 52

Separation options 53
Separation examples 54
Performing a separation 54
Cleaning 55
Media characteristics 56
Chemical stability 56
Storage 56

Sephadex: rapid group separation of high and low molecular weight substances, such as desalting, buffer exchange and sample clean up 57

Separation options 59
Separation examples 61
Performing a separation 61
Scale up and processing large sample volumes 66
Media characteristics 68
Column Packing 68
Cleaning 68
Chemical stability 68
Storage 69

Chapter 3
Gel filtration in theory 71

Defining the process 71
Selectivity curves and media selection 74
Resolution 75

Chapter 4
Molecular weight determination and molecular weight distribution analysis 79

Chapter 5
Sephadex LH-20 81

Separation options 82
Separation examples 82
Packing a column 83
Performing a separation 84
Cleaning 84
Medium characteristics 84
Chemical stability 85
Storage 85
Transferring Sephadex LH-20 between organic solvents 85

Chapter 6
Gel filtration in a Purification Strategy (CIPP) 87

Applying CIPP 87
Selection and combination of purification techniques 88
Gel filtration as a polishing step 91

Appendix 1
Column packing and preparation 93
Columns for packing gel filtration media 93
Checking column efficiency 95
Column packing for high resolution fractionation using Superdex prep grade and Sephacryl High Resolution 95
Column packing for group separations using Sephadex 98

Appendix 2
Sephadex and Darcy's law 102

Appendix 3
Sample preparation 103
Sample stability 103
Sample clarification 104
Specific sample preparation steps 105
Removal of lipoproteins 108
Removal of phenol red 108
Removal of low molecular weight contaminants 108

Appendix 4
Selection of purification equipment 109

Appendix 5
Converting from linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa 110

Appendix 6
Conversion data: proteins, column pressures 111

Appendix 7
Table of amino acids 112

Appendix 8
Analytical assays during purification 114

Appendix 9
Storage of biological samples 116

Additional reading and reference material 117

Ordering information 118

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
Charge	Ion exchange chromatography (IEX)
Hydrophobicity	Hydrophobic interaction chromatography (HIC) Reversed phase chromatography (RPC)
Biorecognition (ligand specificity)	Affinity chromatography (AC)

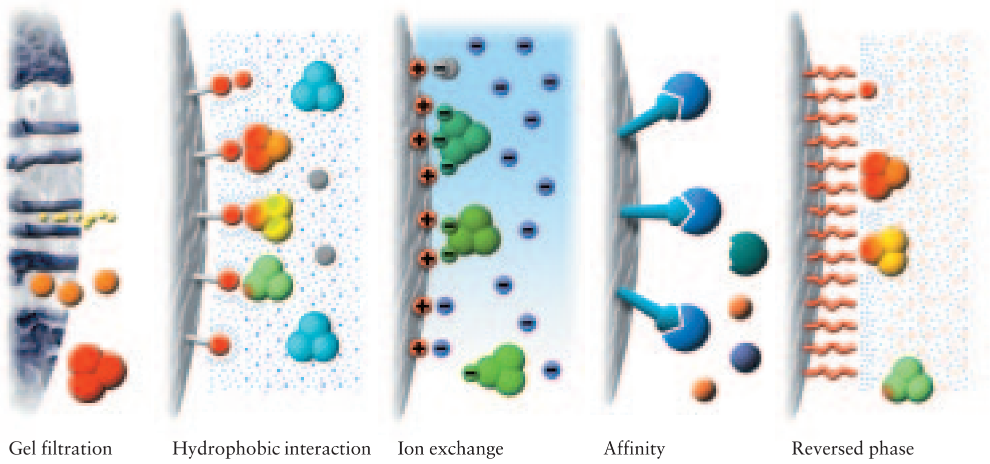


Fig. 1. Separation principles in chromatography purification.

For more than forty years since the introduction of Sephadex™, gel filtration has played a key role in the purification of enzymes, polysaccharides, nucleic acids, proteins 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 to desalt and exchange buffers.
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, to determine molecular weight or to perform a molecular weight distribution analysis.

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

Gel filtration is a robust technique that is well suited to handling biomolecules that are 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.

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.

The biocompatibility, stability and utility of gel filtration media from Amersham Biosciences have made these products the standard choice in practically every laboratory using the technique. A wide variety of prepacked columns and ready to use media is available.

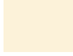
The illustration on the inside cover shows the range of handbooks from Amersham Biosciences that have been produced to ensure that chromatography and other separation techniques are used easily and effectively at any scale, in any laboratory and for any application.

Symbols

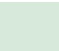
this symbol indicates general advice which can improve procedures or provide recommendations for action under specific situations.

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

this symbol highlights troubleshooting advice to help analyse and resolve difficulties that may occur.



chemicals, buffers and equipment.



experimental protocol.

Common abbreviations

In chromatography

GF: gel filtration (sometimes referred to as SEC: size exclusion chromatography)

IEX: ion exchange chromatography (also seen as IEC)

AC: affinity chromatography

RPC: reverse phase chromatography

HIC: hydrophobic interaction chromatography

CIPP: Capture, Intermediate Purification and Polishing

MPa: megapascals

psi: pounds per square inch

SDS: sodium dodecyl sulphate

CIP: cleaning in place

A_{280nm}, A_{214nm}: UV absorbance at specified wavelength

M_r: relative molecular weight

N: column efficiency expressed as theoretical plates per meter

V_e: elution volume is measured from the chromatogram and relates to the molecular size of the molecule.

V_o: void volume is the elution volume of molecules that are excluded from the gel filtration medium because they are larger than the largest pores in the matrix and pass straight through the packed bed

V_t: total column volume is equivalent to the volume of the packed bed (also referred to as CV)

R_s: resolution, the degree of separation between peaks

K_{av} and $logM_r$: partition coefficient and log molecular weight, terms used when defining the selectivity of a gel filtration medium

In product names

HMW: high molecular weight

LMW: low molecular weight

HR: high resolution

pg: prep grade

PC: precision column

SR: solvent resistant

Chapter 1
Gel filtration in practice

Introduction

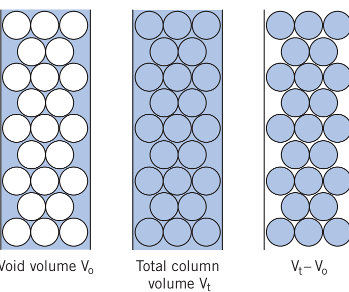
Gel filtration separates molecules according to differences in size as they pass through a gel filtration medium packed in a column. Unlike ion exchange or affinity chromatography, molecules do not bind to the chromatography medium so buffer composition does not directly affect resolution (the degree 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 to select the correct medium, so selection guides for the most up-to-date gel filtration media and prepacked columns are included. Other application examples and product-specific information are found in Chapter 2.

Separation 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* in the form of spherical *particles* that have been chosen for their 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 in between the particles. The liquid inside the pores is sometimes referred to as the *stationary phase* and this liquid is in equilibrium with the liquid outside the particles, referred to as the *mobile phase*. It should be noted that samples are eluted *isocratically*, i.e. 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 facilitate the removal of any molecules that may have been retained on the column and to prepare the column for a new run. Figure 2 shows the most common terms used to describe the separation and Figure 3 illustrates the separation process of gel filtration.



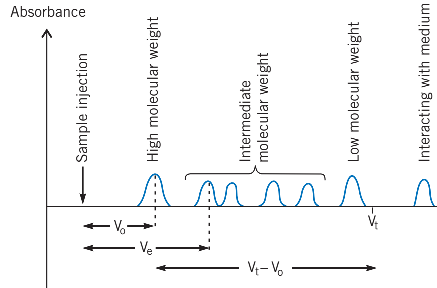


Fig. 2. Common terms in gel filtration.

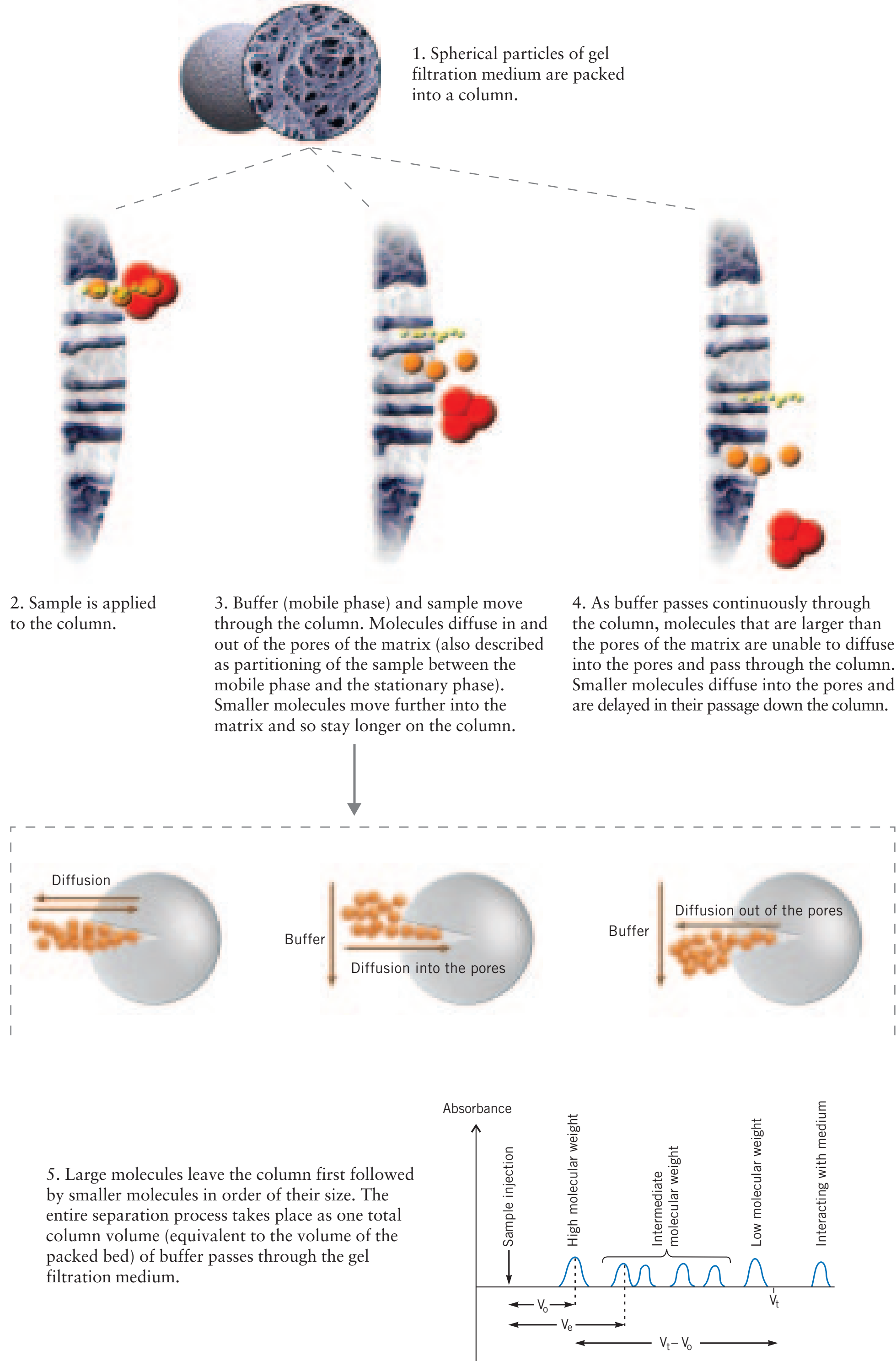


Fig. 3. Process of gel filtration.

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 (desalting) or free labels are easily separated. 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. For further details refer to Chapter 2, page 57 and the *Protein Purification Handbook* from Amersham Biosciences.

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 4 shows the elution profile (chromatogram) of a typical group separation. Large molecules are eluted in or just after the *void volume*, V_o 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 A_{280nm}, and the salts are detected by monitoring the conductivity of the buffer.

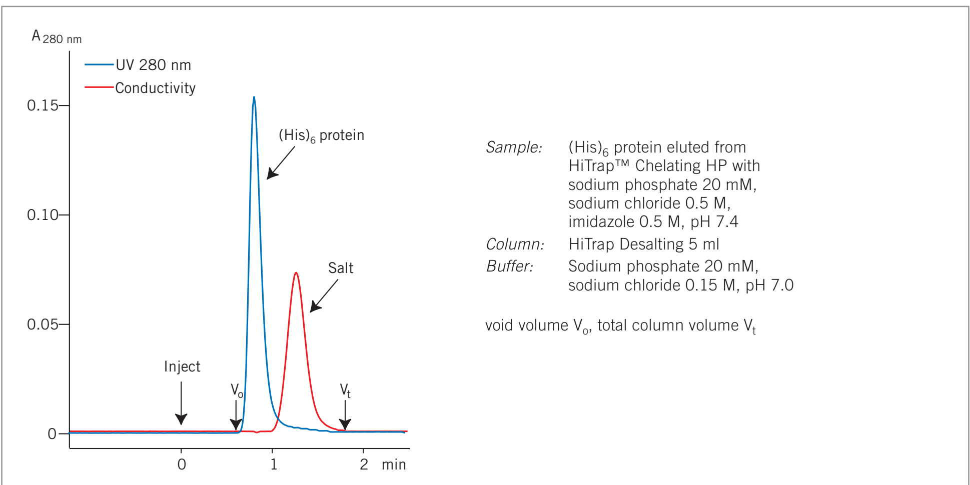


Fig. 4. Typical chromatogram of a group separation. The UV (protein) and conductivity (salt) traces enable pooling of the desalted fractions and facilitate optimization of the separation.

Refer to Chapter 2, page 57 for detailed information on group separation of high and low molecular weight substances, i.e. desalting, buffer exchange and sample clean up using Sephadex.

Refer to Chapter 3 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, to determine molecular weight, or to analyze the molecular weight distribution in the sample (refer to Chapter 4 for details of molecular weight determination and distribution analysis). 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 (in order to eliminate proteins of similar size that are not of interest).

High resolution fractionation by gel filtration is well suited for the final polishing step in a purification scheme. Monomers can be separated from aggregates (difficult to achieve by any other technique) and samples can be transferred to a suitable buffer for assay or storage. Gel filtration can be used directly after any of the chromatography techniques such as ion exchange, chromatofocusing, hydrophobic interaction or affinity since the components from any elution buffer will not affect the final separation. For further details on using gel filtration in a purification strategy, refer to Chapter 6 and the *Protein Purification Handbook* from Amersham Biosciences.

Figure 5 shows the theoretical elution profile (chromatogram) of a high resolution fractionation. Molecules that do not enter the matrix are eluted in the *void volume*, V_o 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 (packed bed). 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.

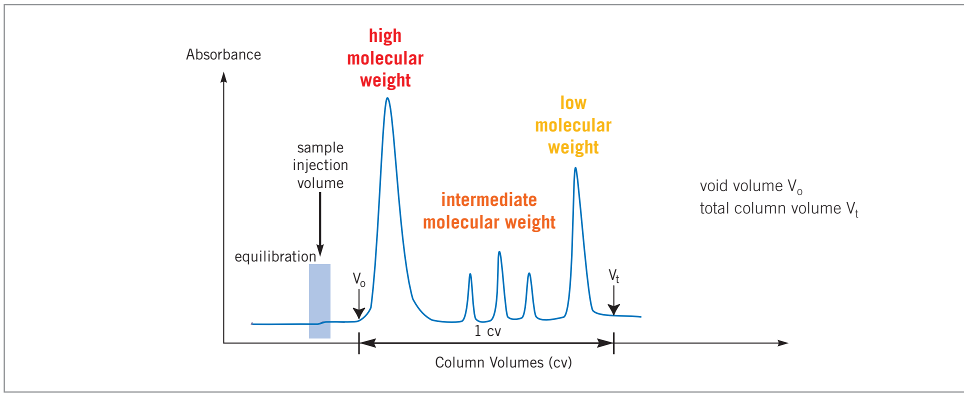


Fig. 5. Theoretical chromatogram of a high resolution fractionation (UV absorbance).

Resolution in gel filtration

Many factors influence the final resolution (the degree of separation between peaks of a gel filtration separation): sample volume, the ratio of sample volume to column volume, column dimensions, particle size, particle size distribution, packing density, pore size of the particles, flow rate, and viscosity of the sample and buffer. The molecular weight range over which a gel filtration medium can separate molecules is referred to as the selectivity of the medium (see selection guide for gel giltration media on page 18). 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 6. The success of gel filtration depends primarily on choosing conditions that give sufficient selectivity and counteract peak broadening effects during the separation.

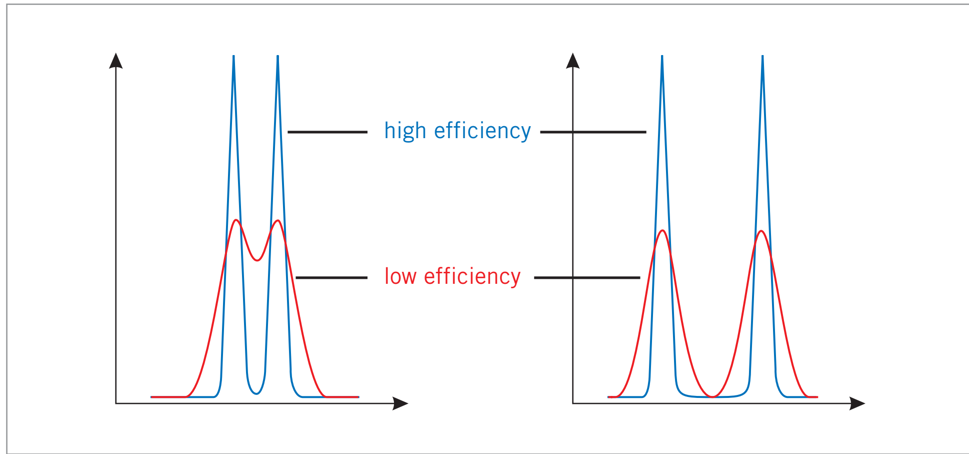


Fig. 6. Dependence of resolution on selectivity and the counteraction of peak broadening.

After selecting a gel filtration medium with the correct selectivity, sample volume and column dimensions become two of the most critical parameters that will affect the resolution of the separation.

Sample volume and column dimensions

Sample volumes are expressed as a percentage of the total column volume (packed bed). Using smaller sample volumes helps to avoid overlap if closely spaced peaks are eluted. Figure 7 illustrates how sample volume can influence a high resolution fractionation.

1) 25 µl,1.0 ml/min (76 cm/h)

2) 250 µl,1.0 ml/min (76 cm/h)

3) 1 000 µl,1.0 ml/min (76 cm/h)

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

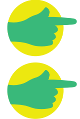
Sample concentration: 15.2 mg/ml

Sample volumes:
1) 25 µl (0.1% × V_t)
2) 250 µl (1% × V_t)
3) 1000 µl (4.2% × V_t)

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

Flow: 1.0 ml/min (76.4 cm/h)

Fig. 7. Influence of sample volume on resolution.



For group separations sample volumes up to 30% of the total column volume can be applied.

For high resolution fractionation a sample volume from 0.5–4% 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.

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

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

Sample dilution is inevitable because diffusion occurs as sample passes through the column. In order to minimize sample dilution use a maximum sample volume that gives the resolution required between the peaks of interest.

The *ratio of sample volume to column volume* influences resolution, as shown in Figures 8a and 8b, where higher sample volume to column volume ratios give lower resolution. Column volumes are normally selected according to the sample volumes to be processed. However, since larger sample volumes may require significantly larger column volumes, there may be occasions when it is more beneficial to repeat the separation several times on a smaller column and pool the fractions of interest or to concentrate the sample (see Appendix 3 on sample preparation).

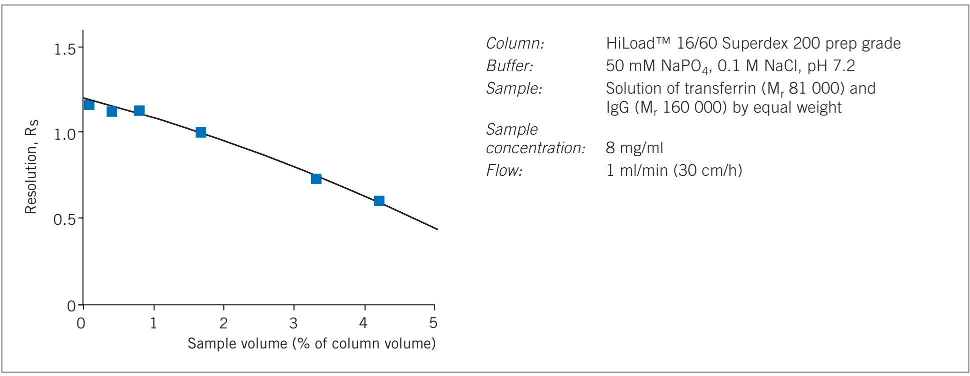


Fig. 8a. Influence of sample volume on the resolution of transferrin and IgG on prepacked HiLoad 16/60 Superdex 200 prep grade.

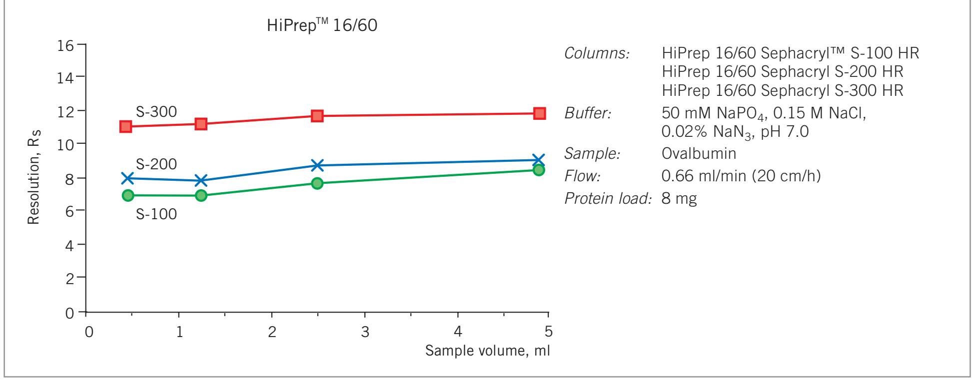


Fig. 8b. Influence of sample volume on the resolution of ovalbumin and IgG on different prepacked columns of HiPrep 16/60 Sephacryl High Resolution.

The *height of the packed bed* affects both resolution and the time taken for elution. The resolution in gel filtration increases as 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 fractionation long columns will give the best results and a bed height between 30–60 cm should be satisfactory. Sufficient bed height together with a low flow rate allows time for all 'intermediate' molecules to diffuse in and out of the matrix pores and give sufficient resolution.

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

Refer to Chapter 3 for detailed information on the theory of gel filtration.

Media selection

Chromatography media for gel filtration are made from porous matrices chosen for their inertness and chemical and physical stability. The size of the pores within a particle and the particle size distribution are carefully controlled to produce a variety of media with different selectivities. Today's gel filtration media cover a molecular weight range from 100 to 80 000 000, 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 on their selectivity, as shown for Superdex in Figure 9. The curve has been obtained by plotting a partition coefficient K_{av} against the log of the molecular weight for a set of standard proteins (see Chapter 3 Gel filtration in theory for calculation of K_{av}).

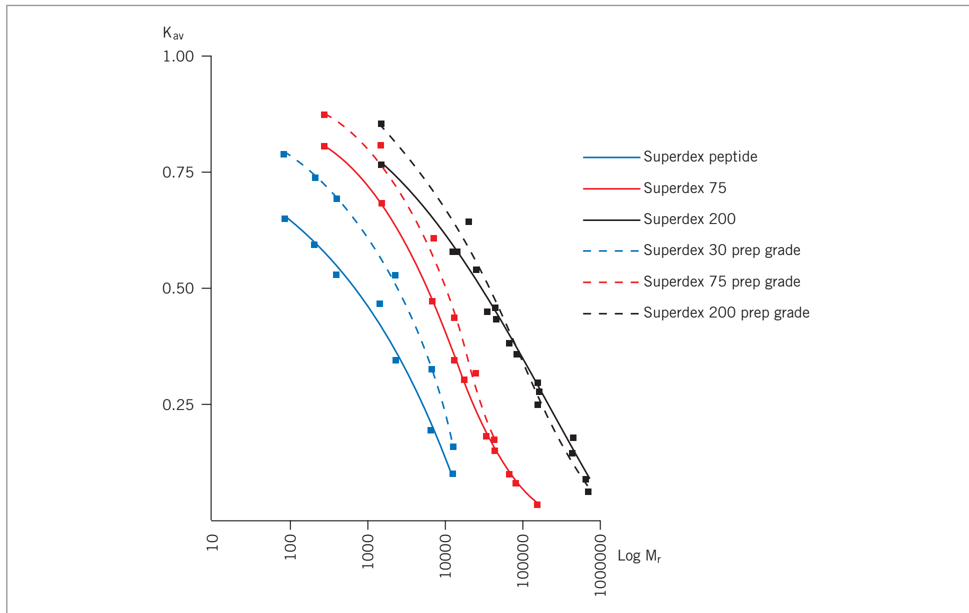


Fig. 9. Selectivity curves for Superdex.

Selectivity curves are usually quite linear over the range K_{av} = 0.1 to K_{av} = 0.7 and it is this part of the curve that is used to determine the *fractionation range* of a gel filtration medium (Figure 10).

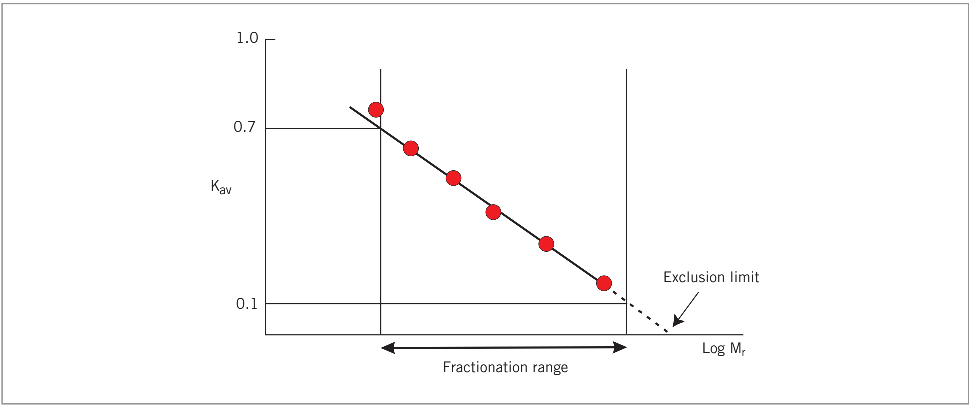


Fig. 10. Defining fractionation range and exclusion limit from a selectivity curve.

The fractionation range defines the range of molecular weights that have partial access to the pores of the matrix, i.e. 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 an appropriate 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 11 on the next page gives a step by step guide to media selection.

Fig. 11. Gel filtration media selection guide.

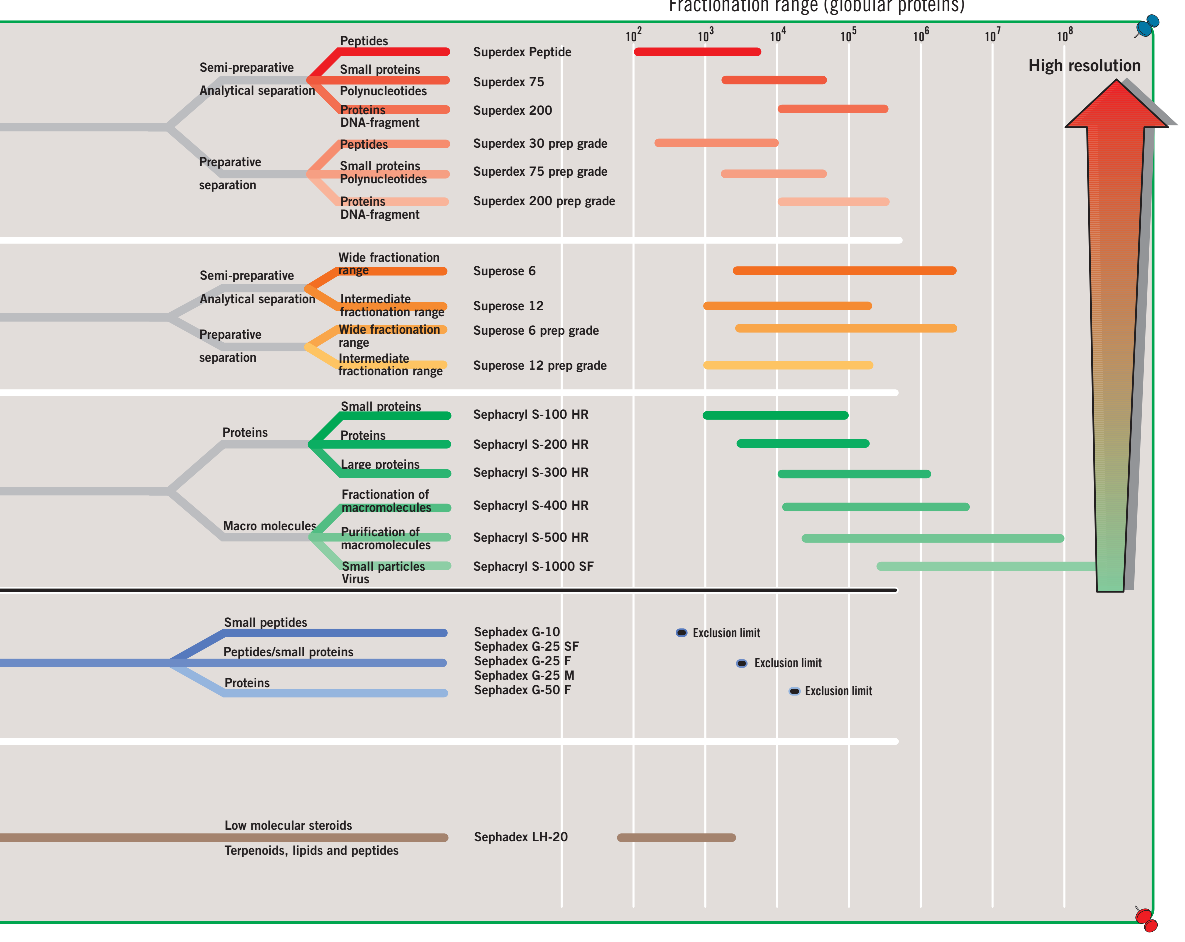
Superdex is the first choice for high resolution, short run times and high recovery.

Sephacryl is suitable for fast, high recovery separations at laboratory and industrial scale.

Superose offers a broad fractionation range, but is not suitable for large scale or industrial scale separations.

After deciding upon Superdex, Sephacryl or Superose, select the medium with the fractionation range that covers the molecular weight values of interest in your sample. In cases where two media have a similar fractionation range: select the medium with the steepest selectivity curve for best resolution of all components in the sample. When you are interested in a specific component, select the medium where the log molecular weight of the target component falls in the middle of the selectivity curve.

Sephadex is ideal 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.



- Sephadex G-25 is recommended for the majority of group separations involving globular proteins. This medium is excellent for removing salt and other small contaminants away from molecules that are greater than M_r 5 000.
- 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 free label.

For group separations select gel filtration media so that 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.

Sample preparation

Correct sample preparation is extremely important for gel filtration. 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 packed chromatography medium.

Samples must be clear and free from particulate matter, particularly 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 12. Hence high resolution can be maintained despite high sample concentration and, with the appropriate medium, high flow rates.

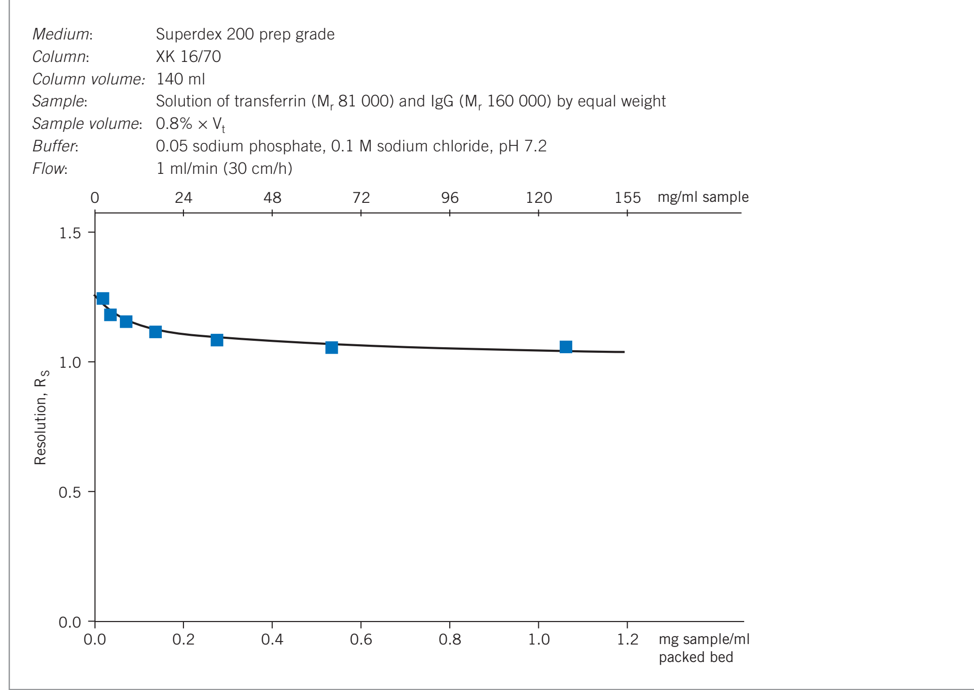


Fig. 12. Influence of sample concentration on the resolution of transferrin and IgG on Superdex 200 prep grade.

However, the solubility or the viscosity of the sample may 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 haemoglobin and NaCl at different sample viscosities in Figure 13.

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

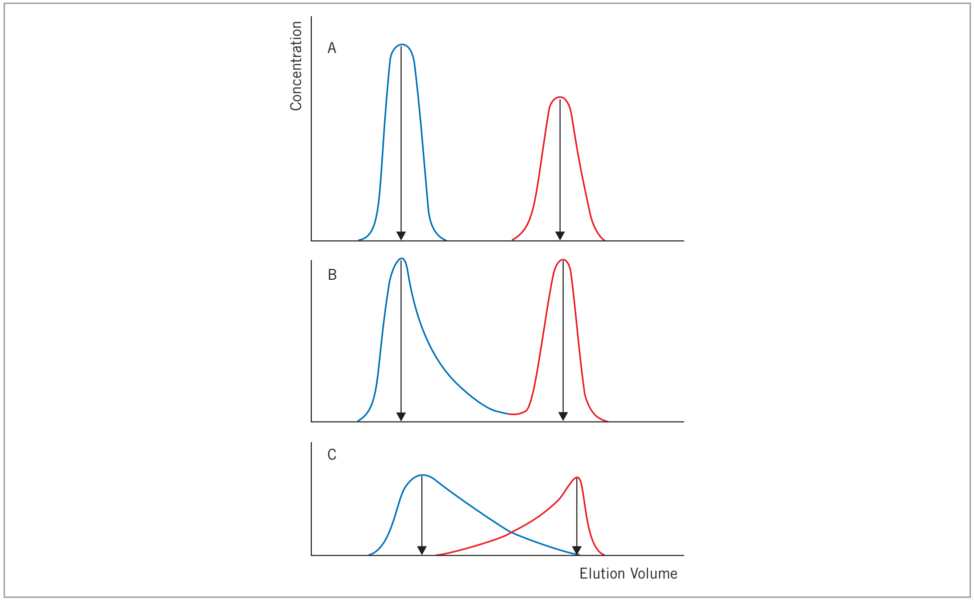


Fig. 13. Elution diagrams obtained when haemoglobin (blue) and NaCl (red) were separated. Experimental conditions were identical except that the viscosities were altered by the addition of increasing amounts of dextran. A deterioration of the separation becomes apparent. (A lower flow rate will not improve the separation.)

Samples should generally not exceed 70 mg/ml protein. Dilute viscous samples, but note sample volume (refer to page 14 for more information on the importance of sample volume). Remember that viscosity varies with temperature.

Sample volume

Sample volume is one of the most important parameters in gel filtration. Refer to page 14 for more information.

Buffer composition and preparation

Buffer composition does not directly influence the resolution obtained in gel filtration since the separation should depend only on the sizes of the different molecules. The most important consideration is the effect of buffer composition on the shape or biological activity of the molecules of interest. For example, the pH and ionic strength of the buffer and the presence of denaturing agents or detergents can cause conformational changes, dissociation of proteins into subunits, dissociation of enzymes and cofactors, or dissociation of hormones and carrier proteins.

Select a buffer and pH that are compatible with protein stability and activity and in which the product of interest should be collected. Use a buffer concentration that is sufficient to

maintain buffering capacity and constant pH. Use up to 0.15 M NaCl to avoid non-specific ionic interactions with the matrix (shown by delays in peak elution). Note that some proteins may precipitate in low ionic strength solutions. Use volatile buffers such as ammonium acetate, ammonium bicarbonate or ethylenediamine acetate if the separated product is to be lyophilized.

Use high quality water and chemicals. Solutions should be filtered through 0.45 µm or 0.22 µm filters. It is essential to degas buffers before any gel filtration separation as air bubbles can significantly affect performance. Buffers will be 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 such as 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 a 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 and in gel filtration buffers in order to maintain solubility of the sample. However, because they will denature the protein, they should be avoided unless denaturation is required.

Modern media such as Superdex, Sephacryl and Superose 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.

If denaturing agents or detergents are necessary to maintain the solubility of the sample, they should be present in both the running buffer and the sample buffer. Note that high concentrations of detergent will increase the viscosity of the buffer so that lower flow rates may be necessary to avoid over-pressuring the column packing.

If proteins that have been solubilized in a denaturant or detergent are seen to precipitate, elute later than expected or be poorly resolved during gel filtration, add a suitable concentration of the denaturing agent or detergent to the running buffer.

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

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

Gel filtration can be used to exchange a protein solubilized initially in, for example SDS, into a more gentle 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. 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 through the column and hence affects the shape and eventual peak width. Gel filtration media with *high uniformity* (lower particle size distribution) facilitate the elution of molecules in narrow peaks. Refer to Chapter 3 Gel filtration in theory and Appendix 1 for further information on column efficiency and column packing.

Efficiency can be improved by decreasing the particle size of a medium. However, using a smaller particle size may increase back pressure so that flow rate needs to be decreased, lengthening the run time.

Using prepacked columns is highly recommended to ensure the best performance and reproducible results. An evenly packed column ensures that, as the sample passes down the column, the component peaks are not unnecessarily broadened. Uneven packing causes peak broadening and high resolution results become impossible.

Allow buffers, media or prepacked columns to reach the same temperature before beginning preparation. 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

The choice of sample application method depends largely on the volume to be applied and on the equipment available. Ensure that the sample is not diluted on the way to the column and that the top of the column bed is not disturbed during sample application. Samples can be applied automatically or manually.

Apply samples directly to the column via a chromatography system, a peristaltic pump or a syringe. The choice of equipment depends largely on the size of column, the type of gel filtration medium and the sample volume. For example, a chromatography system will be required for a Superdex column whereas a syringe can be used with small prepacked columns such as HiTrap Desalting. Note that samples are 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.

Use flow rates that allow time for molecules to diffuse in and out of the matrix (partitioning between the mobile phase and the stationary phase) in order to achieve a separation.

The goal for any separation is to achieve the highest possible resolution in the shortest possible time. Figures 14a, 14b and 14c show that resolution decreases as flow rate increases and 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.

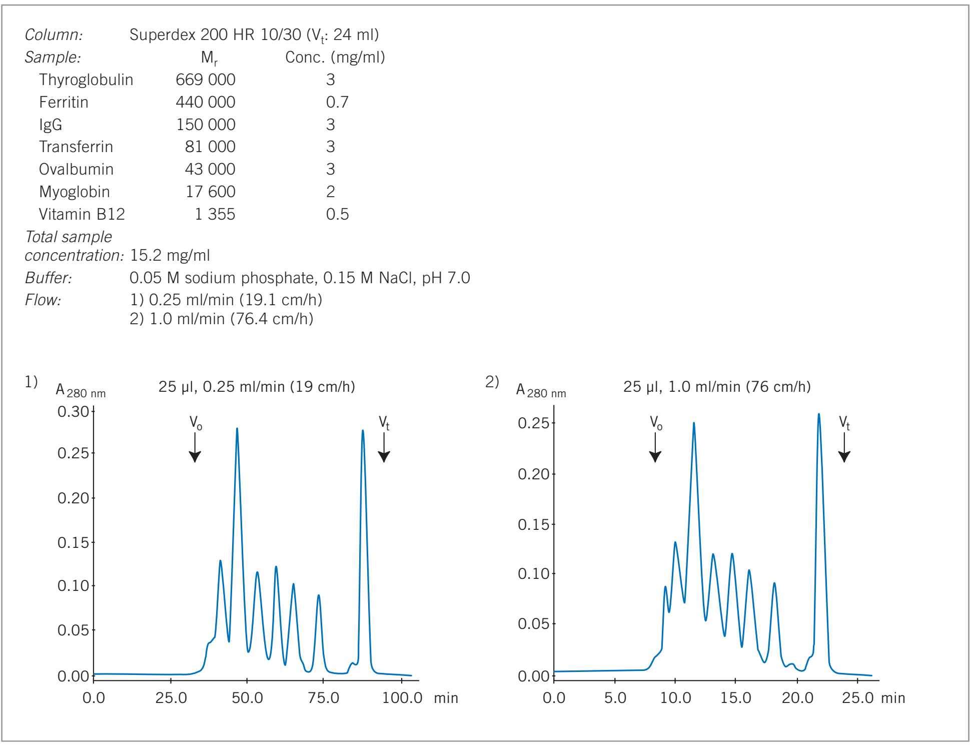


Fig. 14a. Influence of flow rate on resolution.

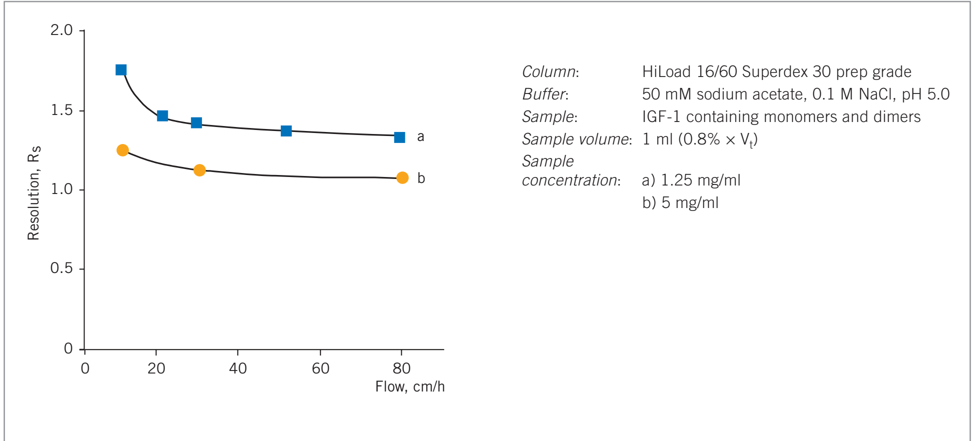


Fig. 14b. Resolution between two different concentrations of IGF-1 containing monomers and dimers at different flow rates.

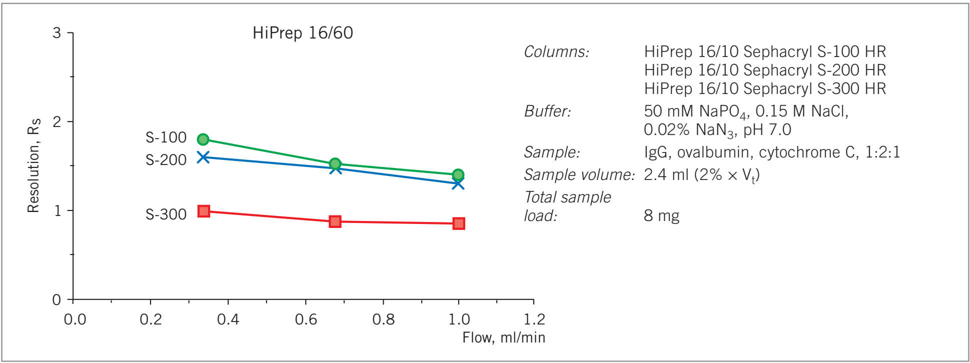


Fig. 14c. Resolution between IgG, ovalbumin and cytochrome C at different flow rates.

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 simple volume terms, e.g. ml/min, but when comparing results between columns of different sizes it is useful to use the linear flow, cm/hour (see Appendix 5). Results obtained at the same linear flow on different size 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 the correct balance between flow rate and resolution. Smaller particles of the same medium can increase column efficiency, improve resolution and may 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 facilitate the removal of 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. A pump is used to control liquid flow for most gel filtration separations, although gravity feed has been used in the past (see Appendix 1).

Use a pump within 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 onto 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 flow rate for separation that is less than the flow rate used for column packing.

Use a syringe or pump with small prepacked columns such as HiTrap Desalting or 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.

Reversing flow through a gel filtration column 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.

Method development for high resolution fractionation

Steps are given in order of priority.

1. Select the medium that will give the best resolution of the target protein(s), see media selection guide, page 18.
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 Chapter 2 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 on this page.

Care of gel filtration media

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. The need for cleaning may show itself as the appearance of a colored band at top of the column, as a space between the upper adaptor and the bed surface, as a loss in resolution or as a significant increase in back pressure. Detailed cleaning procedures for each gel filtration medium are given in Chapter 2. 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. A dirty on-line filter 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 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 systems recommended for gel filtration separation.

Scaling up

After establishing a high resolution or group separation on a small column, it may be preferred to pack a larger column in order to process larger sample volumes in a single step. General guidelines for scaling up are shown below.

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

When scaling up a gel filtration column, follow the points below:

1. Optimize the separation at small scale (see method development page 26).
2. Maintain the sample volume: column volume ratio and the sample concentration.
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 equipment factors may affect performance after scale-up. If the large scale column has a less efficient flow distribution system, or the large scale system introduces large 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 certain media, e.g. Superdex, Superose or Sephadex, it is usually recommended to select a larger particle size. 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 15 shows an example of a large scale buffer exchange step used during the production of albumin and IgG from human plasma.

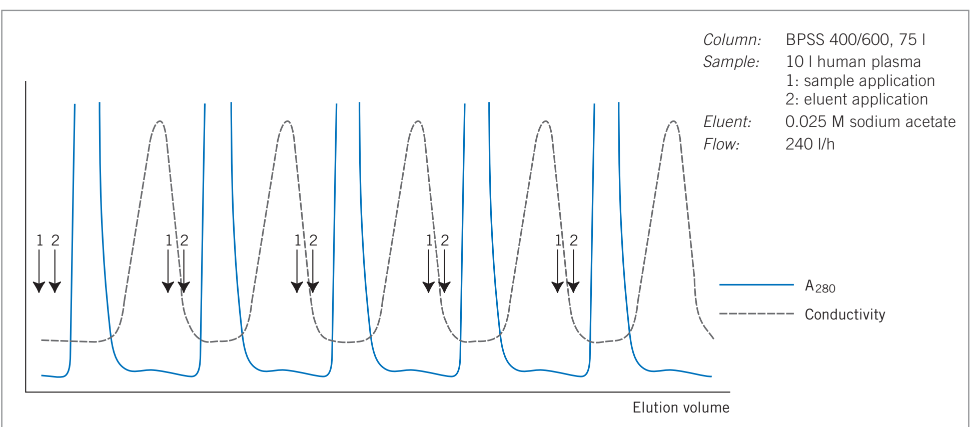


Fig. 15. Chromatogram of the buffer exchange step on Sephadex G-25 Coarse during production of albumin and IgG from human plasma.

BioProcess Media for large-scale production

Specific BioProcess™ Media have been designed for each chromatography stage in a process from Capture to Polishing. Large capacity production integrated with clear ordering and delivery routines ensure that BioProcess media are available in the right quantity, at the right place and at the right time. Amersham Biosciences can assure future supplies of BioProcess Media, making them a safe investment for long-term production. The media are produced following validated methods and tested under strict control to fulfill high performance specifications. A certificate of analysis is available with each order.

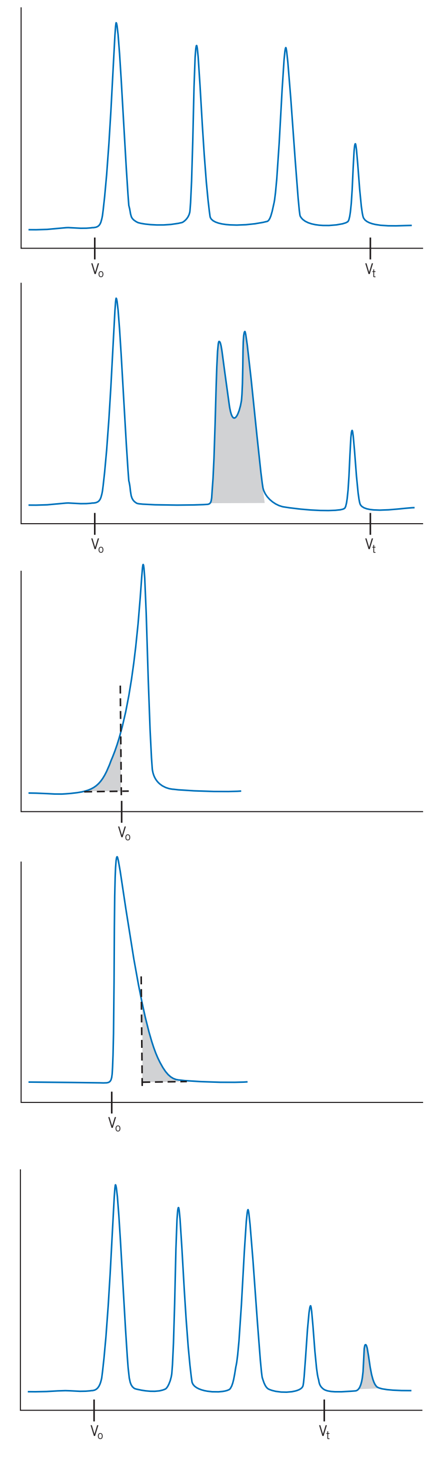
Regulatory Support Files (RSF) contain details of performance, stability, extractable compounds and analytical methods. The essential information in these files gives an invaluable starting point for process validation, as well as providing support for submissions to regulatory authorities. Using BioProcess Media for every stage results in an easily validated process. High flow rate, high capacity and high recovery contribute to the overall economy of an industrial process.

All BioProcess Media have chemical stability to allow efficient cleaning and sanitization procedures. Packing methods are established for a wide range of scales and compatible large-scale columns and equipment are available.

Troubleshooting

This section focuses on practical problems that may occur when running a chromatography column. The diagrams indicate how a chromatogram may deviate from ideal behavior during a gel filtration separation. The following pages contain suggestions of possible causes and their remedies.

Highly acidic or basic substances at low ionic strength or aromatic materials may behave differently during gel filtration, interacting with the matrix. For some applications this can be an advantage. For example aromatic peptides and other substances that differ only slightly in molecular weight can be separated on Sephadex. However this is not a true gel filtration separation.



Satisfactory separation
Well resolved, symmetrical peaks.

Poor resolution
Review factors affecting resolution (see page 13), 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 to overpacking of column (packed at too high pressure or flow rate). Column may need to be repacked.

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

Molecules may be binding non-specifically to gel filtration medium. If the interaction is ionic in nature, increasing the concentration of sodium chloride (up to 150 mM) may help. If the interaction is hydrophobic in nature, reducing salt concentration, increasing pH or adding a detergent or organic solvent may help.

Situation	Cause	Remedy
Peak of interest is poorly resolved from other major peaks.	Sample volume is too high or sample has been incorrectly applied.	Decrease sample volume and apply carefully. Check bed surface and top filter for possible contamination.
	Sample is too viscous.	Dilute with buffer, but check maximum sample volume. Maintain protein concentration below 70 mg/ml.
	Sample filtered incorrectly.	Reequilibrate 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 reequilibrate.
	Incorrect medium.	Check selectivity curve. Check for adsorption effects. Consider effects of denaturing agents or detergents if present.
	Large dead volumes.	Minimize dead volumes in tubings and connections.
	Column too short.	See Appendix 1 for recommended bed heights.
	Flow rate too high.	Check recommended flow rates. Reduce flow.
	Uneven temperature.	Use a column with a water jacket.
Protein does not elute as expected.	Sample volume is different from previous runs.	Keep sample volume constant. Resolution is dependent on sample volume.
	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.	Clean the column or use a new column.
	Column is overloaded with sample.	Decrease the sample load.
	Microbial growth has occurred in the column.	Microbial growth rarely occurs in columns during use. To prevent infection of packed columns, store in 20% ethanol when possible.
Molecular weight or shape is not as expected.	Protein has changed during storage.	Prepare fresh samples.
	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. 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.
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 reequilibrate.
Protein elutes earlier than expected (before the void volume)	Channelling in the column.	Repack column using a thinner slurry of medium. Avoid introduction of air bubbles.

Situation	Cause	Remedy
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.
Leading peaks.	Column is 'over' packed.	Check column efficiency (see Appendix 1). Repack using a slower flow rate. Use prepacked columns.
Medium/beads appears 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 fractions and repeating 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.
	Non-specific adsorption.	Reduce salt concentration to minimize hydrophobic interaction. Increase pH. Add suitable detergent or organic solvent e.g. 5% isopropanol. If necessary, add 10% ethylene glycol to running buffer.
Peaks too small.	Sample absorbs poorly at chosen wavelength.	Check absorbance range on monitor. If possible, use a different wavelength, e.g. 214 nm instead of 280 nm.
	Excessive band broadening.	Check column packing. Repack if necessary.
More sample is recovered than expected.	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, but, to prevent infection of packed columns, store in 20% ethanol when possible.
	Medium not fully swollen (Sephadex).	See Appendix 1 for reswelling conditions.

Situation	Cause	Remedy
	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.
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.

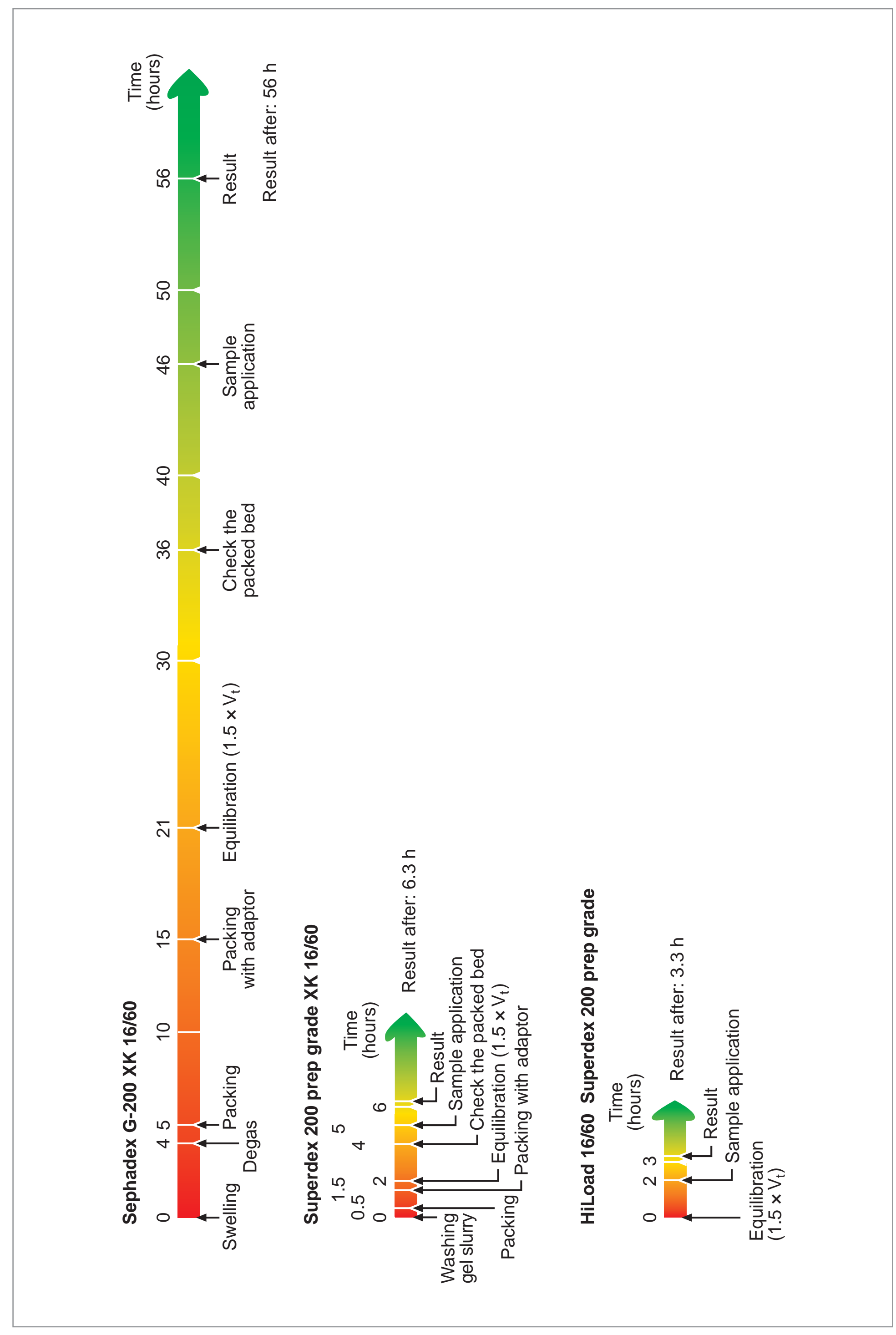


Fig. 16. Time-line for a gel filtration separation on Sephadex G-200 compared to the latest Superdex 200 prep grade (which has replaced Sephadex G-200) and a prepacked HiLoad 16/60 Superdex prep grade column.

Chapter 2
Gel filtration media

Components of gel filtration media

The development of gel filtration media has been driven by the need to achieve the highest flow rates while retaining the highest resolution. As a sample passes through a gel filtration column the separation or resolution of the different components is affected by several parameters: flow rate, particle size, particle size distribution, packing density, porosity of the particle and viscosity of the mobile phase. Attempts to optimize each parameter has led to the development of a series of gel filtration media.

The earliest gel filtration matrices were formed by cross-linking polymers to form a three-dimensional network, for example Sephadex is formed by cross-linking dextran. Controlling the degree of cross-linking and particle size made it possible to produce a broad range of media, each one having a high selectivity over a narrow range of molecular weight values.

However, to increase the speed of a separation the medium must withstand higher flow rates and so alternative polymers such as agarose were investigated. This resulted in gel filtration media based on Sepharose and, later, the more highly cross-linked Superose. Matrices based on agarose are, in general, more porous than those based on dextran so that, although the speed of a separation could be increased there was less selectivity when compared to Sephadex. The porosity of Sepharose makes it highly suitable for binding techniques such as affinity chromatography where the high porosity facilitates a high binding capacity.

A major advance in gel filtration technology occurred when composite gels could be prepared by grafting a second polymer onto a pre-formed matrix, for example Sephacryl (cross-linking allyl dextran with N,N'-methylene bisacrylamide) and the most recent, Superdex. In the case of Superdex, with the dextran chains covalently bonded to a highly cross-linked agarose matrix, it has been possible to create a range of media with the same high selectivity as Sephadex, but with the mechanical strength of a highly cross-linked agarose-based matrix. The selectivity curves and pressure-flow relationship curves shown for Superose, Sephacryl and Superdex in the following sections show how the performance of gel filtration media has developed. Figure 16 clearly illustrates the significant increase in speed without loss of performance that has been achieved with the development of Superdex. For Superose and Superdex, manipulation of the particle size (Superose prep grade or Superdex prep grade) is also used to allow higher flow rates when running larger columns.

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

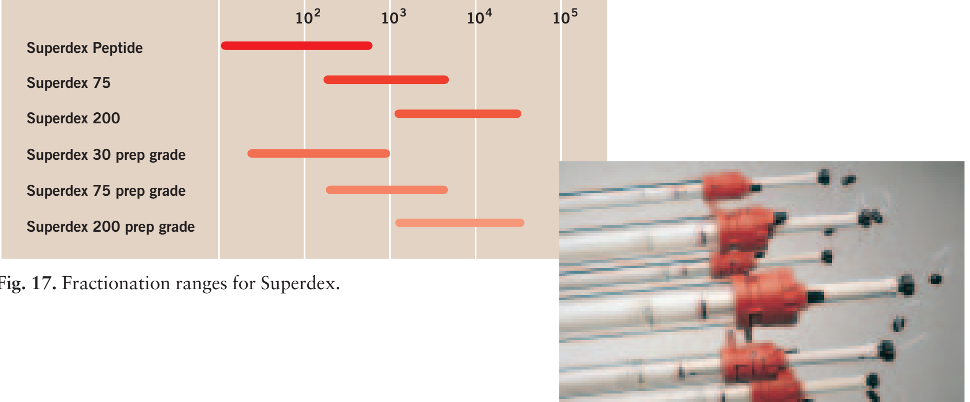


Fig. 17. Fractionation ranges for Superdex.

Fig. 18. Prepacked HiLoad Superdex prep grade columns.

From laboratory to process scale applications, Superdex is the first choice for a high resolution fractionation with short run times and good recovery. The success of Superdex is clearly demonstrated by the hundreds of scientific publications in which the use of Superdex has been described. Reference lists highlighting the use of the prepacked columns HiLoad Superdex 200 prep grade, HiLoad Superdex 75 prep grade and HiLoad Superdex 30 prep grade are available at *www.chromatography.amershambiosciences.com*.

Selectivity curves and pressure-flow relationship curves for Superdex are shown in Figures 19a and 19b. A typical linear flow is up to 75 cm/h.

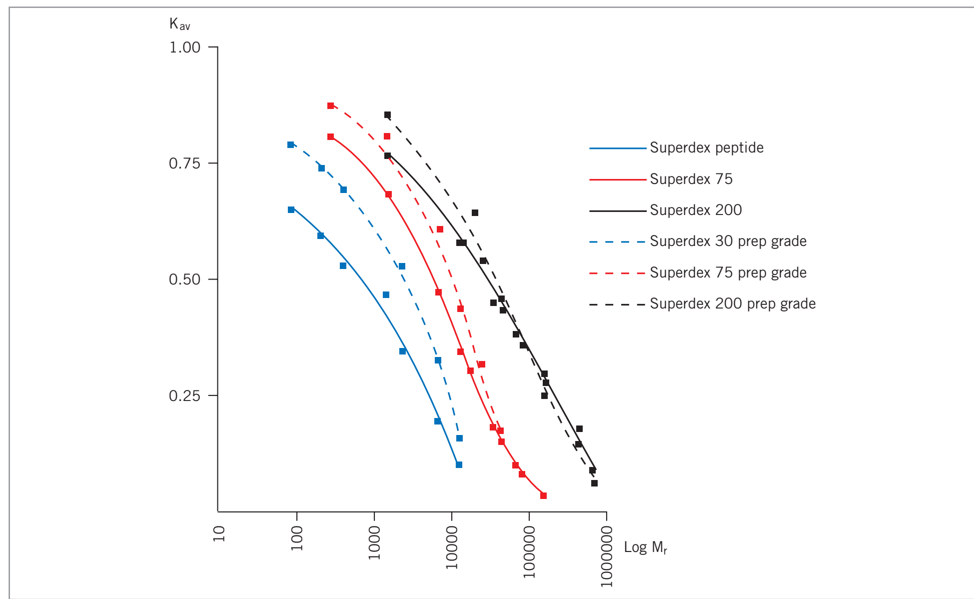


Fig. 19a. Selectivity curves for Superdex (13 μm) and Superdex prep grade (34 μm) media.

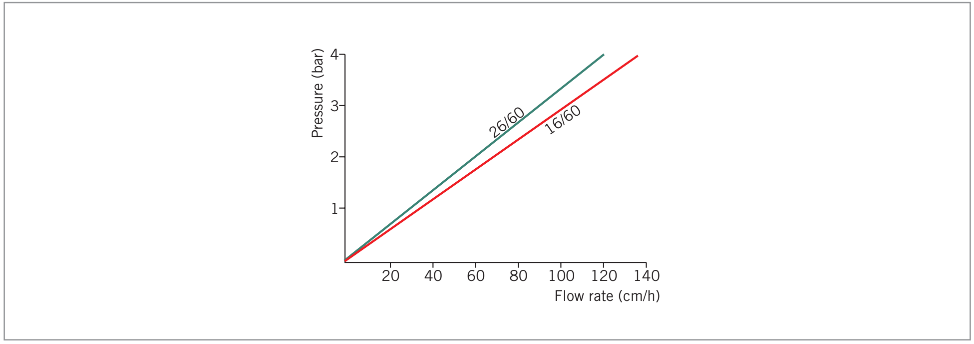


Fig. 19b. Pressure drop as a function of flow rate for HiLoad columns packed with Superdex prep grade. Bed height approximately 60 cm in distilled water at +25 °C. To calculate volumetric flow rate, multiply linear flow by cross-sectional area of column (2 cm^2 for XK 16, 5.3 cm^2 for XK 26).See Appendix 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 20. The result is media with high physical and chemical stability, due mainly 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 non-specific interactions between proteins and Superdex are negligible when using buffers with ionic strengths in the range 0.15 M to 1.5 M.

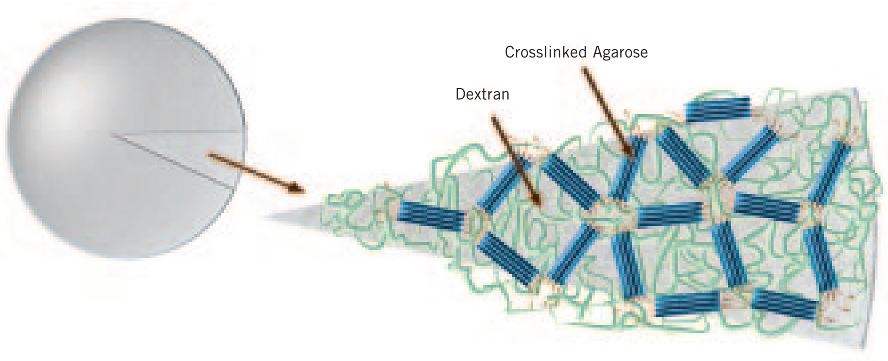


Fig. 20. In Superdex the dextran chains are covalently linked to a highly cross-linked agarose matrix. The figure shows a schematic of a section through a Superdex particle.

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).

- 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 loose media) for larger scale applications.

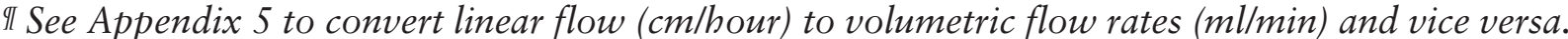
Product †	**Fractionation range, M_r (globular proteins)**	**Sample loading capacity ‡**	**Maximum operating back pressure**	**Recommended operating flow ¶**
Superdex Peptide PC 3.2/30	1×10^2–7×10^3	25–250 µl	1.8 MPa, 18 bar, 260 psi	<0.15 ml/min
Superdex Peptide HR 10/30	1×10^2–7×10^3	25–250 µl	1.8 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*	$<1 \times 10^4$	0.5–4% of total column volume	0.5 MPa, 5 bar, 70 psi	10–50 cm/h
Superdex 75 PC 3.2/30	3×10^3–7×10^4	<50 µl	2.4 MPa, 24 bar, 350 psi	<0.1 ml/min
Superdex 75 HR 10/30	3×10^3–7×10^4	25–250 µl	1.8 MPa, 18 bar, 260 psi	<1.5 ml/min
HiLoad 16/60 Superdex 75 pg*	3×10^3–7×10^4	≤5 ml	0.3 MPa, 3 bar, 42 psi	≤1.6 ml/min
HiLoad 26/60 Superdex 75 pg*	3×10^3–7×10^4	≤13 ml	0.3 MPa, 3 bar, 42 psi	≤4.4 ml/min
Superdex 75 pg*	3×10^3–7×10^4	0.5–4% of total column volume	0.5 MPa, 5 bar, 70 psi	10–50 cm/h
Superdex 200 PC 3.2/30	1×10^4–6×10^5	<50 µl	1.5 MPa, 15 bar, 220 psi	<0.1 ml/min
Superdex 200 HR 10/30	1×10^4–6×10^5	25–250 µl	1.5 MPa, 15 bar, 220 psi	0.25–0.75 ml/min
HiLoad 16/60 Superdex 200 pg*	1×10^4–6×10^5	≤5 ml	0.3 MPa, 3 bar, 42 psi	≤1.6 ml/min
HiLoad 26/60 Superdex 200 pg*	1×10^4–6×10^5	≤13 ml	0.3 MPa, 3 bar, 42 psi	≤4.4 ml/min
Superdex 200 pg*	1×10^4–6×10^5	0.5–4% of total column volume	0.5 MPa, 5 bar, 70 psi	10–50 cm/h

** prep grade.*

† HR and PC columns are packed with Superdex and HiLoad columns are packed with Superdex prep grade.

‡ For maximum resolution apply as small a sample volume as possible, but note that sample volumes less than 0.5% do not normally improve resolution.

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



Start with Superdex 200 when the molecular weight of the protein of interest is unknown. Superdex 200 or Superdex 200 prep grade (pg) 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 re-packed.

Separation examples

The figures below illustrate examples of separations performed on Superdex Peptide, Superdex 75 and Superdex 200.

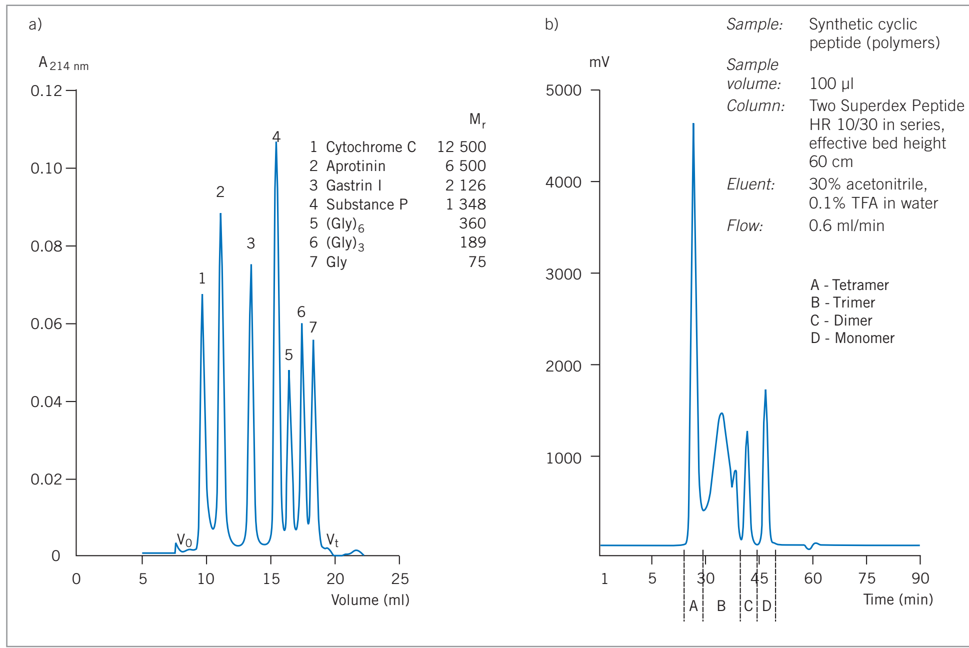


Fig. 21. a) Separation of standard peptides on Superdex Peptide HR 10/30. **b)** Separation of peptide polymers (monomer M_r ~1 000). The fractions indicated were analyzed by off-line mass spectrometry. Courtesy of K. Walhagen, Ferring Research Institute, Sweden.

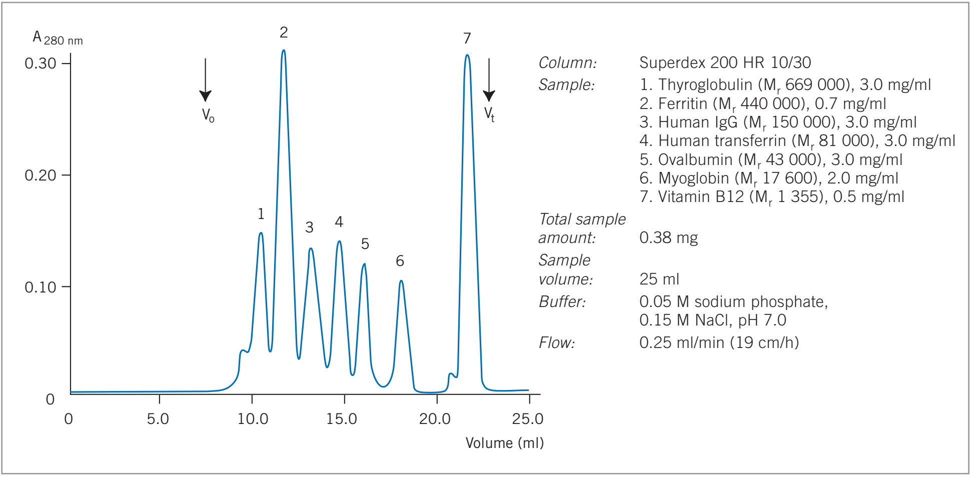


Fig. 22. Separation of standard proteins on Superdex 200 HR 10/30.

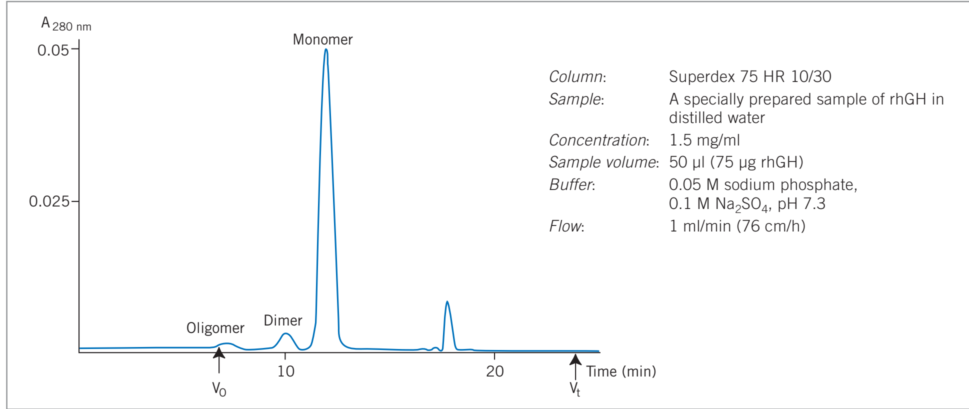


Fig. 23. Separation of growth hormone oligomers on Superdex 75 HR 10/30.

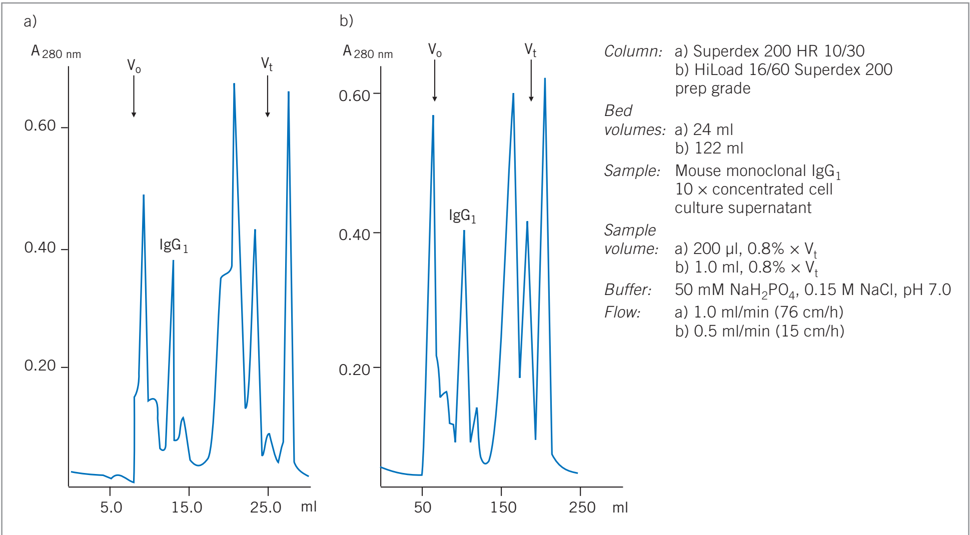


Fig. 24. Scale up (five times) of a mouse monoclonal IgG_1 purification from Superdex 200 HR 10/30 (a) onto HiLoad 16/60 Superdex 200 prep grade (b).

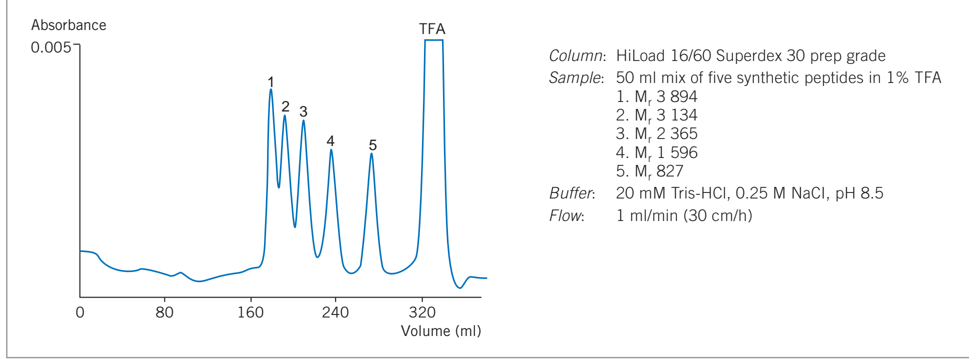


Fig. 25. Separation of test substances on HiLoad 16/60 Superdex 30 prep grade.

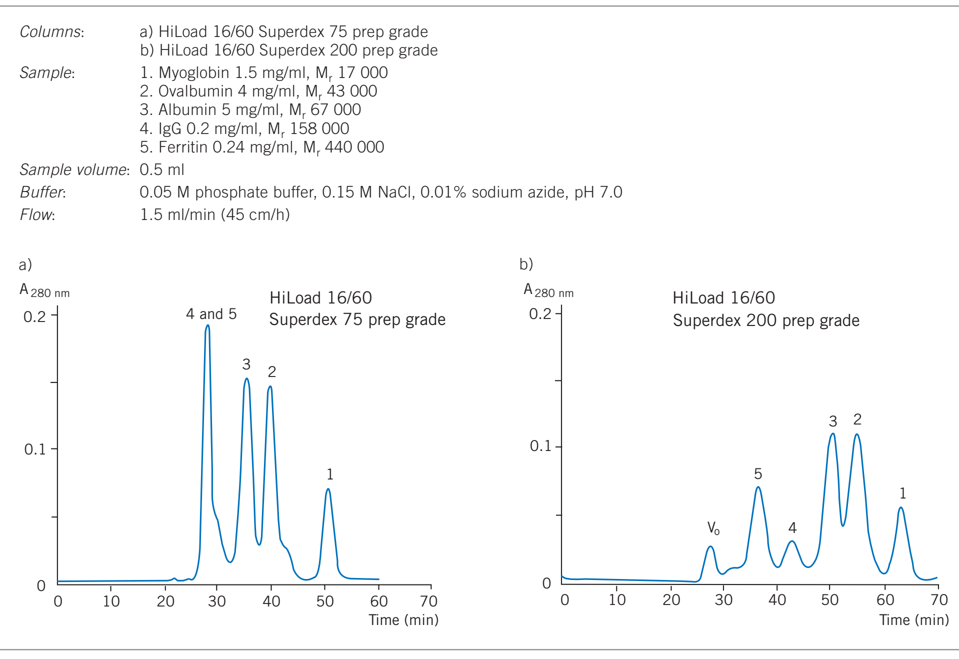


Fig. 26. Comparison of the selectivity of Superdex 75 prep grade and Superdex 200 prep grade for model proteins. Superdex 75 prep grade (a) gives excellent resolution of the three proteins in the M_r range 17 000 to 67 000 while the two largest proteins elute together in the void volume. Superdex 200 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_o) peak at 28 minutes in (b) is caused by protein aggregates.

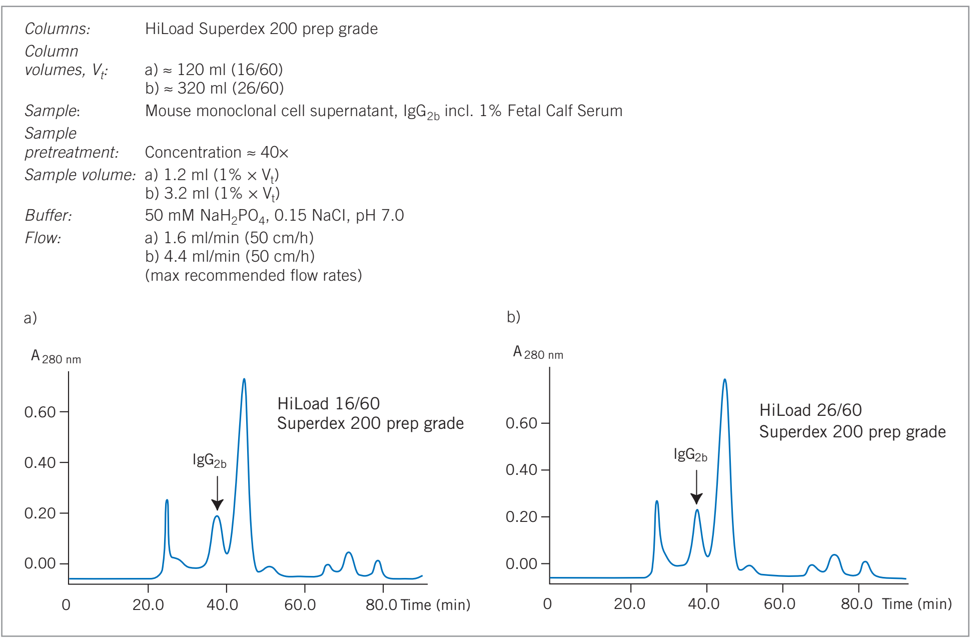


Fig. 27. Purification of mouse monoclonal IgG_{2b} from cell supernatant using a) HiLoad 16/60 Superdex 200 prep grade, column volume 120 ml and b) HiLoad 26/60 Superdex 200 prep grade, column volume 320 ml. Almost identical separations are the result, even using prepacked columns of different sizes.

Protein refolding

After solubilization, recombinant proteins must be properly refolded to regain function. Denaturing agents must be removed to allow refolding of the protein and formation of the correct intramolecular associations. Critical parameters during refolding include pH, presence of thiol reagents, the speed of denaturant removal, and the relative concentrations of host proteins and recombinant protein. The table below compares the advantages and disadvantages of the alternative methods for refolding of recombinant proteins. Different proteins require different conditions for successful refolding. Gel filtration provides an alternative method that can be tried if on-column refolding during affinity purification is not possible. For further details about on-column refolding using affinity chromatography refer to *The Recombinant Protein Handbook, Amplification and Simple Purification*, available from Amersham Biosciences and Febs Letters 345 (1994) 125–130.

Refolding technique	Advantages/Disadvantages
Step dialysis	Takes several days. Uses large volumes of buffer.
Dilution into near neutral pH	Dilutes the protein of interest.
On-column gel filtration	Slower than on-column refolding by affinity chromatography. Requires a second column to be run. Only small volumes can be processed per column.
On-column affinity chromatography	Fast and simple. No sample volume limitations.

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 non-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.

1. For first time use, or after long term storage, equilibrate the column with 1 column volume of buffer, but containing 0.05 M NaCl at 30 cm/h (0.4 ml/min for HR 10/30, 1 ml/min for XK 16/60 or 2.6 ml/min for XK 26/60).
2. Equilibrate with 2 column volumes of buffer containing 0.15 M NaCl at 50 cm/h (0.65 ml/min for HR 10/30, 1.6 ml/min for XK 16/60 or 4.3 ml/min for XK 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 HR 10/30, 1.2 ml for XK 16/60 or 3.2 ml for XK 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 95 for how to check column efficiency.

See page 26 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 re-packed.

Cleaning

1. Wash with 1 column volume of 0.5 M NaOH at a flow of 25 cm/h (0.33 ml/min for HR 10/30, 0.8 ml/min for XK 16/60 or 2.2 ml/min for XK 26/60) to remove most non-specifically adsorbed proteins.
2. Wash with 1 column volume of distilled water at 25 cm/hr.
3. Re-equilibrate with 2 column volumes of buffer at a flow of 50 cm/hr (0.4 ml/min for HR 10/30, 1.6 ml/min XK 16/60 or 4.3 ml/min for XK 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.

To remove 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.

Reversing flow through a gel filtration column 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

Composition: Superdex is formed from dextran bound covalently to highly cross-linked agarose.

Product	Efficiency: theoretical plates per meter (prepacked columns only)	pH stability*	Particle size	Mean particle size
Superdex Peptide	≥30 000 m^{-1}	Long term: 1–14 Short term: 1–14	13–15 µm	13 µm
Superdex 75	≥30 000 m^{-1}	Long term: 3–12 Short term: 1–14	13–15 µm	13 µm
Superdex 200	≥30 000 m^{-1}	Long term: 3–12 Short term: 1–14	13–15 µm	13 µm
Superdex 30 prep grade	>13 000 m^{-1}	Long term: 3–12 Short term: 1–14	22–44 µm	34 µm
Superdex 75 prep grade	>13 000 m^{-1}	Long term: 3–12 Short term: 1–14	22–44 µm	34 µm
Superdex 200 prep grade	>13 000 m^{-1}	Long term: 3–12 Short term: 1–14	22–44 µm	34 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 Amersham Biosciences.

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 +25 °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 +25 °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.

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 28. High chemical stability and tolerance of high flow rates make Sephacryl well suited for industrial use.

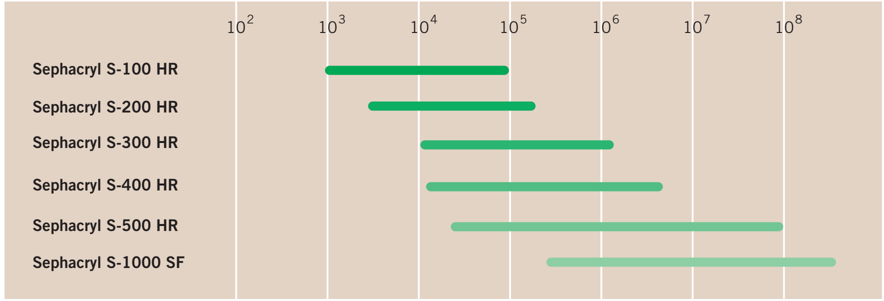


Fig. 28. Fractionation ranges for Sephacryl High Resolution (HR).

Fig. 29. Sephacryl is available as loose media and in prepacked columns.

Reference lists highlighting the use of HiPrep Sephacryl S-300 HR, HiPrep Sephacryl S-200 HR and HiPrep Sephacryl S-300 HR are available at *www.chromatography.amershambiosciences.com*.

Figure 30 shows a comparison of the different selectivities of Sephacryl High Resolution.

Typical selectivity and pressure-flow relationship curves for Sephacryl are shown in Figures 31a and 31b.

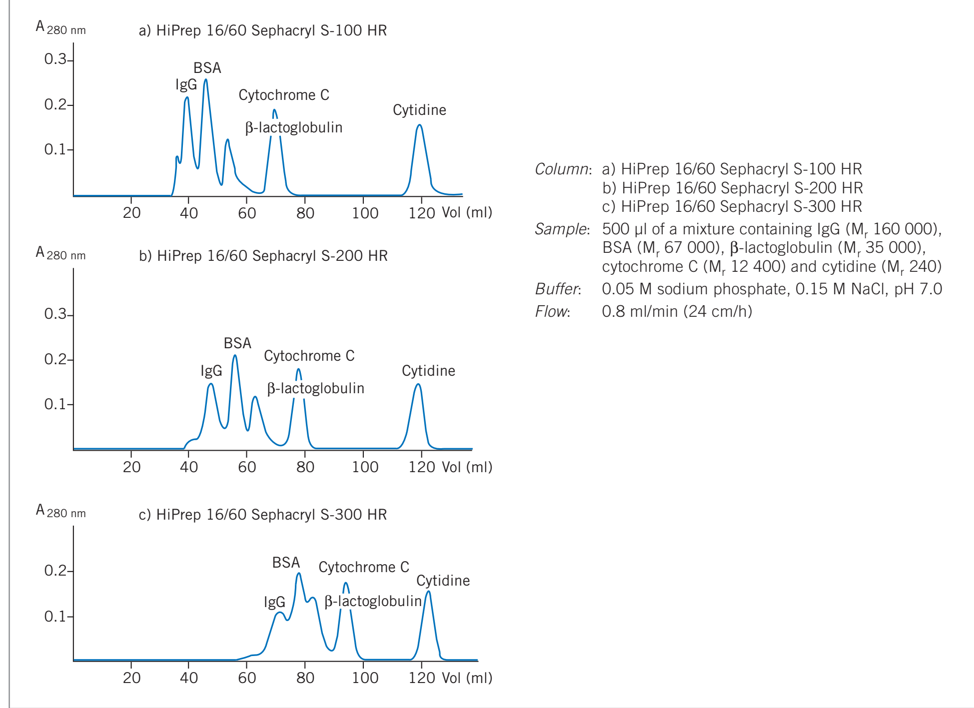


Fig. 30. Comparison of the selectivity of HiPrep 16/60 Sephacryl S-100 HR, HiPrep 16/60 Sephacryl S-200 HR, and HiPrep 16/60 Sephacryl S-300 HR columns.

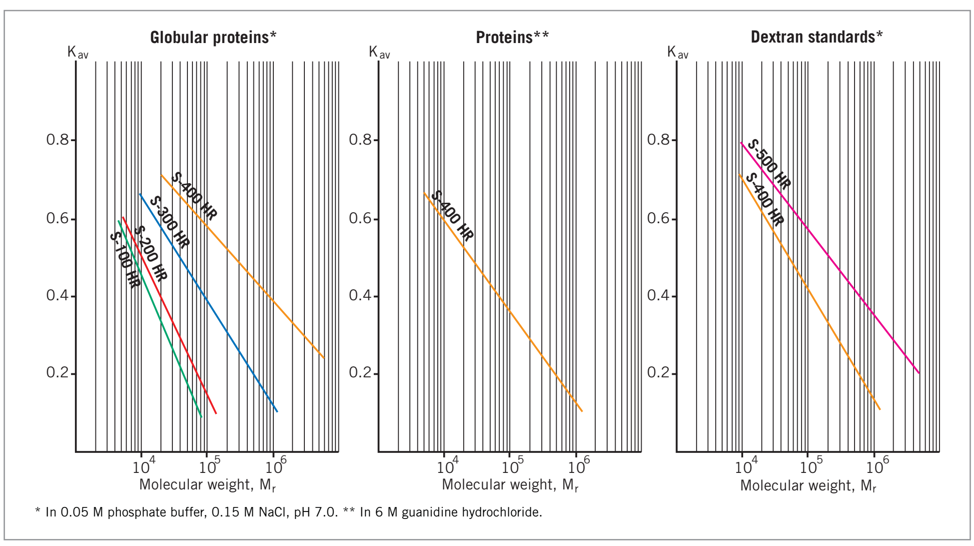


Fig. 31a. Selectivity curves for Sephacryl High Resolution media.

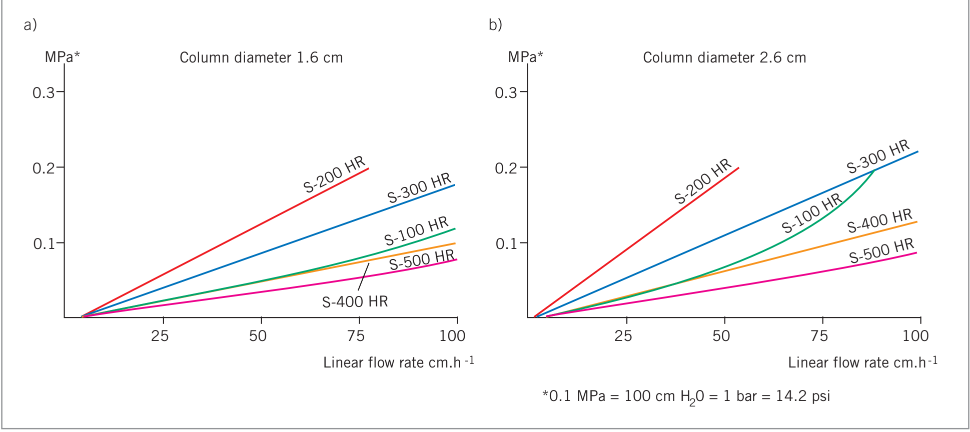


Fig. 31b. Pressure drop as a function of flow rate for Sephacryl High Resolution. Bed height approximately 60 cm, distilled water, temperature +25 °C. To calculate the volumetric flow rate, multiply the linear flow by the cross-sectional area of the column (2 cm^2 for XK 16 or 5.3 cm^2 for XK 26).

Sephacryl High Resolution (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, illustrated in Figure 32. 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.

Fig. 32. Partial structure of Sephacryl High Resolution.

Separation options

Product †	Fractionation range, M_r (globular proteins)	Sample loading capacity †	Maximum operating back pressure	Recommended operating flow ‡
HiPrep 16/60 Sephacryl S-100 HR*	1×10^3–1×10^5	≤5 ml	0.15 MPa, 5 bar, 21 psi	0.5 ml/min
HiPrep 26/60 Sephacryl S-100 HR*	1×10^3–1×10^5	≤13 ml	0.15 MPa, 5 bar, 21 psi	1.3 ml/min
Sephacryl S-100 HR*	1×10^3–1×10^5	0.5–4% of total column volume	0.2 MPa, 2 bar, 28 psi	10–35 cm/h
HiPrep 16/60 Sephacryl S-200 HR*	5×10^3–2.5×10^5	≤5 ml	0.15 MPa, 5 bar, 21 psi	0.5 ml/min
HiPrep 26/60 Sephacryl S-200 HR*	5×10^3–2.5×10^5	≤13 ml	0.15 MPa, 5 bar, 21 psi	1.3 ml/min
Sephacryl S-200 HR*	5×10^3–2.5×10^5	0.5–4% of total column volume	0.2 MPa, 2 bar, 28 psi	10–35 cm/h
HiPrep 16/60 Sephacryl S-300 HR*	1×10^4–1.5×10^6	≤5 ml	0.15 MPa, 5 bar, 21 psi	0.5 ml/min
HiPrep 26/60 Sephacryl S-300 HR*	1×10^4–1.5×10^6	≤13 ml	0.15 MPa, 5 bar, 21 psi	1.3 ml/min
Sephacryl S-300 HR*	1×10^4–1.5×10^6	0.5–4% of total column volume	0.2 MPa, 2 bar, 28 psi	10–35 cm/h
Sephacryl S-400 HR*	2×10^4–8×10^6	0.5–4% of total column volume	0.2 MPa, 2 bar, 28 psi	10–35 cm/h
Sephacryl S-500 HR*	–	0.5–4% of total column volume	0.2 MPa, 2 bar, 28 psi	10–35 cm/h
Sephacryl S-1000 SF (Superfine)	–	0.5–4% of total column volume	not determined	2–30 cm/h

** High Resolution.*

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

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

Separation examples

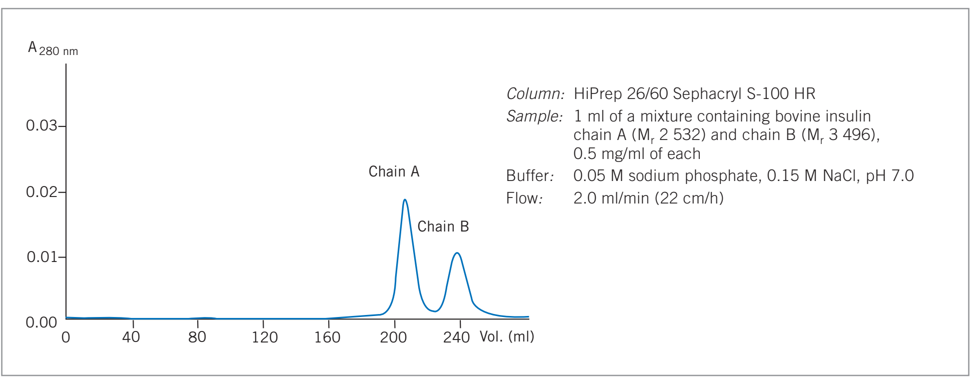


Fig. 33. Separation of insulin chains on HiPrep 26/60 Sephacryl S-100 HR.

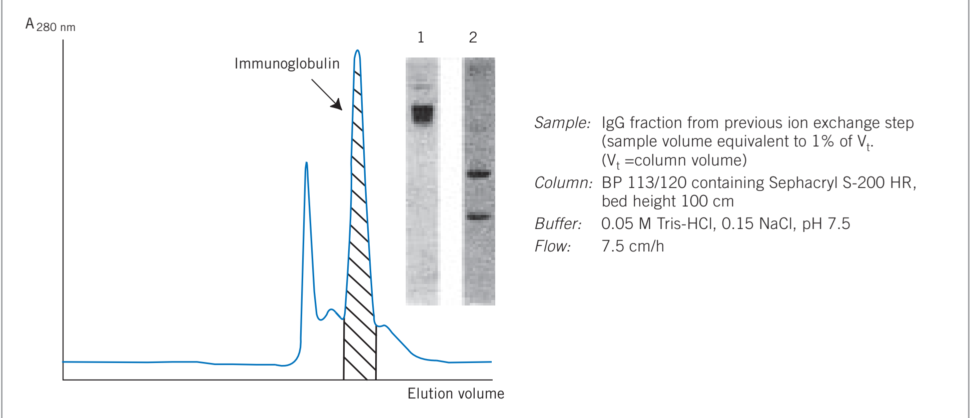


Fig. 34. Purification of monoclonal antibodies on Sephacryl S-200 HR. Inset shows analysis by gradient SDS-PAGE of the immunoglobulin pool. Lane 1, native sample; lane 2, sample reduced with 2-mercaptoethanol.

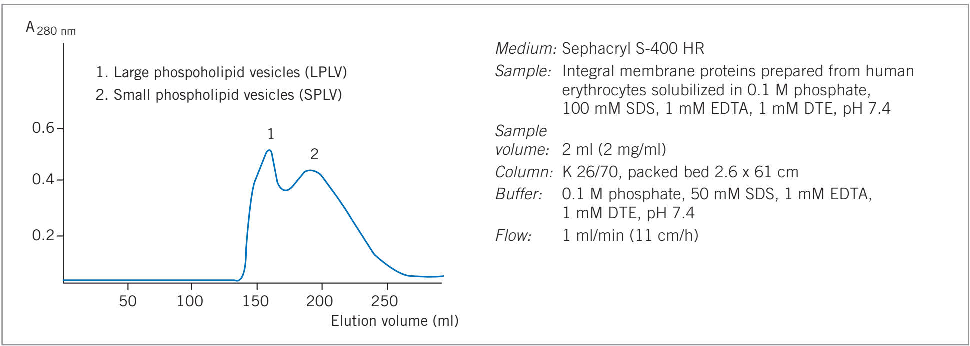


Fig. 35. Gel filtration on Sephacryl S-400 HR quickly separates phospholipid vesicles (liposomes) into large (LPLV) and small (SPLV) phospholipid vesicles. (Data provided by E. Greijer and P. Lundahl, Dept. of Biochemistry, Biomedical Centre, University of Uppsala, Sweden.)

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 non-ionic 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.

1. For first time use, or after long term storage, 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 0.5–4% 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 95 on how to check column efficiency.

See page 26 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 re-packed.

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 non-specifically 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 High Resolution may be autoclaved repeatedly at +121 °C, pH 7 for 30 minutes without significantly affecting its chromatography properties. The medium must be removed from a column before autoclaving as certain column components cannot tolerate such high temperatures.

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 gel filtration column 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

Composition: Sephacryl 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 is determined by the dextran component.

Product	Efficiency: theoretical plates per meter (prepacked columns only)	pH stability †	Particle size	Mean particle size
Sephacryl S-100 HR*	>5 000 m^{-1}	Long term: 3–11 Short term: 2–13	25–75 µm	47 µm
Sephacryl S-200 HR*	>5 000 m^{-1}	Long term: 3–11 Short term: 2–13	25–75 µm	47 µm
Sephacryl S-300 HR*	>5 000 m^{-1}	Long term: 3–11 Short term: 2–13	25–75 µm	47 µm
Sephacryl S-400 HR*	‡	Long term: 3–11 Short term: 2–13	25–75 µm	47 µm
Sephacryl S-500 HR*	‡	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

** High Resolution.*

† 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 Amersham Biosciences.

‡ Efficiencies of 9 000 m^{-1} have been achieved, but depend significantly on how well the column is packed.

Chemical stability

Sephacryl High Resolution 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 +25 °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 +25 °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.

Superose: broad fractionation range, but not suitable for industrial scale separations

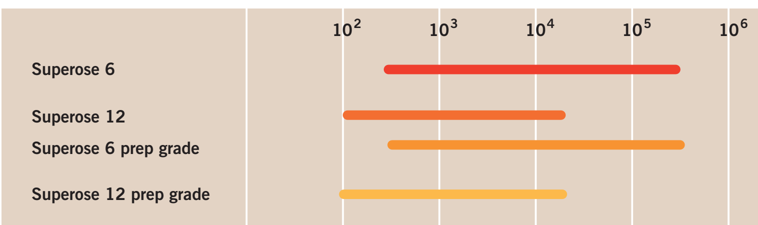


Fig. 36. Fractionation ranges of Superose.

Superose is a medium with high physical and chemical stability based on highly cross-linked porous agarose particles. Typical fractionation ranges for Superose are shown in Figure 36.

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 non-specific interactions between proteins and Superose are negligible when using buffers with ionic strengths in the range 0.15 M to 1.5 M.

Some hydrophobic interactions have been noted, particularly for compounds such as smaller hydrophobic and/or aromatic peptides, membrane proteins and/or lipoproteins which 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 37.

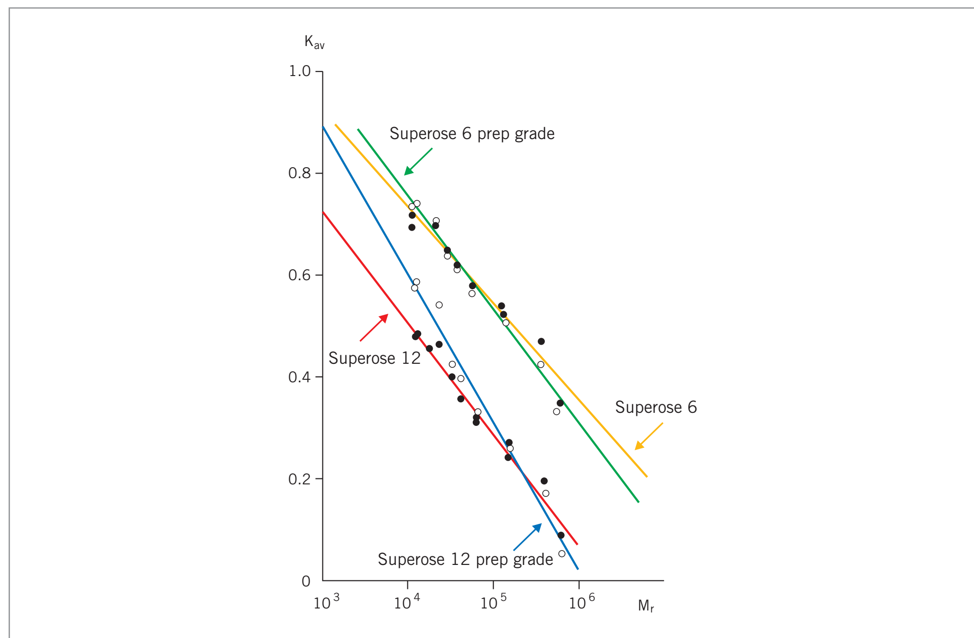


Fig. 37. Selectivity curves of Superose for globular proteins.

Figure 38 gives a comparison of the different selectivities of Superose 6 and Superose 12 prepacked columns.

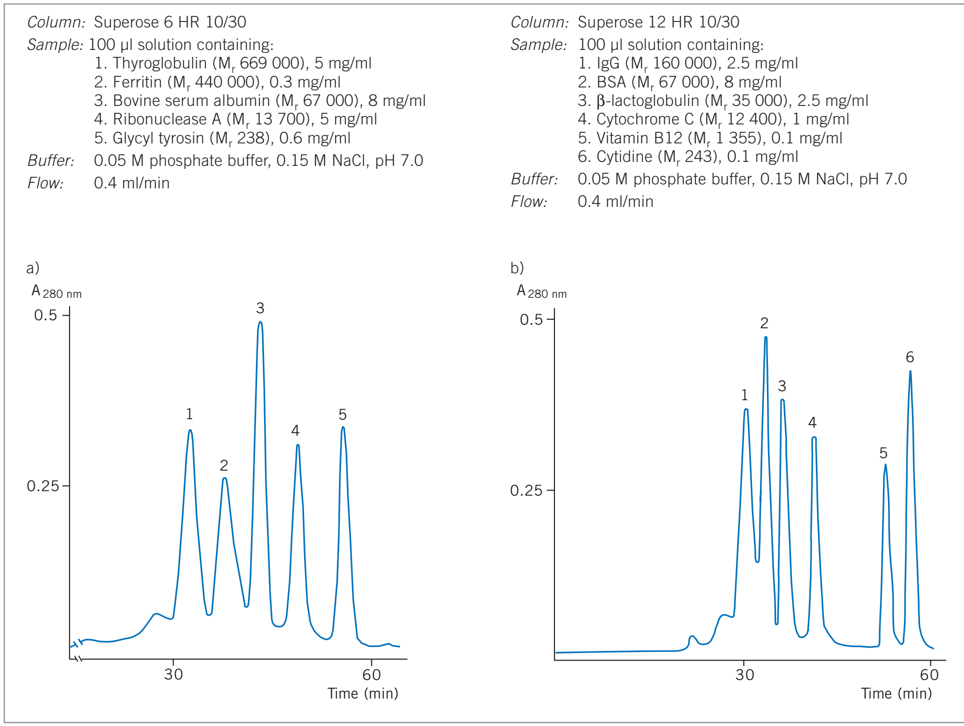


Fig. 38. a) Standard proteins separated on Superose 6 HR 10/30, M_r range: 5 000–5 000 000. b) Standard proteins separated on Superose 12 HR 10/30, M_r range: 1 000–300 000.

Separation options

Superose is produced in different particle sizes (11 µm, 13 µm and 30 µm) and with two different selectivities (Superose 6 and Superose 12).

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

Product*	Fractionation range, M_r (globular proteins)	Sample loading capacity*	Maximum operating back pressure	Recommended operating flow †
Superose 6 PC 3.2/30	5×10^3–5×10^6	200 µl	1.2 MPa, 12 bar, 175 psi	<0.1 ml/min
Superose 6 HR 10/30	5×10^3–5×10^6	25–250 µl	1.5 MPa,15 bar, 220 psi	0.3–0.5 ml/min
Superose 6 prep grade	5×10^3–5×10^6	0.5–4% of total column volume	0.4 MPa, 4 bar, 58 psi	<40 cm/h
Superose 12 PC 3.2/30	1×10^3–3×10^6	200 µl	2.4 MPa,24 bar, 350 psi	<0.1 ml/min
Superose 12 HR 10/30	1×10^3–3×10^6	25–250 µl	3 MPa, 30 bar, 435 psi	0.5–1.0 ml/min
Superose 12 prep grade	1×10^3–3×10^6	0.5–4% of total column volume	0.7 MPa, 7 bar, 101 psi	<40 cm/h

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

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

Separation examples

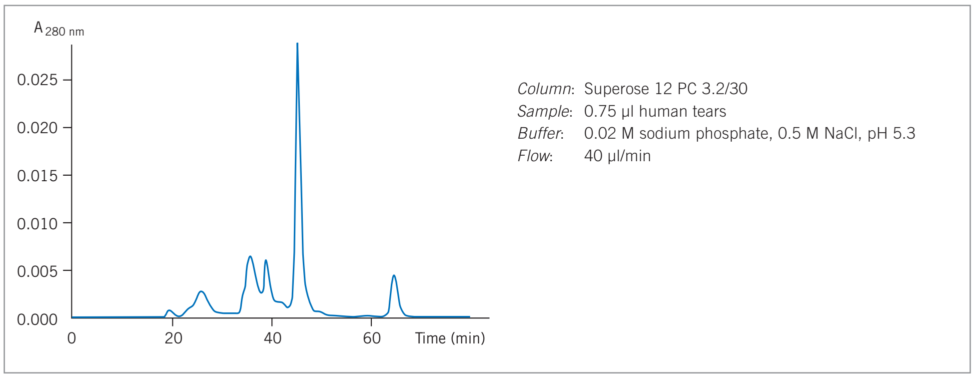


Fig. 39. Microfractionation of 0.75 µl of human tears.

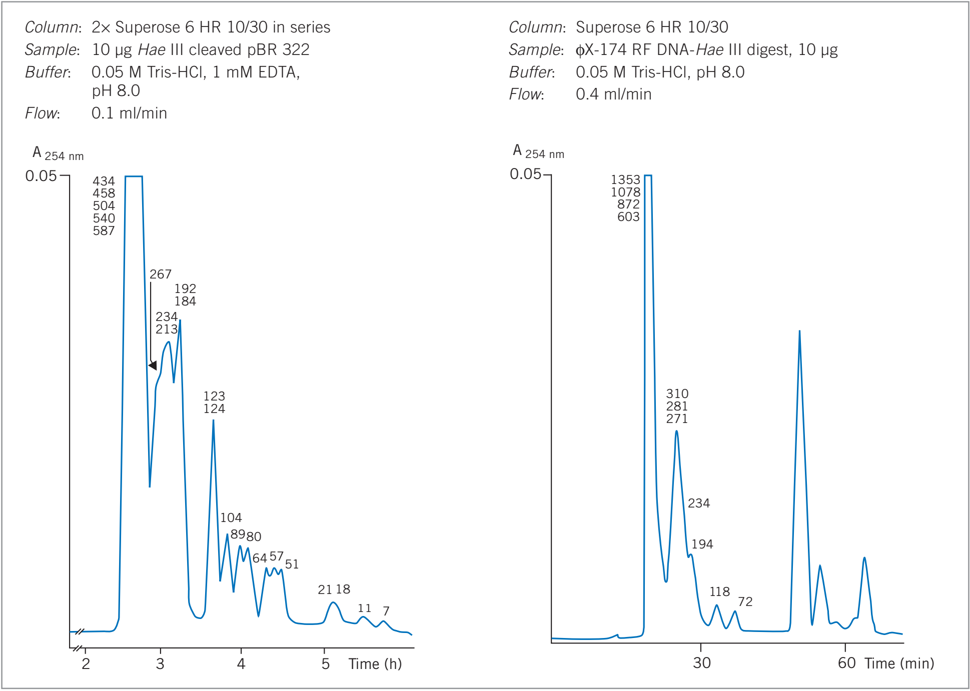


Fig. 40. Separation of DNA fragments on Superose 6 HR 10/30. Peak figures correspond to number of base pairs.

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 non-ionic 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.

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 96 for how to check column efficiency.

See page 26 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 re-packed.

Cleaning

1. Wash with 1 column volume 0.5 M NaOH at 40 cm/h (0.5 ml/min for HR 10/30 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 only, 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 carried out extremely carefully to avoid serious loss of resolution.

Reversing flow through a gel filtration column 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.

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 a column before autoclaving as certain column components cannot tolerate such high temperatures.

Media characteristics

Composition: Superose is formed from highly 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.

Product	Efficiency: theoretical plates per meter* (prepacked columns only)	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	9–13 µm	11 µm
Superose 12 prep grade	*	Long term: 3–12 Short term: 1–14	20–40 µm	30 µm

** A minimum column efficiency of 10 000 m^{-1} should be expected for a well packed column.*

† 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 Amersham Biosciences.

Chemical stability

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 +25 °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 +25 °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.

Sephadex: rapid group separation of high and low molecular weight substances, such as desalting, buffer exchange and sample clean up

Sephadex is prepared by cross-linking dextran with epichlorohydrin, illustrated in Figure 41.

Fig. 41. Partial structure of Sephadex.

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, as shown later on page 68 Media characteristics.

- 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 5 000. Using different particle sizes enables columns to be packed according to application requirements, see below. 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.

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 flow rate at a low operating pressure is essential, e.g. large scale
Coarse	For batch procedures

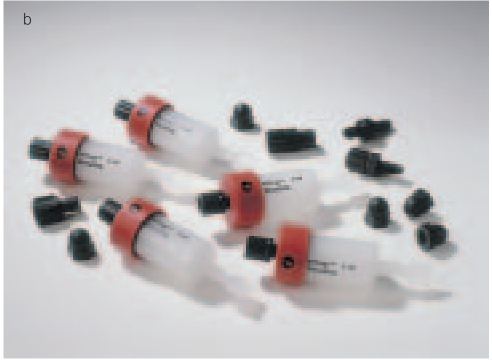


Fig. 42. Prepacked columns: a) HiPrep 26/10 Desalting, b) HiTrap Desalting 5 ml.

Dialysis is frequently mentioned in the literature as a technique to remove salt or other small molecules and to change the buffer composition of a sample. However, dialysis is generally a very slow technique, requiring large volumes of buffer. During handling or as a result of proteolytic breakdown or non-specific binding to the dialysis membranes, there is a risk of losing material. A simpler and much faster technique is to use a desalting column, packed with Sephadex G-25, for group separation between high and low molecular weight substances. Proteins are separated from salts and other small molecules.

In a fast, single step, the sample is desalted, transferred into a new buffer and low molecular weight materials are removed. The high speed and high volume capacity of the separation enables even large sample volumes to be processed rapidly and efficiently. Sample volumes up to 30% of the total volume of the desalting column can be applied and separated at much higher flow rates than those used for high resolution fractionation, as illustrated in Figure 43.

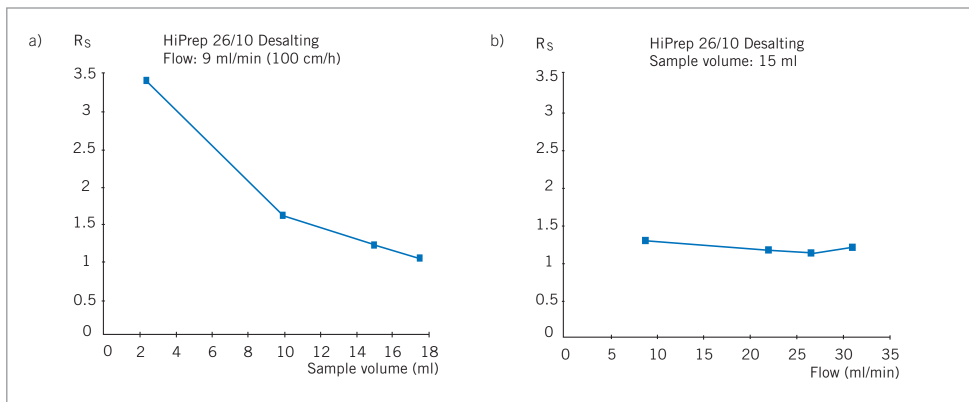


Fig. 43. a) Influence of sample volume on resolution. b) Influence of flow rate on resolution.

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.

Product	Exclusion limit	Sample loading capacity	Sample elution volume (for maximum sample loading)	Maximum operating back pressure (MPa, bar, psi)	Recommended operating flow
HiTrap Desalting 5 ml (Sephadex G-25 Superfine)	$>5\times10^3$	0.25–1.5 ml	1.0–2.0 ml	0.3 MPa, 3 bar, 42 psi	1–10 ml/min
HiPrep 26/10 Desalting (Sephadex G-25 Fine)	$>5\times10^3$	2.5–15 ml	7.5–20 ml	0.15 MPa, 1.5 bar, 22 psi	9–31 ml/min
PD-10 (Sephadex G-25 Medium)	$>5\times10^3$	1.5–2.5 ml	2.5–3.5 ml	Run under gravity	Run under gravity
NICK*	$>3\times10^4$	<0.1 ml	0.4 ml	Run under gravity	Run under gravity
MicroSpin™ G-25†	$>5\times10^3$	10–100 µl	–	Centrifuge	Centrifuge
NAP-5*	$>5\times10^3$	<0.5 ml	1.0 ml	Run under gravity	Run under gravity
NAP-10*	$>5\times10^3$	<1.0 ml	1.5 ml	Run under gravity	Run under gravity
NAP-25*	$>5\times10^3$	<2.5 ml	3.5 ml	Run under gravity	Run under gravity
Sephadex G-25 Superfine	$>5\times10^3$	n.a.	n.a.	Darcy's law applies‡ applies‡	Darcy's law
Sephadex G-25 Fine	$>5\times10^3$	n.a.	n.a.	Darcy's law applies‡	Darcy's law applies‡
Sephadex G-25 Medium	$>5\times10^3$	n.a.	n.a.	Darcy's law applies‡	Darcy's law applies‡
Sephadex G-25 Coarse	$>5\times10^3$	n.a.	n.a.	Darcy's law applies‡	Darcy's law applies‡
Sephadex G-50 Fine	$>3\times10^4$	n.a.	n.a.	Darcy's law applies‡	Darcy's law applies‡
Sephadex G-10	>700	n.a.	n.a.	Darcy's law applies‡	Darcy's law applies‡

** NICK columns are packed with Sephadex G-50 Fine DNA Grade and NAP columns are packed with Sephadex G-25 Medium DNA Grade.*

† A range of MicroSpin columns is available for desalting of proteins and purification of labeled DNA fragments and PCR products. Refer to the BioDirectory catalogue from Amersham Biosciences for further details.

‡ 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 the flow rate doubles the column pressure. See Appendix 2 for an explanation of Darcy's law.

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

Reference lists highlighting the use of HiPrep 26/10 Desalting and HiTrap Desalting are available at *www.chromatography.amershambiosciences.com*.

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 44.

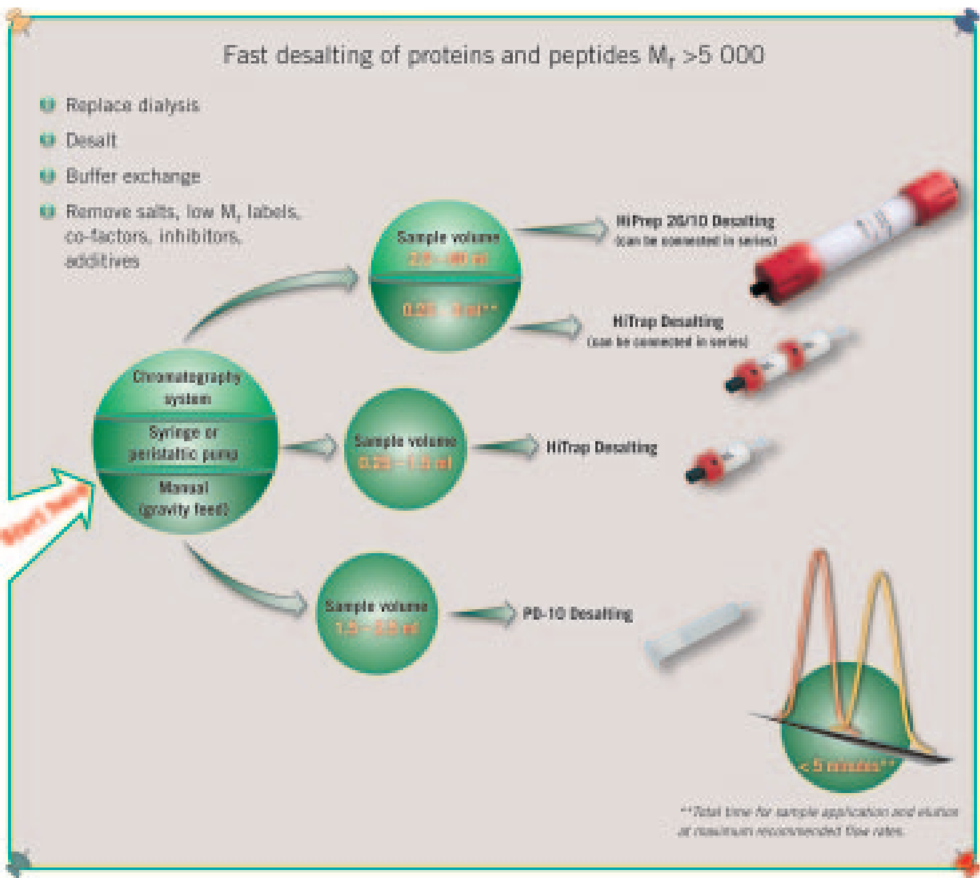


Fig. 44. Selecting prepacked columns for desalting and buffer exchange.

Separation examples

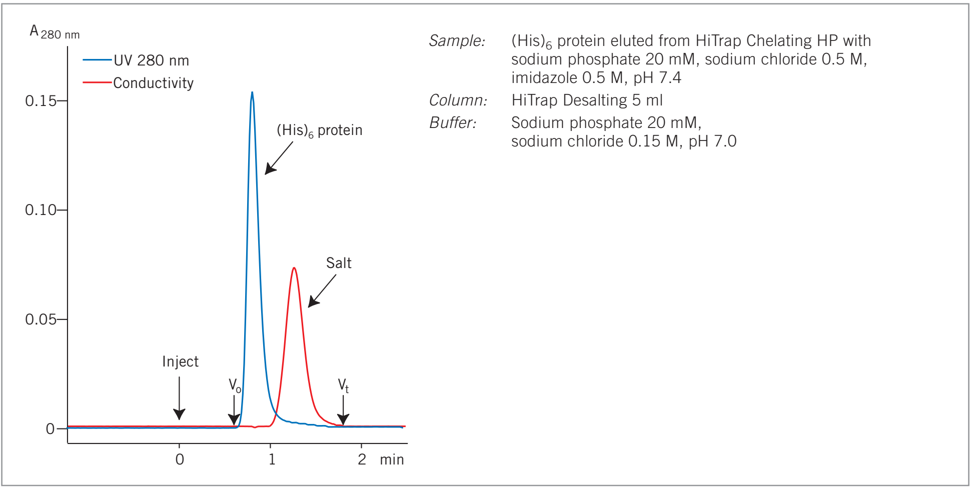


Fig. 45. Desalting a $(His)_6$ fusion protein using HiTrap Desalting 5 ml on ÄKTA™prime. The UV (protein) and conductivity (salt) traces enable pooling of the desalted fractions and facilitate optimization of the separation.

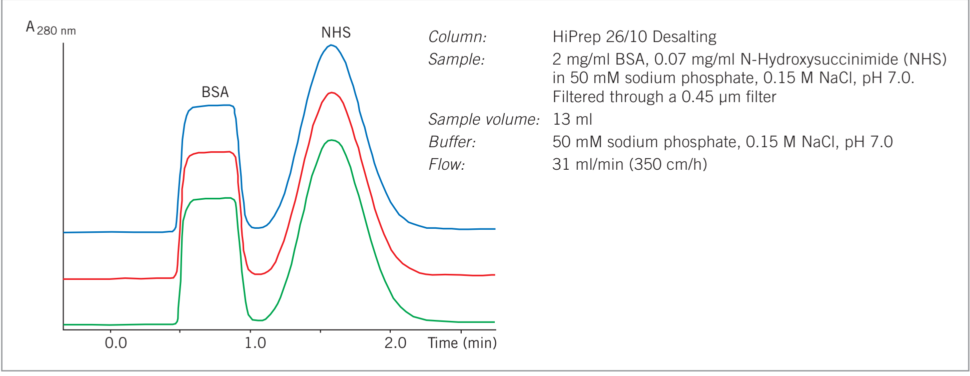


Fig. 46. Reproducible removal of N-Hydroxysuccinimide from bovine serum albumin.

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 47.

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.

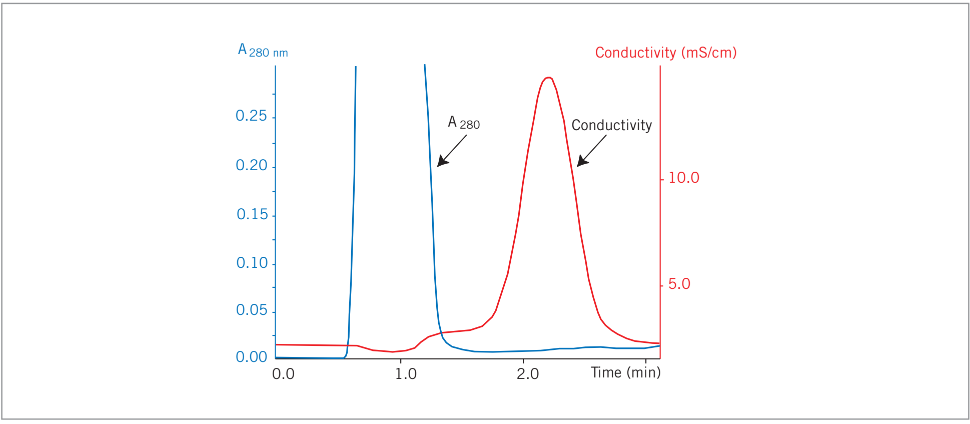


Fig. 47. Buffer exchange of mouse plasma on HiPrep 26/10 Desalting.

Alternative 1: Using a HiTrap column with a syringe

The maximum recommended sample volume is 1.5 ml. The table below shows the effect of reducing the sample volume applied to the column.

Table 1. Recommended sample and elution volumes using a syringe or Multipipette™ with HiTrap Desalting 5 ml

Sample load	Add buffer	Elute and collect	Yield %	Remaining salt %	Dilution factor
0.25 ml	1.25 ml	1.0 ml	> 95	0.0	4.0
0.50 ml	1.0 ml	1.5 ml	> 95	< 0.1	3.0
1.00 ml	0.5 ml	2.0 ml	> 95	< 0.2	2.0
1.50 ml	0 ml	2.0 ml	> 95	< 0.2	1.3

Step 3

Step 4

Step 6

1. Fill the syringe with buffer. Unscrew the stop plug at the top of the column. To avoid introducing air into the column, connect the column "drop to drop" to the syringe (via the adapter provided).
2. Remove the twist-off end.
3. Wash the column with 25 ml buffer at 5 ml/min to completely remove the 20% ethanol (supplied as storage buffer). If air is trapped in the column, wash with degassed buffer until the air disappears. Air bubbles introduced onto the column by accident during sample application do not influence the separation.
4. Apply the sample using a 2–5 ml syringe at a flow rate between 1–10 ml/min. Discard the liquid eluted from the column.
5. If the sample volume is less than 1.5 ml, change to buffer and proceed with the injection until a total of 1.5 ml has been eluted. Discard the eluted liquid.
6. Elute the protein with the appropriate volume selected from Table 1.

Collect the desalted protein in the volume indicated.

Note: 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column.

A simple peristaltic pump can also be used to apply sample and buffers.

Alternative 2: Simple desalting with ÄKTAprime

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

Buffer Preparation

Prepare at least 500 ml of the required buffer

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

Alternative 3: 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 48 shows an example of the influence of flow rate on group separation.
4. Determine the maximum sample volume that can be loaded. Figure 49 shows an example of the influence of sample volume on group separation.

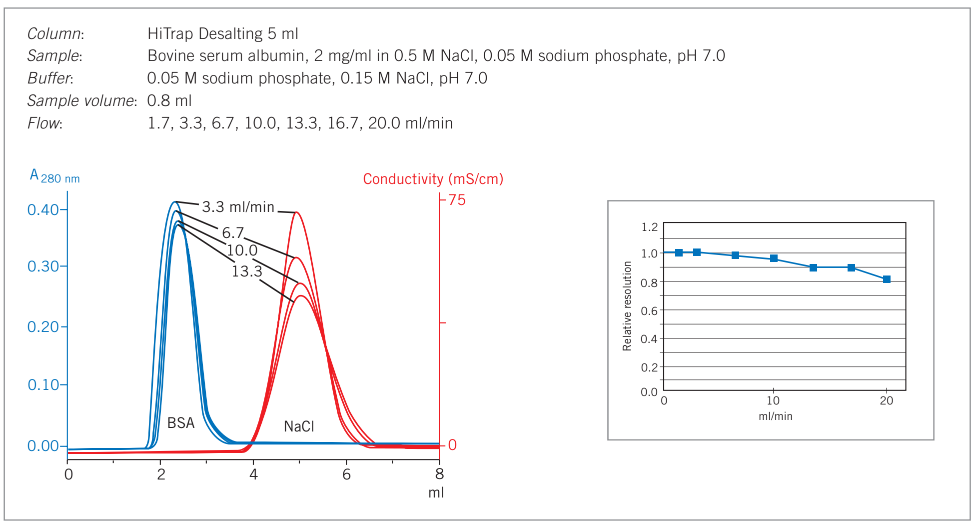


Fig. 48. Influence of flow rate on separation using a HiTrap Desalting column.

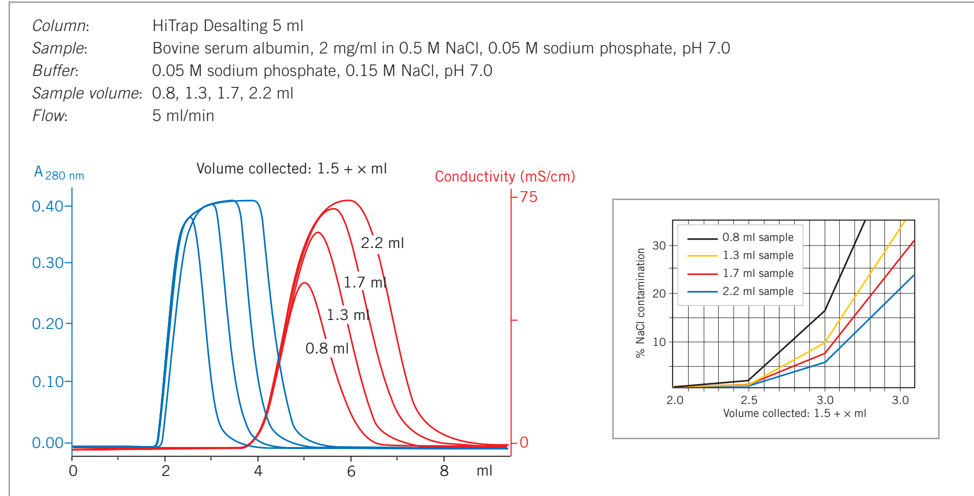


Fig. 49. Influence of sample volume on separation using a HiTrap Desalting column.

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. Table 1 on page 63 illustrates this effect when using a HiTrap Desalting 5 ml column.

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 large sample volumes

Connecting columns in series increases the effective column volume and so increases sample loading capacity. Table 2 shows the sample loading capacities and dilution factors when using prepacked desalting columns alone or in series, see also Figure 50 for HiTrap application examples.

Table 2. Selection guide for desalting/buffer exchange columns

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

Increasing sample loading capacity from 1.5 ml up to 7.5 ml

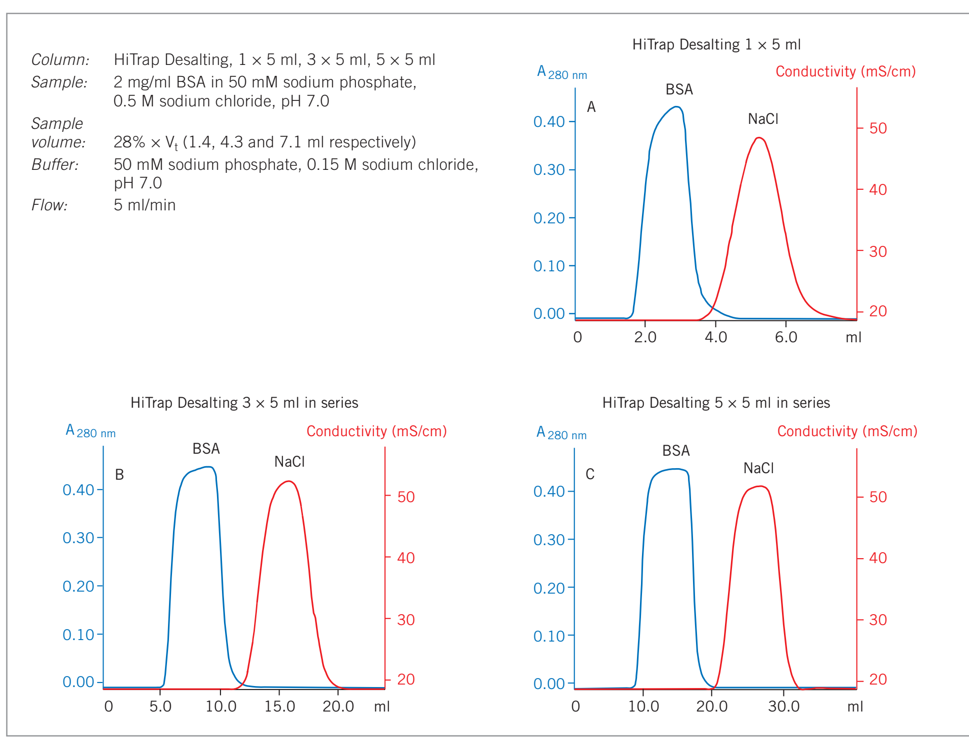


Fig. 50. Scale up using HiTrap columns connected in series.

Increasing sample loading capacity from 15 ml up to 60 ml

Connect HiPrep 26/10 Desalting columns in series, e.g. 2 columns: sample volume 30 ml, 4 columns: sample volume 60 ml, as shown in Figure 51. 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.

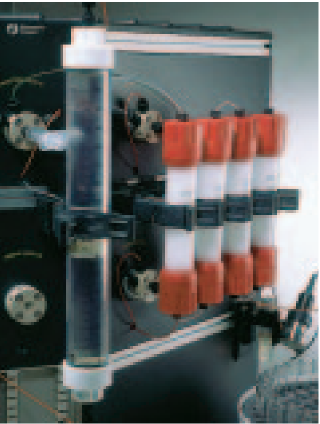


Fig. 51. Four HiPrep 26/10 Desalting columns connected in series.

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 52.

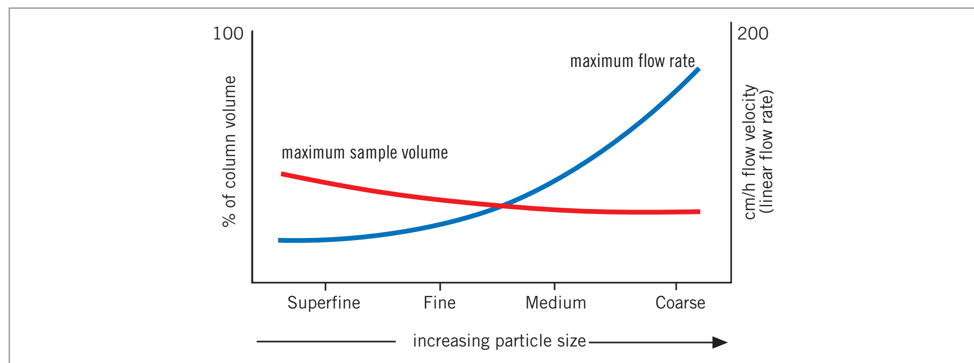


Fig. 52. Sephadex G-25: recommended sample volumes and flow rates vary with particle size.

- 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 Coarse and Medium 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.

Product	Fractionation range, M_r (globular proteins)	pH stability*	Bed volume ml/g dry Sephadex	Maximum operating flow	Particle size, wet
Sephadex G-10	<7×10^2	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†	170–520 µm
Sephadex G-25 Medium	1×10^3–5×10^3	Long term: 2–13 Short term: 2–13	4–6	Darcy's law†	85–260 µm
Sephadex G-25 Fine	1×10^3–5×10^3	Long term: 2–13 Short term: 2–13	4–6	Darcy's law†	35–140 µm
Sephadex G-25 Superfine	1×10^3–5×10^3	Long term: 2–13 Short term: 2–13	4–6	Darcy's law†	17–70 µm
Sephadex G-50 Fine	1×10^3–3×10^4	Long term: 2–10 Short term: 2–13	9–11	Darcy's law†	40–160 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 Amersham Biosciences.*

† 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, NAP, NICK 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 +25 °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 +25 °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 3
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 at A_{280}nm) of sample components as they elute from the column in order of their molecular size (Figure 53). Molecules that do not enter the matrix are eluted in the *void volume*, V_o as they pass directly through the column at the same speed as the flow of buffer. Molecules with partial access to the pores of the matrix elute from the column in order of decreasing size. Molecules with 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.

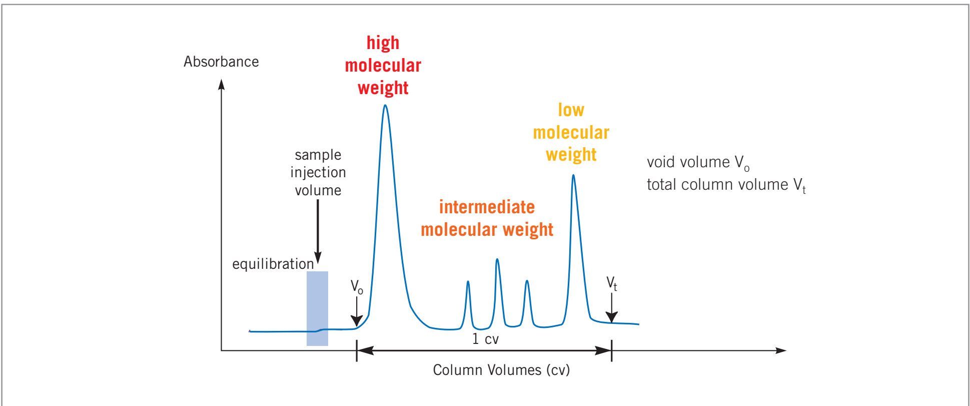


Fig. 53. Theoretical chromatogram of a high resolution fractionation.

The behavior of each component can be expressed in terms of its *elution volume*, V_e, determined by direct measurement from the chromatogram.

As shown in Figure 54 there are three different ways of measuring V_e, dependent on the volume of sample applied to the column.

A. When very small samples are applied (small enough to be neglected compared to the elution volume), take the position of the peak maximum in the elution diagram as V_e.

B. If the sample volume cannot be neglected compared with the elution volume, measure the elution volume from half the sample volume to the position of the peak maximum.

C. When very large sample volumes are used (giving a plateau region in the elution curve), take the volume eluted from the start of sample application to the inflexion point (or half height) of the rising part of the elution peak as V_e.

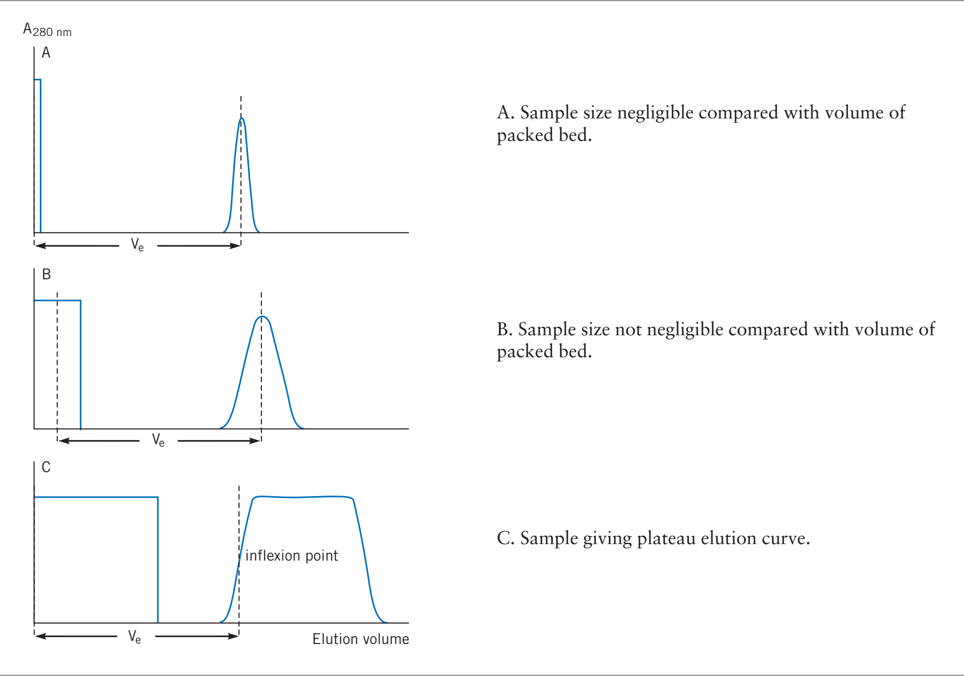


Fig. 54. Measurement of elution volume, V_e.

Since symmetrical peaks are common in gel filtration, elution volumes are easily determined. However, V_e does not completely define the behavior of the sample since 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) derived as follows:

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

The *volume of the stationary phase*, V_s, is equal to V_i, *the volume of buffer inside the matrix which is available to very small molecules*, i.e. the elution volume of molecules that distribute freely between the mobile and stationary phases minus the void volume.

Since, in practice, V_s or V_i are difficult to determine, it is more convenient to substitute 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.

K_d represents *the fraction of the stationary phase that is available for diffusion of a given molecular species*. The stationary phase V_s can be can be substituted by the term $(V_t - V_o)$ in order to obtain a value K_{av}.

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

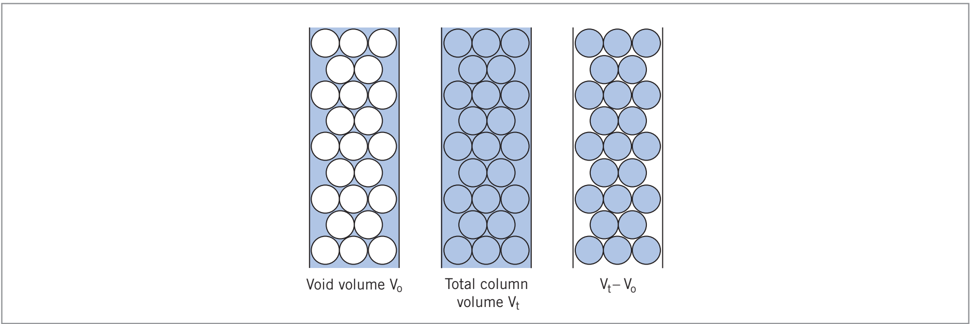


Fig. 55. Diagrammatic representation of V_t and V_o. Note that $V_t - V_o$ will include the volume of the solid material which forms the matrix (Fischer, L. Laboratory Techniques in Biochemistry and Molecular Biology. Vol. 1 part II. An Introduction to Gel Chromatography. North Holland Publishing Company, Amsterdam. Reproduced by kind permission of the author and publisher).

Since $(V_t - V_o)$ 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 56.

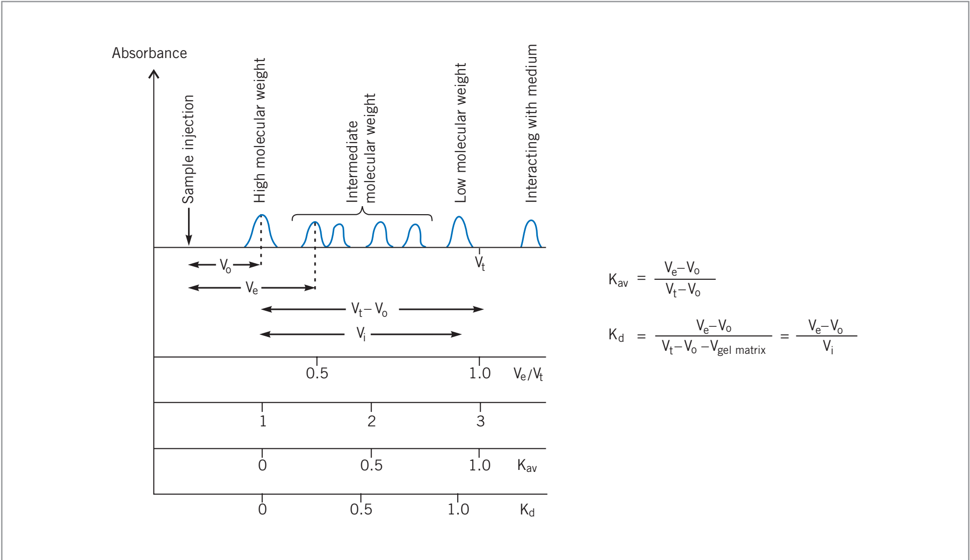


Fig. 56. Relationship between several expressions used for normalizing elution behavior.

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 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 57.

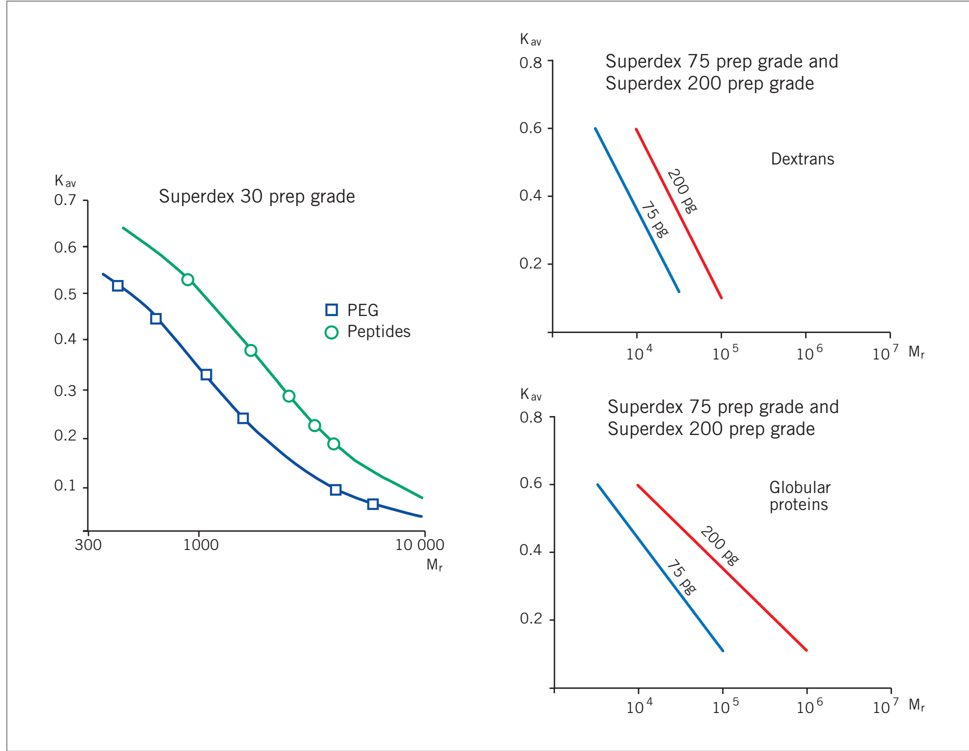


Fig. 57. Selectivity curves for Superdex 30 prep grade, Superdex 75 prep grade and Superdex 200 prep grade.

Gel filtration media should be selected so that the high molecular weight molecules are eluted at the void volume (K_{av} = 0) 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 non-specifically to the medium.

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

Resolution

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 (R_s) is defined by the following expression:

$$R_s = \frac{V_{r2} - V_{r1}}{\frac{(W_1 + W_2)}{2}}$$

V_{r1} and V_{r2} 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.

$(V_{r2} - V_{r1})$ 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 58.

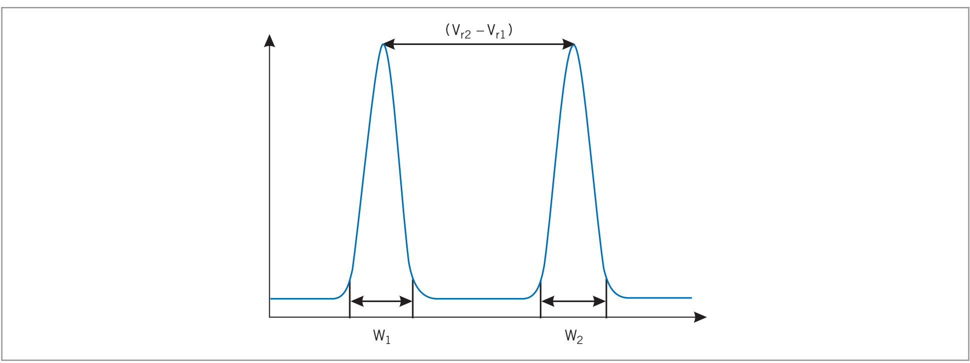


Fig. 58. Parameters used to define resolution (R_s).

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 59.

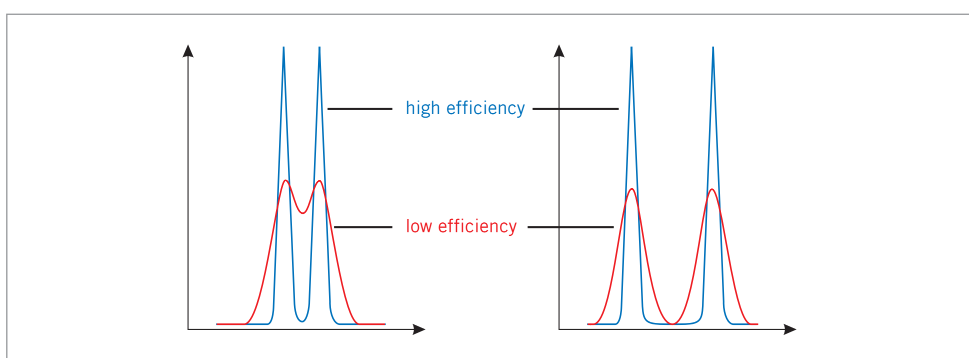


Fig. 59. Dependence of resolution on selectivity and the counteraction of peak broadening.

The efficiency of a packed column defines its ability to produce narrow symmetrical peaks during elution. Refer to Appendix 1 for column packing and preparation and determination of column efficiency. Efficiency is particularly important in gel filtration in which separation takes place as only a single column volume of buffer passes through the column. 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

Efficiency can be improved by decreasing the particle size of the medium. However, using a smaller particle size may create an increase in back pressure so that flow rate needs to be decreased, lengthening the run time.

The *uniformity 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* (lower particle size distribution) facilitate the elution of molecules in narrow 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 sample passes through the column and diffusion occurs. In order to minimize sample dilution a maximum sample volume is used within the limits set by the separation distance i.e. the resolution required between the peaks of interest. The sample can be regarded as a zone passing down the column. Figure 60 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.

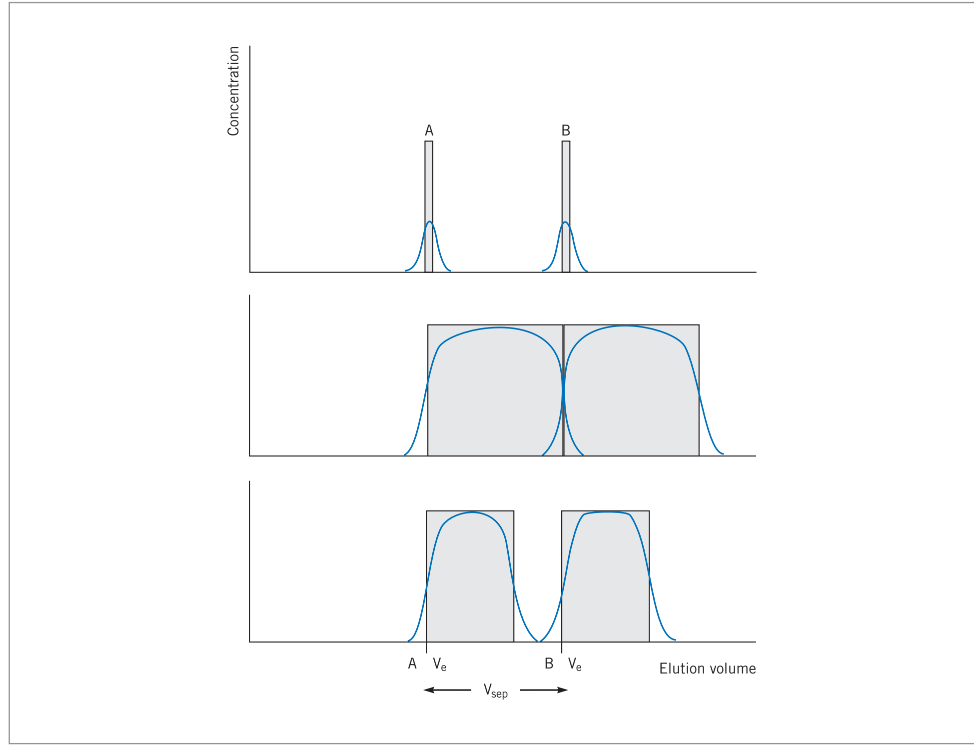


Fig. 60. Elution curves for different sample sizes. The top diagram corresponds to the application of a small sample. The center diagram corresponds to the maximum sample volume that gives complete separation if there is no zone broadening. The bottom diagram corresponds to the maximum sample volume 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 4
Molecular weight determination 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 3 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 sigmodial 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 accurate determination 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 Amersham Biosciences provide well-characterized, globular protein standards for protein molecular weight determination. The Low Molecular Weight Gel Filtration Calibration Kit contains 4 individually lyophilized proteins with molecular weights in the range 13 700–67 000 and Blue Dextran 2000. The High Molecular Weight Gel Filtration Calibration Kit contains 4 individually lyophilized proteins with molecular weights in the range 158 000–669 000 and Blue Dextran 2000.

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 selection guide page 18).

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, 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 proteins 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

   On semilogarithmic graph paper, plot the K_{av} value for each protein standard (on the linear scale) against the corresponding molecular weight (on the logarithmic scale). Draw the straight line which best fits the points on the graph. Alternatively, use a statistics package 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.

Chapter 5
Sephadex LH-20

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. The partial structure of Sephadex LH-20 is shown in Figure 61.

Fig. 61. Partial structure of Sephadex LH-20.

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.

For more detailed information on gel filtration in organic solvents refer to *Preparative Gel Chromatography on Sephadex LH-20 by H. Henke*, available from Amersham Biosciences.

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.

Separation options

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/hr) (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 62.

Fig. 62. Structure of free acid form of repandusinic acid A.

Table 3 shows the recovery of active inhibitor from an analytical separation on Sephadex LH-20.

Table 3. Summary of data for the isolation of repandusinic acid A from *P. niruri*

Purification step	Yield (mg)	ID50* (μg/ml)	Specific activity ($\times 10^2$ IU/mg)	Total activity ($\times 10^3$ IU) †
H_2O extract	6 600	50	4	2 640
MeOH insoluble	2 500	20	10	2 500
Sephadex LH-20 Fr. 4–11‡	247	3.0–3.6	56–67	1 616
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 63 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.

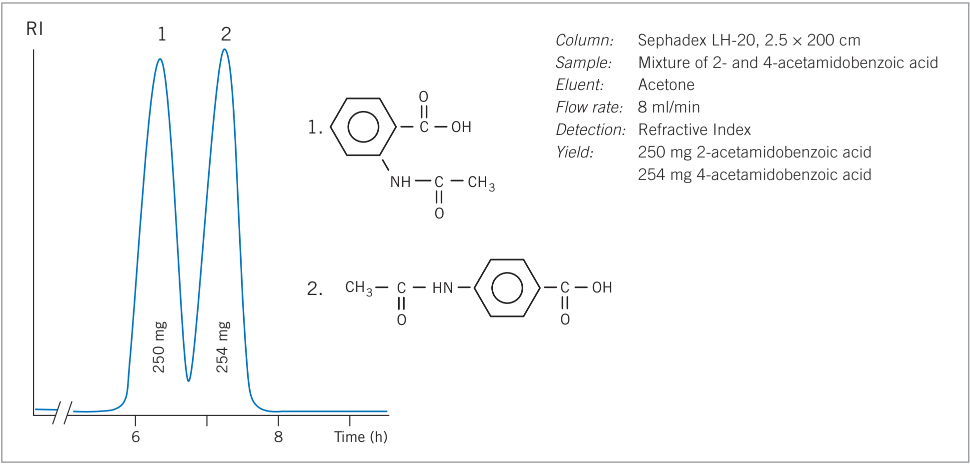


Fig. 63. Separation of 2- and 4-acetamidobenzoic acid on Sephadex LH-20.

Packing a column

Sephadex LH-20 should be packed in a solvent resistant column selected from Table 4 according to the column volume required for the separation.

Table 4. Solvent resistant columns (*SR 10/50J includes a borosilicate glass jacket. Jackets are not available for other SR columns. All SR columns are supplied with two adaptors for top and bottom assembly)

Column	Volume (ml)	Bed height (cm)
SR 10/50	16–39	20–50
SR 10/50J*	16–39	20–50
SR 25/45	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 5, page 85, 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.

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. The lower the flow, the 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.

Medium characteristics

	pH stability	Bed volume ml/g dry Sephadex LH-20	Particle size
Sephadex LH-20	Working: 2–11 *Short term: 2–13	Depends on solvent, see table 5	Dry: 18–111 μm Wet: depends on solvent

** 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 Amersham Biosciences.*

The wet particle size for Sephadex LH-20 varies according to the solvent used for swelling, as shown in Table 5.

Table 5. 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 ml/g 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 +25 °C. Store packed columns and used medium at +4 °C to +8 °C in the presence of a bacteriostatic agent.

Transferring Sephadex LH-20 between 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 64.

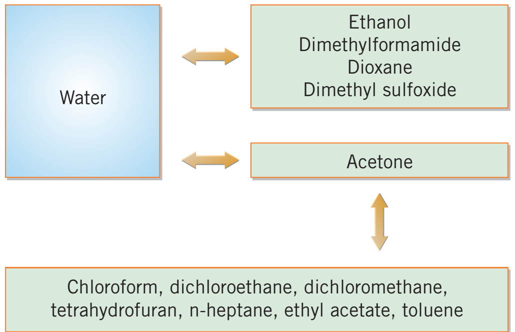


Fig. 64. Suggested routes for changing to organic solvents.

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 10/50, SR 25/45 or SR 25/100 columns.

Chapter 6
Gel filtration in a Purification Strategy (CIPP)

For a high degree of purity, or when a suitable ligand is unavailable for a single step affinity purification, an efficient multi-step process must be developed using the purification strategy of Capture, Intermediate Purification and Polishing (CIPP).

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 multi-step protein purification. *The Protein Purification Handbook* from Amersham Biosciences is an ideal guide for planning efficient and effective protein purification strategies and for the selection of the correct medium for each step and scale of purification. As shown in Figure 65, 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), when 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.

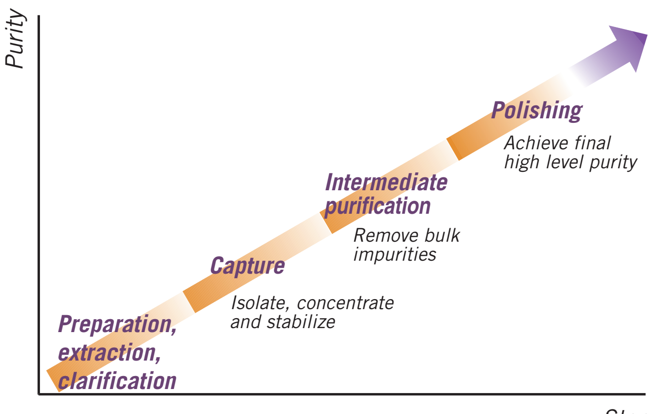


Fig. 65. Preparation and CIPP.

Applying CIPP

Imagine the purification has three phases: Capture, Intermediate Purification and Polishing.

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

The purification problem associated with a particular 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 objectives are 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.

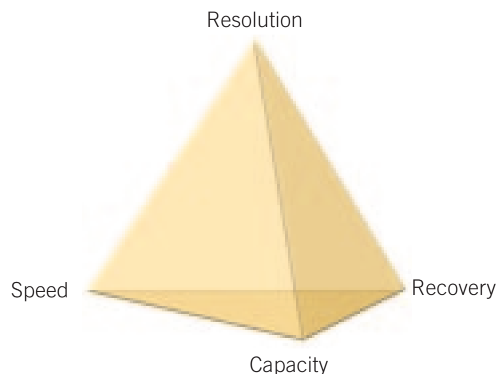
The optimal selection and combination of purification techniques for *Capture, Intermediate Purification and Polishing* is crucial for an efficient purification.

Selection and combination of purification techniques

Proteins are purified using purification techniques that separate according to differences in specific properties, as shown in Table 6.

Table 6. Protein properties used during purification

Protein property	Technique
Size	Gel filtration (GF)
Charge	Ion exchange (IEX)
Hydrophobicity	Hydrophobic interaction (HIC), Reversed phase (RPC)
Biorecognition (ligand specificity)	Affinity (AC)



Every technique offers a balance between resolution, capacity, speed and recovery.

Capacity, in the simple model shown, refers to the amount of target protein loaded during purification. In some cases the amount of sample that can be loaded will be limited by volume (as in gel filtration) or by large amounts of contaminants rather than the amount of the target protein.

Speed is most important at the beginning of purification where contaminants, such as proteases, must be removed as quickly as possible.

Recovery becomes increasingly important as the purification proceeds because of the increased value of the purified product. Recovery is influenced by destructive processes in the sample and by unfavourable conditions on the column.

Resolution is achieved by the selectivity of the technique and the efficiency of the chromatography matrix in producing narrow peaks. In general, resolution is most difficult to achieve in the final stages of purification when impurities and target protein are likely to have very similar properties.

Select a technique to meet the objectives for the purification step. Choose logical combinations of purification techniques based on the main benefits of the technique and the condition of the sample at the beginning or end of each step.

A guide to the suitability of each purification technique for the stages in CIPP is shown in Table 7.

Table 7. Suitability of purification techniques for CIPP

Technique	Main features	Capture	Intermediate	Polishing	Sample start condition	Sample end condition
IEX	high resolution high capacity high speed	★★★	★★★	★★★	low ionic strength sample volume not limiting	high ionic strength or pH change **concentrated sample**
HIC	good resolution good capacity high speed	★★	★★★	★	high ionic strength sample volume not limiting	low ionic strength **concentrated sample**
AC	high resolution high capacity high speed	★★★	★★★	★★	specific binding conditions sample volume not limiting	specific elution conditions **concentrated sample**
GF	high resolution using Superdex media		★	★★★	limited sample volume (<5% total column volume) and flow rate range	buffer exchanged (if required) diluted sample
RPC	high resolution		★	★★★	sample volume usually not limiting additives may be required	in organic solvent, risk loss of biological activity

Minimize sample handling between purification steps by combining techniques to avoid the need for sample conditioning. The product should be eluted from the first column in conditions suitable for the start conditions of the next column (see Table 7).

Ammonium sulfate, often used for sample clarification and concentration (see Appendix 3), leaves the sample in a high salt environment. Consequently HIC, which requires high salt to enhance binding to the media, becomes the ideal choice as the capture step. The salt concentration and the total sample volume will be significantly reduced after elution from the HIC column. Dilution of the fractionated sample or rapid buffer exchange using a desalting column will prepare it for the next IEX or AC step.

Gel filtration is a non-binding technique unaffected by buffer conditions, but with limited volume capacity. GF is well suited for use after any of the concentrating techniques (IEX, HIC, AC) since the target protein will be eluted in a reduced volume and the components from the buffer will not affect the gel filtration process.

Selection of the final strategy will always depend upon specific sample properties and the required level of purification. Logical combinations of techniques are shown in Figure 66.

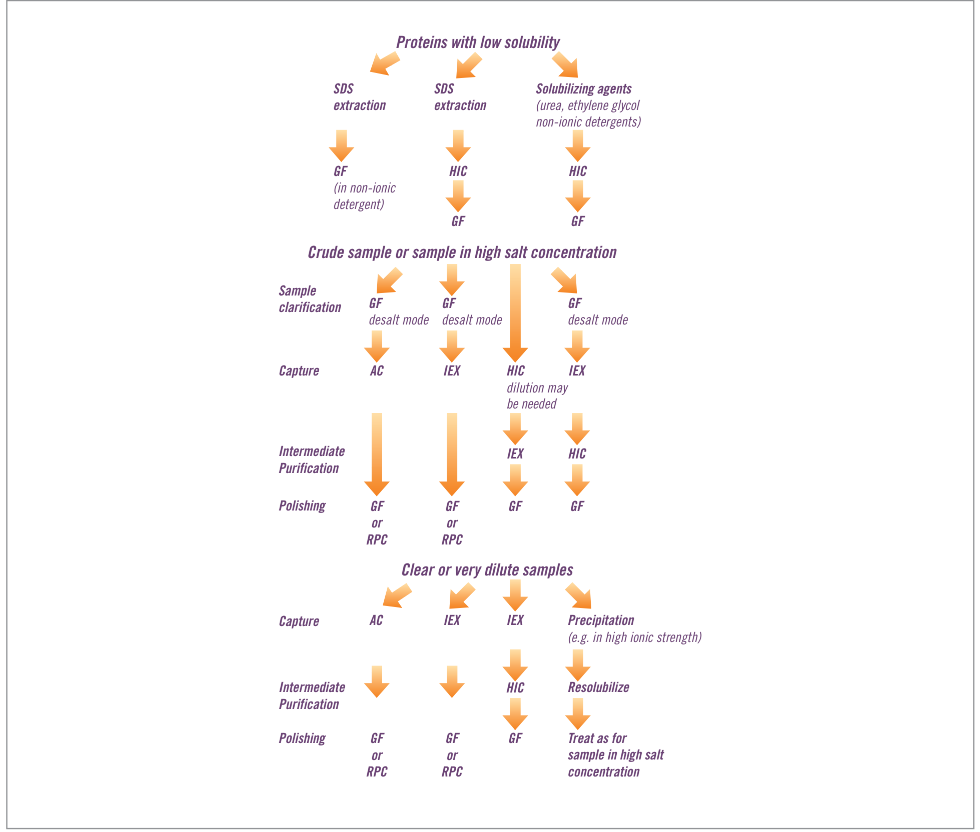


Fig. 66. Logical combinations of chromatography techniques.

For any capture step, select the technique showing the most effective binding to the target protein while binding as few of the contaminants as possible, i.e. the technique with the highest selectivity and/or capacity for the target protein.

A sample is purified using a combination of techniques and alternative selectivities. For example, in an IEX-HIC-GF strategy, the capture step selects according to differences in charge (IEX), the intermediate purification step according to differences in hydrophobicity (HIC) and the final polishing step according to differences in size (GF).

If nothing is known about the target protein use IEX-HIC-GF. This combination of techniques can be regarded as a standard protocol.

IEX is a technique which offers different selectivities using either anion or cation exchangers. The pH of the purification can be modified to alter the charge characteristics of the sample components. It is therefore possible to use IEX more than once in a purification strategy, for capture, intermediate purification or polishing. IEX can be used effectively in the same purification scheme for rapid purification in low resolution mode during capture and in high resolution mode during polishing.

Consider the use of both anion and cation exchange chromatography to give different selectivities within the same purification strategy.

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 67.

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.

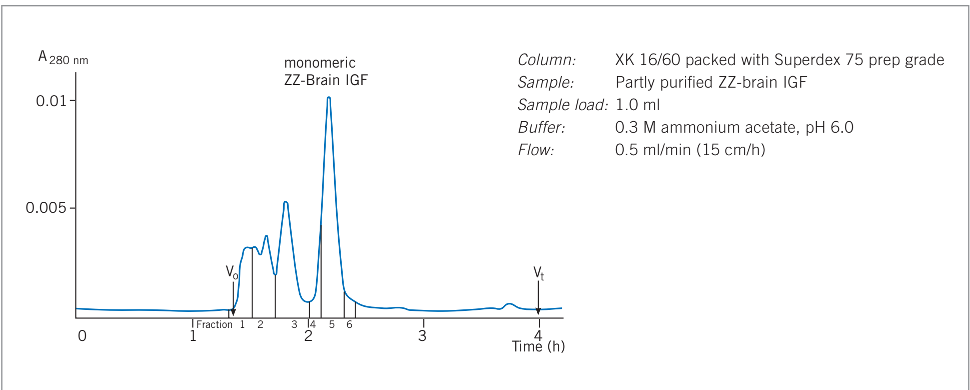


Fig. 67. Final polishing step: separation of dimers and multimers on Superdex 75 prep grade.

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.

RPC can also be considered for a polishing step, provided that the target protein can withstand the run conditions. Reversed phase chromatography (RPC) separates proteins and peptides on the basis of hydrophobicity. RPC is a high selectivity (high resolution) technique, usually requiring the use of organic solvents. The technique is widely used for purity check analyses when recovery of activity and tertiary structure are not essential. Since many proteins are denatured by organic solvents, RPC is not generally recommended for protein purification because recovery of activity and return to a correct tertiary structure may be compromised. However, in the polishing phase, when the majority of protein impurities have been removed, RPC can be an excellent technique, particularly for small target proteins that are not often denatured by organic solvents.

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 humanised IgG_4 monoclonal antibody

A humanized IgG_4 monoclonal antibody was expressed in a myeloma cell culture and purified by a combination of affinity chromatography and gel filtration. The antibody was captured by affinity chromatography using MabSelect™. Gel filtration on Superdex 200 prep grade was then used to separate the monomer from the dimer and larger polymers.

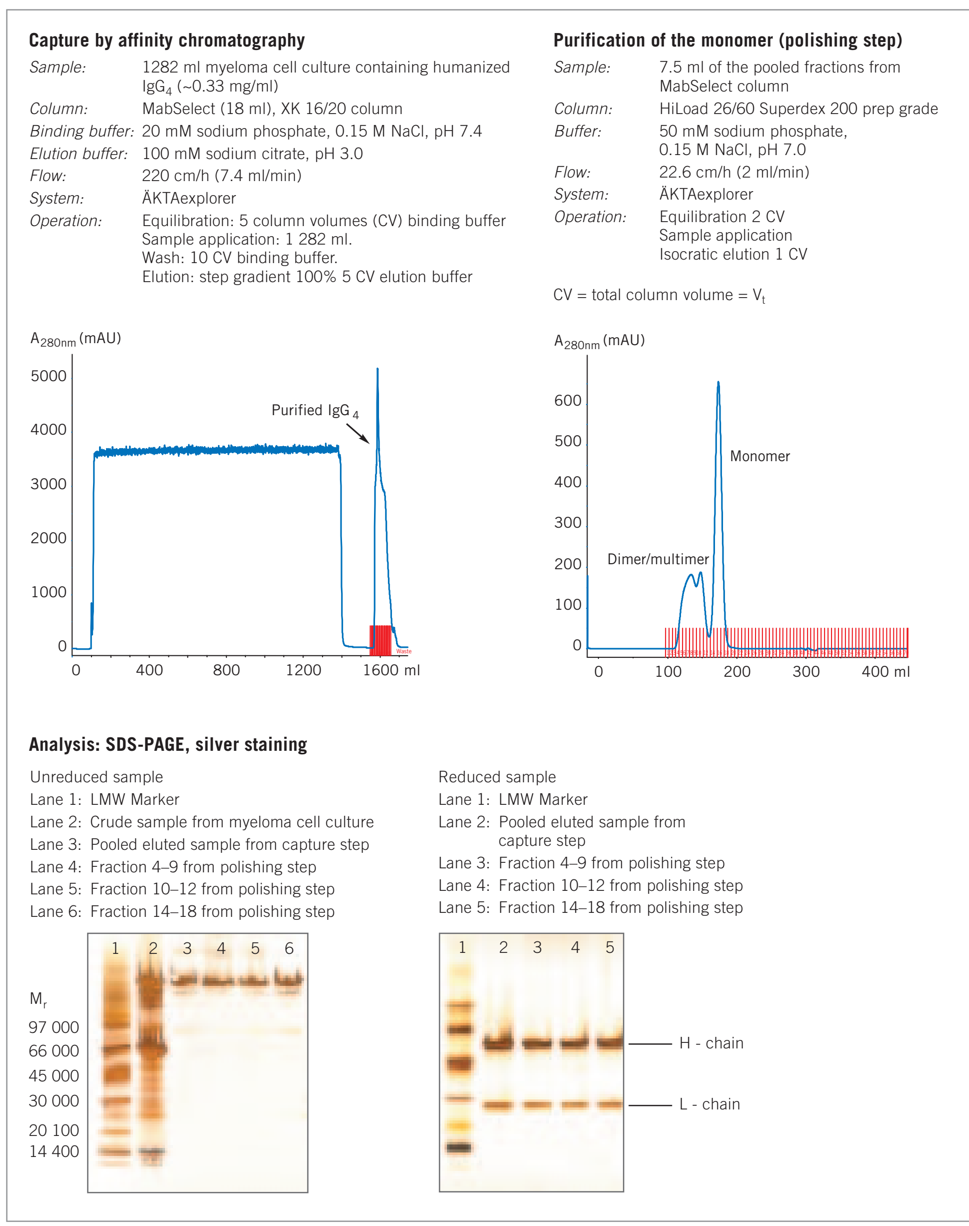


Fig. 68. Two step purification of humanized IgG_4.

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 Amersham Biosciences will ensure reproducible results and the highest performance. If the column volume or medium you require is not available as a prepacked column, contact you local Amersham Biosciences 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, loss of resolution and 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.

A Column Packing Video is available to demonstrate how to produce a well-packed column (see Ordering information). The video 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

XK columns are fully compatible with the high flow rates achievable with modern media and a broad range of column dimensions is available. Ordering information for XK columns and main accessories can be found at the back of this handbook. For a complete listing of all spare parts refer to the Amersham Biosciences BioDirectory catalogue or web catalogue (*www.chromatography.amershambiosciences.com*).

Table 8. XK columns for packing gel filtration media

Column	Column volume (ml) with one adaptor
XK 16/20	2–34
XK 16/40	42–74
XK 16/70	102–135
XK 16/100	163–195
XK 26/20	0–80
XK 26/40	122–196
XK 26/70	281–356
XK 26/100	440–315
XK 50/20	0–275
XK 50/30	330–510
XK 50/60	785–1099
XK 50/100	1570–1884

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.

All XK columns are delivered with one AK adaptor, but a second adaptor can be used instead of a column end piece if a shorter bed height is required. TEFZEL tubing with M6 connectors, a thermostatic jacket, snap-on support net rings, dismantling tool and instructions are supplied with the XK column.

Longer XK 50 columns can be difficult to pack under normal laboratory conditions. As alternatives, use our column packing services or connect two or more shorter XK 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

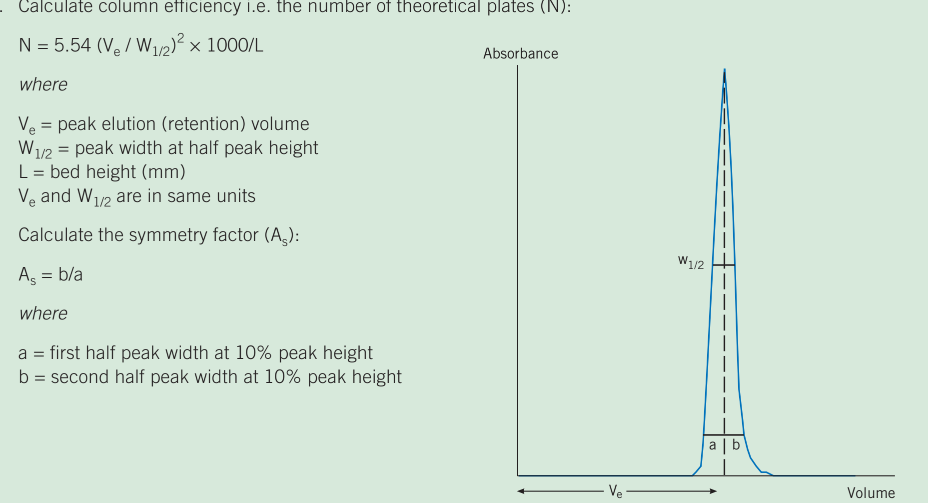
   V_e = peak elution (retention) volume
   $W_{1/2}$ = peak width at half peak height
   L = bed height (mm)
   V_e and $W_{1/2}$ are in same units

   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



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 simple 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 good resolution can be maintained.

The settled medium should have a volume of 1.15 times that of the required packed column volume, see Table 8 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 9 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 minutes. Table 9 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 9. 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 adapter 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 9 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 9. Recommended flow rates for packing Sephacryl HR and Superdex prep grade

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

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 ÄKTAdesign chromatography system. Accurate and reproducible flow control is particularly important for efficient column packing and when repeating experiments or performing routine preparative work.

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.

Do not exceed the maximum recommended values for pressure or linear flow for the medium (see Chapter 2). 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.

A peristaltic pump cannot achieve the highest flow rates or back pressures tolerated by Superdex and Sephacryl and so is not recommended for column packing or running high resolution fractionation on larger columns.

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 10). This also serves to degas the suspension. Allow the suspension to cool before use.

Table 10. Bed volume and swelling times for Sephadex

Medium	Approx. bed volume (ml/g)	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 matrix.
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.

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.

3. If the slurry volume is greater than the volume of the column, attach a packing reservoir to the column (Figure 69).
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 (Figure 70).

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. 69. Using a packing reservoir.

Fig. 70. Adding the top adaptor.

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 ÄKTAdesign 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.

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.

Packing under gravity

Sephadex can be packed using a gravity feed system in which flow rates are controlled by differences in hydrostatic pressure, that is the operating pressure created by the difference between the free surface of the buffer in the buffer container and the column outlet.

Use a safety loop as shown in Figure 71 to prevent air from entering the column.

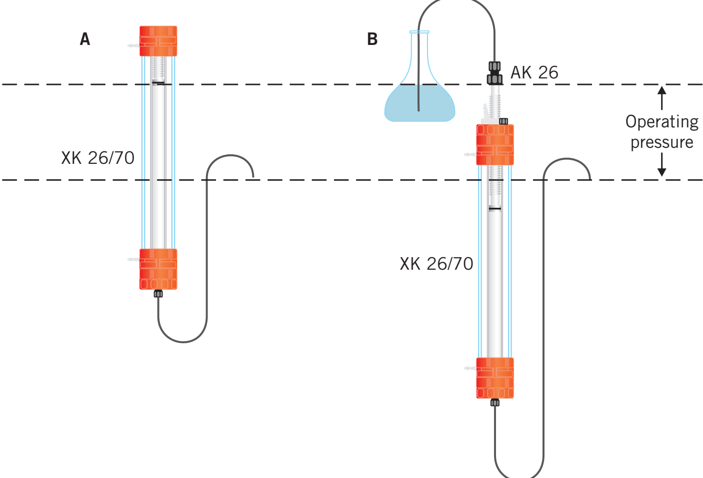


Fig. 71a. Definition of operating pressure A and B. Pressure (cm water) is measured as the distance between the free surface in the column or reservoir and the end of the outlet tubing.

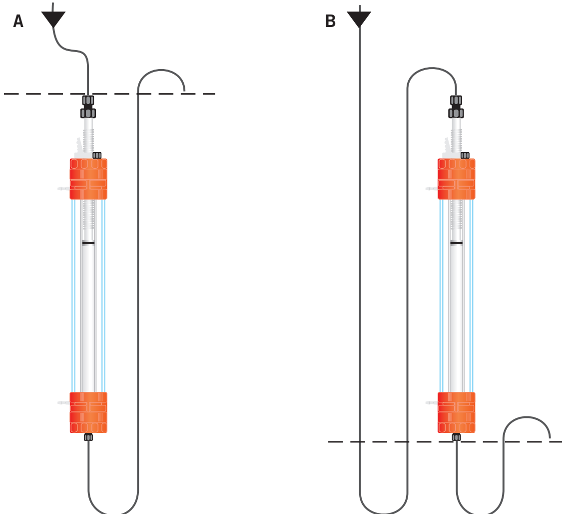


Fig. 71b. Alternative safety loop arrangements:

A. Place the safety loop after the column and place the end of the outlet tubing above the column. The flow stops when the buffer in the inlet tubing reaches the level of the outlet.

B. Place the safety loop before the column with the column outlet tubing in any position above the lower loop on the inlet side. The flow stops when the buffer in the inlet tubing reaches the level of the outlet.

Temperature influences the viscosity of the buffer. For a given pressure head, lower flow rates will be reached in a cold room than at room temperature.

Custom Designed Products

The Custom Products (CP) Group at Amersham Biosciences supplies prepacked columns, made according to the client's choice from our range of columns and media. Custom Designed Media (CDM) can be produced for specific industrial process separations when suitable media are not available from the standard range. The CDM group at Amersham Biosciences works in close collaboration with the user to design, manufacture, test and deliver media for specialized separation requirements. When a chromatography step is developed to be an integral part of a manufacturing process, the choice of column is important to ensure consistent performance and reliable operation. Amersham Biosciences provides a wide range of columns that ensures the highest performance from all our purification media and meets the demands of modern pharmaceutical manufacturing. Please ask your local representative for further details of CP and CDM products or services.

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:

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

U = linear flow rate expressed in cm/h (see Appendix 5).

ΔP = pressure drop over bed expressed in cm H_2O

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: $U = K_o \Delta P L^{-1}$ (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 and so maintaining 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 11.

Table 11. 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, Protein Amplification and Simple Purification* and *Antibody Purification Handbook*, available from Amersham Biosciences.

Sample stability

In the majority of cases, biological activity needs to be retained after purification. Retaining the activity of the target molecule is also an advantage when following the progress of the purification, since detection of the target molecule often relies on its biological activity. Denaturation of sample components often leads to precipitation or enhanced non-specific adsorption, both of which will impair column function. Hence there are many advantages to checking the stability limits of the sample and working within these limits during purification.

Proteins generally contain a high degree of tertiary structure, kept together by van der Waals' forces, ionic and hydrophobic interactions and hydrogen bonding. Any conditions capable of destabilizing these forces may cause denaturation and/or precipitation. By contrast, peptides contain a low degree of tertiary structure. Their native state is dominated by secondary structures, stabilized mainly by hydrogen bonding. For this reason, peptides tolerate a much wider range of conditions than proteins. This basic difference in native structures is also reflected in that proteins are not easily renatured, while peptides often renature spontaneously.

It is advisable to perform stability tests before beginning to develop a purification protocol. The list below may be used as a basis for such testing:

- Test the stability and occurrence of proteolytic activity by leaving an aliquot of the sample at room temperature overnight. Centrifuge each sample and measure activity and UV absorbance at 280 nm in the supernatant.
- Test pH stability in steps of one pH unit between pH 2 and pH 9.
- Test salt stability with 0–2 M NaCl and 0–2 M $(NH_4)_2SO_4$ in steps of 0.5 M.
- Test the stability towards acetonitrile and methanol in 10% steps between 0 and 50%.
- Test the temperature stability in +10 °C steps from +4 to +40 °C.

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 40 000–50 000 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 non-specific 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.

Nominal pore size of filter	Particle size of chromatographic 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 non-specifically to filter surfaces.

Desalting

Desalting columns are suitable for any sample volume and will rapidly remove low molecular weight contaminants in a single step at the same time as transferring the sample into the correct buffer conditions. Centrifugation and/or filtration of the sample before desalting is still recommended. Detailed procedures for buffer exchange and desalting are given in Chapter 2, page 57.

At laboratory scale, when samples are reasonably clean after filtration or centrifugation, the buffer exchange and desalting step can be avoided. For affinity chromatography or hydrophobic interaction chromatography, it may be sufficient to adjust the pH of the sample and, if necessary, dilute to reduce the ionic strength of the solution.

Rapidly process small or large sample volumes. Use before and/or between purification steps, if needed (remember that each extra step can reduce yield and desalting also dilutes the sample).

Remove salts from proteins with molecular weight M_r >5 000.

Use 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate if volatile buffers are required.

Specific sample preparation steps

Specific sample preparation steps may be required if the crude sample is known to contain contamininants 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 frequently used at laboratory scale to remove gross impurities from small sample volumes, and occasionally used in small-scale commercial production. Precipitation techniques separate fractions by the principle of differential solubility. 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 72.

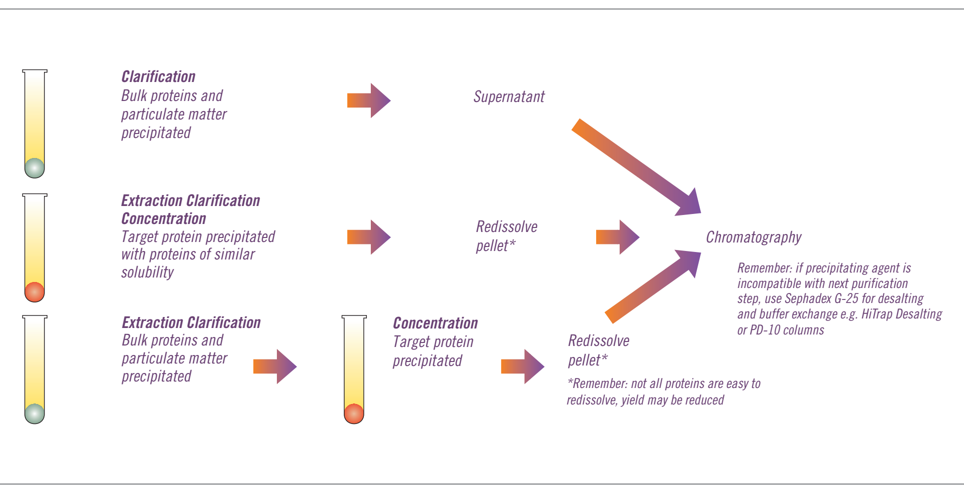


Fig. 72. Three ways to use precipitation.

Examples of precipitation agents are reviewed in Table 12. The most common precipitation method using ammonium sulfate is described in more detail.

Table 12. Examples of precipitation techniques

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulfate	As described below.	>1 mg/ml proteins especially immuno-globulins.	Stabilizes proteins, no denaturation, supernatant can go directly to HIC.
Dextran sulfate	Add 0.04 ml 10% dextran sulfate and 1 ml 1 M $CaCl_2$ per ml sample, mix 15 min, centrifuge 10 000 g, discard pellet.	Samples with high levels of lipoprotein e.g ascites.	Precipitates lipoprotein.
Polyvinylpyrrolidine	Add 3% (w/v), stir 4 hours, centrifuge 17 000 g, discard pellet.	Samples with high levels of lipoprotein e.g ascites.	Alternative to dextran sulfate.
Polyethylene glycol (PEG, M_r > 4 000)	Up to 20% w/v	Plasma proteins.	No denaturation, supernatant goes directly to IEX or AC, complete removal may be difficult.
Acetone (cold)	Up to 80% v/v at $\pm$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		Precipitation of 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).
Personal communications.

Ammonium sulfate precipitation

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 favour of chromatography.

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

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.

1. Filter (0.45 µm) or centrifuge the sample (10 000 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 hour.
4. Centrifuge 20 minutes at 10 000 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 2, page 57).

*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 13 shows the quantities required at +20 °C.

Table 13. 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 litre 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

Resolubilization of protein precipitates

Many proteins are easily resolubilized in a small amount of the buffer to be used in the next chromatographic step. However, a denaturing agent may be required for less soluble proteins. Specific conditions will depend upon the specific protein. These agents must always be removed to allow complete refolding of the protein and to maximize recovery of mass and activity. A chromatographic step often removes a denaturant during purification. Table 14 gives examples of common denaturing agents.

Table 14.

Denaturing agent	Typical conditions for use	Removal/comment
Urea	2 M–8 M	Remove using 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 2, page 57.

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 polyvinylpyrrolidine, described under Fractional precipitation, are recommended to remove high levels of lipoproteins from samples such as ascitic fluid.

Centrifuge samples to avoid the risk of non-specific binding of the target molecule to a filter.

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

Removal of phenol red

Phenol red is frequently used at laboratory scale as a pH indicator in cell culture. Although not directly interfering with purification, phenol red may bind to certain purification media and should be removed as early as possible to avoid the risk of contamination. It is known to bind to anion exchange media at pH >7.

Use a desalting column to simultaneously remove phenol red (a low molecular weight molecule) and transfer sample to the correct buffer conditions for further purification, as described in Chapter 2, page 57.

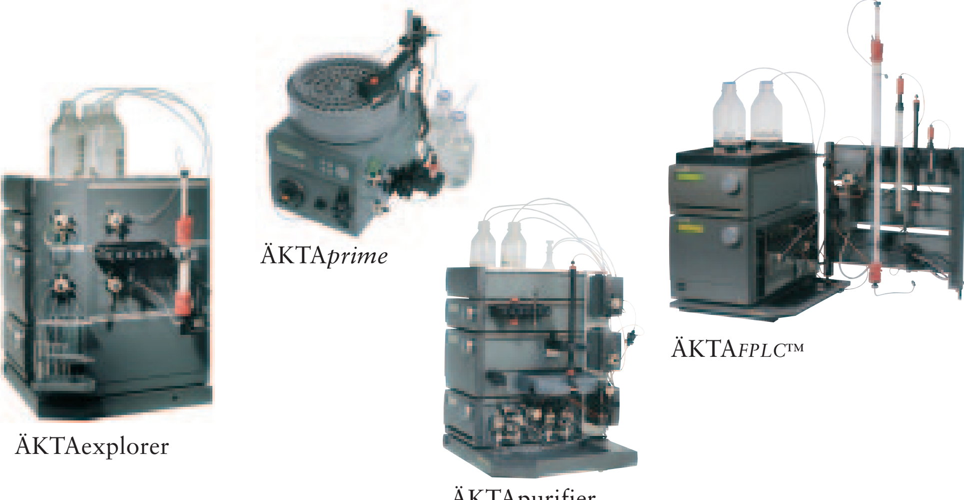
Removal of low molecular weight contaminants

If samples contain a high level of low molecular weight contaminants, use a desalting column before the first chromatographic purification step, as described in Chapter 2, page 57.

Appendix 4
Selection of purification equipment

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

Way of working	Standard ÄKTAdesign configurations				Syringe or peristaltic pump + HiTrap Desalting column	Gravity–fed columns
	Explorer 100	Purifier 10	FPLC	Prime		
Simple, one step desalting, buffer exchange	✓	✓	✓	✓	✓	✓
Reproducible performance for routine separation	✓	✓	✓	✓		
Optimization of one step separation to increase purity	✓	✓	✓	✓		
System control and data handling for regulatory requirements, e.g. GLP	✓	✓	✓			
Automatic method development and optimization	✓	✓	✓			
Automatic buffer preparation	✓	✓				
Automatic pH scouting	✓	✓				
Automatic media or column scouting	✓					
Automatic multi-step purification	✓					
Scale up, process development and transfer to production	✓					



ÄKTA*prime*

ÄKTA*FPLC*™

ÄKTAexplorer

ÄKTApurifier

Appendix 5
Converting from linear flow (cm/hour) 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 (cm/hour). However, flow is usually measured in volumetric flow rate (ml/min). To convert between linear flow and volumetric flow rate use one of the formulae below.

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

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

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

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/hour?

Y = linear flow = 150 cm/h

d = inner diameter of the column = 1.6 cm

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

$$= 5.03 \text{ ml/min}$$

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

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

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

where

Z = volumetric flow rate in ml/min

d = column inner diameter in cm

Example:

What is the linear flow in an HR 5/5 column (i.d. 0.5 cm) when the volumetric flow rate is 1 ml/min?

Z = Volumetric flow rate = 1 ml/min

d = column inner diameter = 0.5 cm

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

$$= 305.6 \text{ cm/h}$$

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, column pressures

Mass (g/mol)	1 µg	1 nmol
10 000	100 pmol; 6 x 10^{13} molecules	10 µg
50 000	20 pmol; 1.2 x 10^{13} molecules	50 µg
100 000	10 pmol; 6.0 x 10^{12} molecules	100 µg
150 000	6.7 pmol; 4.0 x 10^{12} molecules	150 µg

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

1 kb of DNA = 333 amino acids of coding capacity
= 37 000 g/mol
270 bp DNA = 10 000 g/mol
1.35 kb DNA = 50 000 g/mol
2.70 kb DNA = 100 000 g/mol

Average molecular weight of an amino acid = 120 g/mol.

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 megaPascals, bar or pounds per square inch and can be converted as follows: 1MPa = 10 bar = 145 psi

Appendix 7
Table of amino acids

Amino acid	Three-letter code	Single-letter code	Structure
Alanine	Ala	A	$HOOC$, H_2N — CH_3
Arginine	Arg	R	$HOOC$, H_2N — $CH_2CH_2CH_2NHC(NH_2)=NH$
Asparagine	Asn	N	$HOOC$, H_2N — CH_2CONH_2
Aspartic Acid	Asp	D	$HOOC$, H_2N — CH_2COOH
Cysteine	Cys	C	$HOOC$, H_2N — CH_2SH
Glutamic Acid	Glu	E	$HOOC$, H_2N — CH_2CH_2COOH
Glutamine	Gln	Q	$HOOC$, H_2N — $CH_2CH_2CONH_2$
Glycine	Gly	G	$HOOC$, H_2N — H
Histidine	His	H	$HOOC$, H_2N — CH_2 — (ring: N, NH)
Isoleucine	Ile	I	$HOOC$, H_2N — $CH(CH_3)CH_2CH_3$
Leucine	Leu	L	$HOOC$, H_2N — $CH_2CH(CH_3)(CH_3)$
Lysine	Lys	K	$HOOC$, H_2N — $CH_2CH_2CH_2CH_2NH_2$
Methionine	Met	M	$HOOC$, H_2N — $CH_2CH_2SCH_3$
Phenylalanine	Phe	F	$HOOC$, H_2N — CH_2 — (benzene ring)
Proline	Pro	P	$HOOC$, H_2N — (ring: NH)
Serine	Ser	S	$HOOC$, H_2N — CH_2OH
Threonine	Thr	T	$HOOC$, H_2N — $CHCH_3$ (OH)
Tryptophan	Trp	W	$HOOC$, H_2N — CH_2 — (indole ring: NH)
Tyrosine	Tyr	Y	$HOOC$, H_2N — CH_2 — (benzene ring) — OH
Valine	Val	V	$HOOC$, H_2N — $CH(CH_3)_2$

Formula	M_r	Middle unit residue (-H_2O) Formula	M_r	Charge at pH 6.0–7.0	Hydrophobic (non-polar)	Uncharged (polar)	Hydrophilic (polar)
$C_3H_7NO_2$	89.1	C_3H_5NO	71.1	Neutral	■		
$C_6H_{14}N_4O_2$	174.2	$C_6H_{12}N_4O$	156.2	Basic (+ve)			■
$C_4H_8N_2O_3$	132.1	$C_4H_6N_2O_2$	114.1	Neutral		■	
$C_4H_7NO_4$	133.1	$C_4H_5NO_3$	115.1	Acidic(-ve)			■
$C_3H_7NO_2S$	121.2	C_3H_5NOS	103.2	Neutral		■	
$C_5H_9NO_4$	147.1	$C_5H_7NO_3$	129.1	Acidic (-ve)			■
$C_5H_{10}N_2O_3$	146.1	$C_5H_8N_2O_2$	128.1	Neutral		■	
$C_2H_5NO_2$	75.1	C_2H_3NO	57.1	Neutral		■	
$C_6H_9N_3O_2$	155.2	$C6H_7N_3O$	137.2	Basic (+ve)			■
$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	■		
$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	■		
$C_6H_{14}N_2O_2$	146.2	$C_6H_{12}N_2O$	128.2	Basic(+ve)			■
$C_5H_{11}NO_2S$	149.2	C_5H_9NOS	131.2	Neutral	■		
$C_9H_{11}NO_2$	165.2	C_9H_9NO	147.2	Neutral	■		
$C_5H_9NO_2$	115.1	C_5H_7NO	97.1	Neutral	■		
$C_3H_7NO_3$	105.1	$C_3H_5NO_2$	87.1	Neutral		■	
$C_4H_9NO_3$	119.1	$C_4H_7NO_2$	101.1	Neutral		■	
$C_{11}H_{12}N_2O_2$	204.2	$C_{11}H_{10}N_2O$	186.2	Neutral	■		
$C_9H_{11}NO_3$	181.2	$C_9H_9NO_2$	163.2	Neutral		■	
$C_5H_{11}NO_2$	117.1	C_5H_9NO	99.1	Neutral	■		

Appendix 8
Analytical assays during purification

Analytical assays are essential to follow the progress of purification. They are used to assess the effectiveness of each step in terms of yield, biological activity, recovery and to help during optimization of experimental conditions. The importance of a reliable assay for the target molecule cannot be over-emphasized.

When testing chromatographic fractions, ensure that the buffers used for purification do not interfere with the assay.

Total protein determination

Lowry or Bradford assays are used most frequently to determine the total protein content. The Bradford assay is particularly suited to samples where there is a high lipid content that may interfere with the Lowry assay.

Purity determination

Purity is most often estimated by SDS-PAGE. Alternatively, isoelectric focusing, capillary electrophoresis, reversed phase chromatography or mass spectrometry may be used.

SDS-PAGE Analysis

Reagents Required

6X SDS loading buffer: 0.35 M Tris-HCl (pH 6.8), 10.28% (w/v) SDS, 36% (v/v) glycerol, 0.6 M dithiothreitol (or 5% 2-mercaptoethanol), 0.012% (w/v) bromophenol blue. Store in 0.5 ml aliquots at -80 °C.

1. Add 2 µl of 6X SDS loading buffer to 5–10 µl of supernatant from crude extracts, cell lysates or purified fractions as appropriate.
2. Vortex briefly and heat for 5 minutes at +90 to +100 °C.
3. Load the samples onto an SDS-polyacrylamide gel.
4. Run the gel and stain with Coomassie™ Blue (Coomassie Blue R Tablets) or silver (PlusOne™ Silver Staining Kit, Protein).

The percentage of acrylamide in the SDS-gel should be selected according to the expected molecular weight of the protein of interest (see Table 15).

Table 15.

% Acrylamide in resolving gel	Separation size range
Single percentage:	
5%	36 000–200 000
7.5%	24 000–200 000
10%	14 000–200 000
12.5%	14 000–100 000
15%	14 000–60 000[1]
Gradient:	
5–15%	14 000–200 000
5–20%	10 000–200 000
10–20%	10 000–150 000

[1]The larger proteins fail to move significantly into the gel.

Functional assays

Immunospecific interactions have enabled the development of many alternative assay systems for the assessment of active concentration of target molecules.

- Western blot analysis is used when the sensitivity of SDS-PAGE with Coomassie Blue or silver staining is insufficient.

1. Separate the protein samples by SDS-PAGE.
2. Transfer the separated proteins from the gel to an appropriate membrane, such as Hybond™ ECL™ (for subsequent ECL detection) or Hybond P (for subsequent ECL Plus™ detection).
3. Develop the membrane with the appropriate specified reagents.

Electrophoresis and protein transfer may be accomplished using a variety of equipment and reagents. For further details, refer to the *Protein Electrophoresis Technical Manual* and *Hybond ECL instruction manual*, both from Amersham Biosciences.

- ELISAs are most commonly used as activity assays.
- Functional assays using the phenomenon of surface plasmon resonance to detect immunospecific interactions (e.g. using BIACORE™ systems) enable the determination of active concentration, epitope mapping and studies of reaction kinetics.

Detection and assay of tagged proteins

SDS-PAGE, Western blotting and ELISAs can also be applied to the detection and assay of genetically engineered molecules to which a specific tag has been attached. In some cases, an assay based on the properties associated with the tag itself can be developed, e.g. the GST Detection Module for enzymatic detection and quantification of GST tagged proteins. Further details on the detection and quantification of GST and $(His)_6$ tagged proteins are available in *The Recombinant Protein Handbook: Protein Amplification and Simple Purification* and *GST Gene Fusion System Handbook* from Amersham Biosciences.

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, if essential. Stabilizing agents are often required for storage of purified proteins.
- Serum, culture supernatants and ascitic fluid should be kept frozen at -20 °C or -70 °C, in small aliquots.
- Avoid repeated freeze/thawing or freeze drying/re-dissolving that may reduce biological activity.
- Avoid conditions close to stability limits 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. Above 24 hours at +4 °C, add a preserving agent if possible (e.g. merthiolate 0.01%).

Sodium azide can interfere with many coupling methods and some biological assays and can be a health hazard. It can be removed by using a desalting column (see Chapter 2, page 57).

General recommendations for purified proteins

- Store as a precipitate in high concentration of ammonium sulfate, for example 4.0 M.
- Freeze in 50% glycerol, especially suitable for enzymes.
- Avoid the use of 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. Instead store samples in small aliquots and keep frozen.
- Sterile filter to prolong storage time.
- Add stabilizing agents, e.g. glycerol (5–20%), serum albumin (10 mg/ml), ligand (concentration is selected based on concentration of active protein) to help 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/re-dissolving that may reduce biological activity.

Sodium azide can interfere with many coupling methods and some biological assays. It can be removed by using a desalting column (see Chapter 2, page 57).

Cryoproteins are a group of proteins, including some mouse antibodies of the IgG_3 subclass, that should not be stored at +4 °C as they precipitate at this temperature. Keep at room temperature in the presence of a preserving agent.

Additional reading and reference material

	Code No.
Purification	
Antibody Purification Handbook	18-1037-46
Protein Purification Handbook	18-1132-29
Recombinant Protein Handbook: Protein Amplification and Simple Purification	18-1142-75
GST Gene Fusion System Handbook	18-1157-58
Affinity Chromatography Handbook: Principles and Methods	18-1022-29
Ion Exchange Chromatography Handbook: Principles and Methods	18-1114-21
Hydrophobic Interaction Chromatography Handbook: Principles and Methods	18-1020-90
Reversed Phase Chromatography Handbook: Principles and Methods	18-1112-93
Expanded Bed Adsorption Handbook: Principles and Methods	18-1124-26
Protein and Peptide Purification Technique Selection	18-1128-63
Fast Desalting and Buffer Exchange of Proteins and Peptides	18-1128-62
Gel Filtration Columns and Media Selection Guide	18-1124-19
Ion Exchange Columns and Media Selection Guide	18-1127-31
Chromatofocusing with Polybuffer and PBE, Handbook	18-1009-07
HIC Columns and Media Product Profile	18-1100-98
Affinity Columns and Media Product Profile	18-1121-86
Convenient Protein Purification, HiTrap Column Guide	18-1128-81
ÄKTAdesign Brochure	18-1158-77
ÄKTA 3D Kit Brochure	18-1160-45
GST Fusion System Brochure	18-1159-30
Protein Purifier Software	18-1155-49
Protein Purification: Principles, High Resolution Methods and Applications, J-C. Jansson and L.Rydén	18-1128-68
Sephadex LH-20: chromatography in organic solvents	18-1009-74
Preparative Gel chromatography on Sephadex-LH-20, H. Henke	18-1113-89
Column Packing Video (PAL)	17-0893-01
Column Packing Video (NTSC)	17-0894-01
Reference list HiTrap Desalting	18-1156-70*
Reference list HiPrep 26/10 Desalting	18-1156-89*
Reference list HiPrep Sephacryl S-100 HR	18-1156-86*
Reference list HiPrep Sephacryl S-200 HR	18-1156-87*
Reference list HiPrep Sephacryl S-300 HR	18-1156-88*
Reference list HiLoad Superdex 30 prep grade	18-1156-94*
Reference list HiLoad Superdex 75 prep grade	18-1156-95*
Reference list HiLoad Superdex 200 prep grade	18-1156-96*
Analysis	
Protein analysis–using the power of 2-D electrophoresis	18-1124-82
2D Electrophoresis Handbook	80-6429-60
Protein Electrophoresis Technical Manual	80-6013-88
ECL Western and ECL Plus Western Blotting Application Note	18-1139-13

The reference lists are only available at ***www.chromatography.amershambiosciences.com*** and many of the above items can also be downloaded.

Ordering information

Product	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 HR 10/30	1 × 24 ml column	17-1453-01
Superdex 75 HR 10/30	1 × 24 ml column	17-1047-01
Superdex 200 HR 10/30	1 × 24 ml column	17-1088-01
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	25 ml	17-0905-10
Superdex 30 prep grade	150 ml	17-0905-01
Superdex 75 prep grade	25 ml	17-1044-10
Superdex 75 prep grade	150 ml	17-1044-01
Superdex 200 prep grade	25 ml	17-1043-10
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 HR 10/30	1 × 24 ml column	17-0537-01
Superose 12 HR 10/30	1 × 24 ml column	17-0538-01
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
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
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

Product	Quantity	Code No.
Desalting and Group Separations		
HiTrap Desalting	5 × 5 ml columns	17-1408-01
HiPrep 26/10 Desalting	1 × 53 ml column	17-5087-01
PD-10 Desalting Column	30 gravity-fed columns	17-0851-01
Empty PD-10 Desalting Column	50 gravity-fed empty columns	17-0435-01
NICK columns	20 gravity-fed columns	17-0855-01*
NICK columns	50 gravity-fed columns	17-0855-02*
NAP-5 columns	20 gravity-fed columns	17-0853-01*
NAP-5 columns	50 gravity-fed columns	17-0853-02*
NAP-10 columns	20 gravity-fed columns	17-0854-01*
NAP-10 columns	50 gravity-fed columns	17-0854-02*
NAP-25 columns	20 gravity-fed columns	17-0852-01*
NAP-25 columns	50 gravity-fed columns	17-0852-02*
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-25 Superfine	500 g	17-0031-02
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 LMW Calibration Kit *Includes: Ribonuclease A (13 700), chymotrypsinogen A (25 000), ovalbumin (43 000), bovine serum albumin (67 000), Blue Dextran 2000*	1 kit	17-0442-01
Gel Filtration HMW Calibration Kit *Includes: Aldolase (158 000), catalase (232 000), ferritin (440 000), thyroglobulin (669 000), Blue Dextran 2000*	1 kit	17-0441-01
Blue Dextran 2000	10 g	17-0360-01

**Prepacked columns suitable for desalting of oligonucleotides, DNA and proteins.*

Product	Quantity	Code No.
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

All XK columns are delivered with one AK adaptor, TEFZEL tubing (0.8 mm i.d. for XK 16 and XK 26 columns, 1.2 mm i.d. for XK 50 columns, with M6 connectors, thermostatic jacket, support snap-on net rings, dismantling tool (XK 16 and XK 26 only), and instructions.

Accessories and spare parts

Product	Quantity	Code No.
Packing Connector XK 16	1	18-1153-44
Packing Connector XK 26	1	18-1153-45
Solvent resistant columns		
SR 10/50 column	1	19-2638-01
SR 10/50J column*	1	19-1734-01
SR 25/45 column	1	19-0879-01
SR 25/100 column	1	19-0880-01

All SR columns are delivered complete with two SRA adaptors, PTFE tubing (2 × 50 cm), spare bed supports, tubing end fittings, flanging tool and instructions.

**SR 10/50J includes a borosilicate glass jacket. Jackets are not available for other SR columns.*

Accessories

Product	Quantity	Code No.
SRE 10 packing reservoir	1	19-2097-01

For a complete lisiting refer to Amersham Biosciences BioDirectory or *www.chromatography.amershambiosciences.com*

ÄKTA, FPLC, PlusOne, HiLoad , HiTrap, HiPrep, Hybond, ECL, ECL Plus, BioProcess, MabSelect, Sephacryl, Sephadex, Superose, Sepharose, Superdex and Drop Design are trademarks of Amersham Biosciences Limited.

Amersham and Amersham Biosciences are trademarks of Amersham plc.

Coomassie is a trademark of ICI plc.

MicroSpin is a trademark of Lida Manufacturing Corp.

Triton is a registered trademark of Union Carbide Chemicals and Plastics Co.

Tween is a registered trademark of ICI Americas, Inc.

Eppendorf and Multipipette are trademarks of Eppendorf-Netheler-Hinz GmbH.

BIACORE is a trademark of Biacore AB.

All goods and services are sold subject to the terms and conditions of sale of the company within the Amersham Biosciences group that supplies them. A copy of these terms and conditions is available on request.
© Amersham Biosciences AB 2002 – All rights reserved.

Amersham Biosciences AB, Björkgatan 30, SE-751 84 Uppsala, Sweden

Amersham Biosciences UK Limited, Amersham Place, Little Chalfont, Buckinghamshire HP7 9NA, England

Biosciences Corp., 800 Centennial Avenue, PO Box 1327, Piscataway NJ 08855, USA

Amersham Biosciences Europe GmbH, Munzinger Strasse 9, D-79111 Freiburg, Germany

Amersham Biosciences K.K., Sanken Bldg. 3-25-1, Hyakunincho Amersham Shinjuku-ku, Tokyo 169-0073, Japan.

Production: RAK Design AB

www.chromatography.amershambiosciences.com