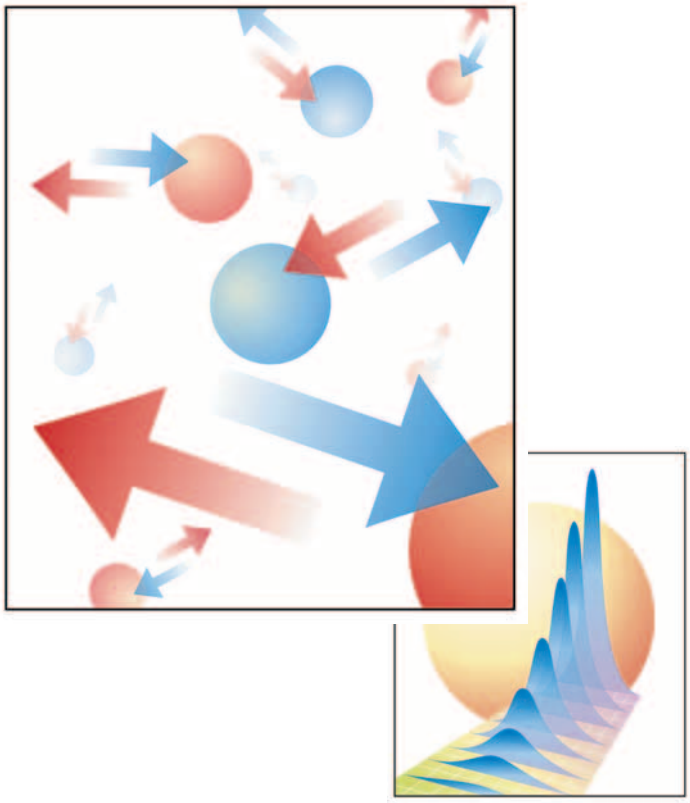
Ion Exchange Chromatography & Chromatofocusing

Principles and Methods



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Ion Exchange Chromatography & Chromatofocusing

Principles and Methods

Contents

Introduction

Biomolecules are purified using chromatography techniques that separate them according to differences in their specific properties, as shown in Figure 1. Ion exchange chromatography (IEX) separates biomolecules according to differences in their net surface charge.

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

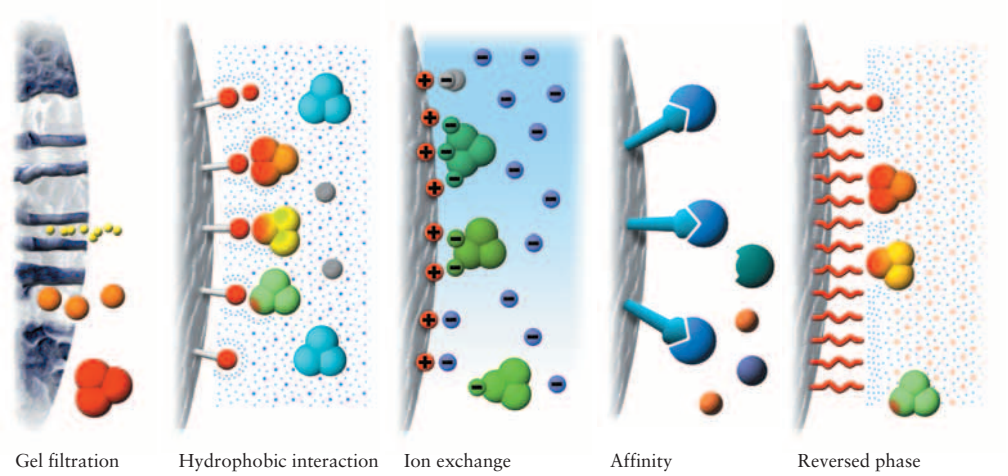


Fig. 1. Separation principles in chromatographic purification.

IEX for the separation of biomolecules was introduced in the 1960s and continues to play a major role in the separation and purification of biomolecules. Today, IEX is one of the most frequently used techniques for purification of proteins, peptides, nucleic acids and other charged biomolecules, offering high resolution and group separations with high loading capacity. The technique is capable of separating molecular species that have only minor differences in their charge properties, for example two proteins differing by one charged amino acid. These features make IEX well suited for capture, intermediate purification or polishing steps in a purification protocol and the technique is used from microscale purification and analysis through to purification of kilograms of product.

This handbook describes both theoretical and practical aspects principles of the technique, the media available and how to select them, application examples and detailed instructions for the most commonly performed procedures. Practical information, with many tips and hints drawn from over forty years of experience in chromatography purification, guides beginners and experts towards obtaining the best possible results from the latest chromatographic media.

The final chapter includes information on *chromatofocusing*, another chromatography technique that separates biomolecules on the basis of charge, but, in this case, according to differences in their isoelectric points. This technique can provide very high resolution separations for specific applications. Proteins with a difference as small as 0.02 pH units in their isoelectric points can be separated in laboratory-scale applications.

Amersham Biosciences offers a wide variety of prepacked columns and ready-to-use chromatography media. A range of handbooks ensure that purification with any chromatographic technique becomes a simple and efficient procedure at any scale and in any laboratory.

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 analyze and resolve difficulties that may occur.

chemicals, buffers and equipment.

experimental protocol.

Common abbreviations

In chromatography

IEX: ion exchange chromatography (also seen as IEC in the literature)

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

AC: affinity chromatography

RPC: reverse phase chromatography

HIC: hydrophobic interaction chromatography

CF: chromatofocusing

CIPP: Capture, Intermediate Purification and Polishing

CV: colume volume

pKa: the pH at which an acid is 50% dissociated

pI: isoelectric point, the pH at which a protein has zero net surface charge

MPa: megaPascal

psi: pounds per square inch

SDS: sodium dodecyl sulfate

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

M_r: relative molecular weight

N: column efficiency expressed as theoretical plates per meter

R_s: resolution, the degree of separation between peaks

Abbreviations found in product names

HMW: high molecular weight

LMW: low molecular weight

Tricorn PE: column manufactured in PEEK (polyetheretherketone)

Tricorn GL: column manufactured in glass

HR: high resolution

PC: precision column

Chapter 1
Principles of ion exchange

This chapter provides a general introduction to the theoretical principles that underlie every ion exchange separation. An understanding of these principles will enable the separation power of ion exchange chromatography (IEX) to be fully appreciated. Practical aspects of performing a separation are covered in Chapter 2.

Net surface charge and pH

IEX separates molecules on the basis of differences in their *net surface charge*. Molecules vary considerably in their charge properties and will exhibit different degrees of interaction with charged chromatography media according to differences in their overall charge, charge density and surface charge distribution. The charged groups within a molecule that contribute to the net surface charge possess different *pKa* values depending on their structure and chemical microenvironment.

Since all molecules with ionizable groups can be titrated, their net surface charge is highly pH dependent. In the case of proteins, which are built up of many different amino acids containing weak acidic and basic groups, their net surface charge will change gradually as the pH of the environment changes i.e. proteins are *amphoteric*. Each protein has its own unique *net charge versus pH relationship* which can be visualized as a *titration curve*. This curve reflects how the overall net charge of the protein changes according to the pH of the surroundings. Figure 2 illustrates several theoretical protein titration curves (these curves can be generated using a combination of isoelectric focusing and electrophoresis, but with modern solutions for rapid method development, actual titration curves are rarely used).

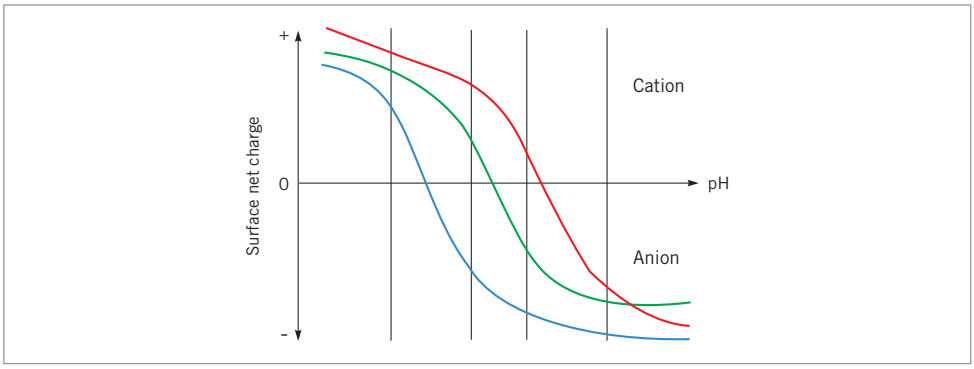


Fig. 2. Theoretical protein titration curves, showing how net surface charge varies with pH.

IEX chromatography takes advantage of the fact that the relationship between net surface charge and pH is unique for a specific protein. In an IEX separation *reversible interactions* between *charged* molecules and *oppositely charged* IEX media are controlled in order to favor binding or elution of specific molecules and achieve separation. A protein that has no net charge at a pH equivalent to its *isoelectric point (pI)* will not interact with a charged medium. However, at a pH above its isoelectric point, a protein will bind to a positively charged medium or *anion exchanger* and, at a pH below its pI, a protein will behind to a negatively charged medium or *cation exchanger*. In addition to the ion exchange interaction, other types of binding may occur, but these effects are very small and mainly due to van der Waals forces and non-polar interactions.

Steps in an IEX separation

An IEX medium comprises a *matrix* of spherical particles substituted with ionic groups that are negatively (cationic) or positively (anionic) charged. The matrix is usually porous to give a high internal surface area. The medium is packed into a column to form a *packed bed*. The bed is then equilibrated with buffer which fills the pores of the matrix and the space in between the particles. For more details on column packing, refer to Appendix 3. Figure 3 illustrates the separation process that follows.

The pH and ionic strength of the equilibration buffer are selected to ensure that, when sample is loaded, proteins of interest bind to the medium and as many impurities as possible do not bind. The proteins which bind are effectively concentrated onto the column while proteins that do not have the correct surface charge pass through the column at the same speed as the flow of buffer, eluting during or just after sample application, depending on the total volume of sample being loaded.

Note that the condition of the sample is very important in order to achieve the most effective high resolution or group separations and make the most of the high loading capacity. Ideally, samples should be in the same conditions as the start buffer (see Appendix 1, Sample preparation and, in particular, *Buffer exchange and desalting*, page 156 for details).

When all the sample has been loaded and the column washed so that all non-binding proteins have passed through the column (i.e. the UV signal has returned to baseline), conditions are altered in order to *elute the bound proteins*. Most frequently, proteins are eluted by *increasing the ionic strength (salt concentration)* of the buffer or, occasionally, by *changing the pH*. As ionic strength increases, the salt ions (typically Na^+ or Cl^-) compete with the bound components for charges on the surface of the medium and one or more of the bound species begin to elute and move down the column. The proteins with the lowest net charge at the selected pH will be the first ones eluted from the column as ionic strength increases. Similarly, the proteins with the highest charge at a certain pH will be most strongly retained and will be eluted last. The higher the net charge of the protein, the higher the ionic strength that is needed for elution. By controlling changes in ionic strength using different forms of gradient, proteins are eluted differentially in a purified, concentrated form.

A wash step in very high ionic strength buffer removes most tightly bound proteins at the end of an elution. The column is then re-equilibrated in start buffer before applying more sample in the next run.

Alternatively, conditions can be chosen to maximize the binding of contaminants and allow the target protein(s) to pass through the column thus *removing contaminants*.

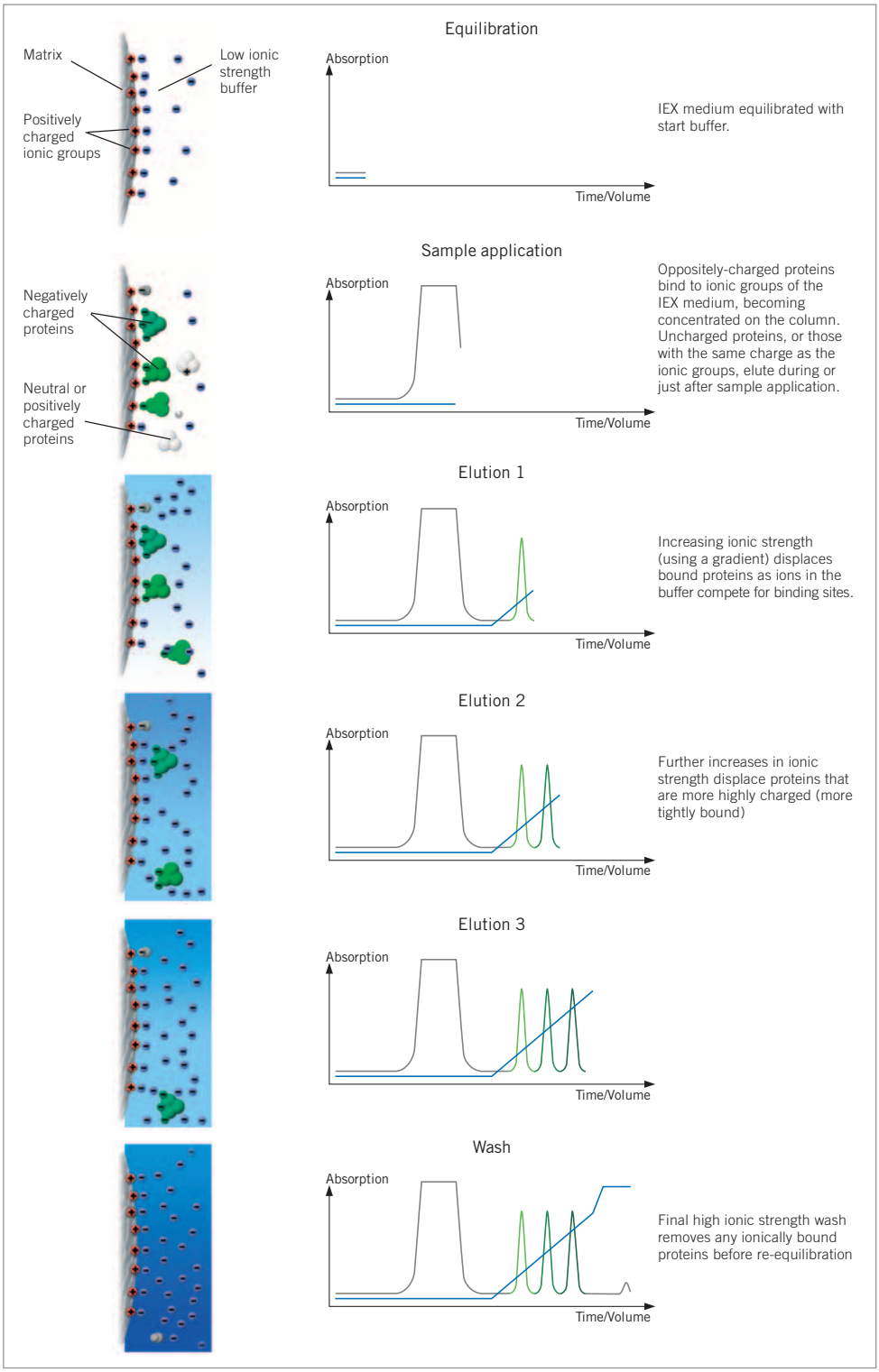


Fig. 3. Principles of an anion exchange separation.

Resolution

The resolution of an IEX separation is a combination of the degree of separation between the peaks eluted from the column (the selectivity of the medium), the ability of the column to produce narrow, symmetrical peaks (efficiency) and, of course, the amount (mass) of sample applied. These factors are influenced by practical issues such as matrix properties, binding and elution conditions, column packing, flow rates which are covered in detail in Chapter 2, Ion exchange in practice.

Resolution (R_s) is defined as the distance between peak maxima compared with the average base width of the two peaks. R_s can be determined from a chromatogram, as shown in Figure 4.

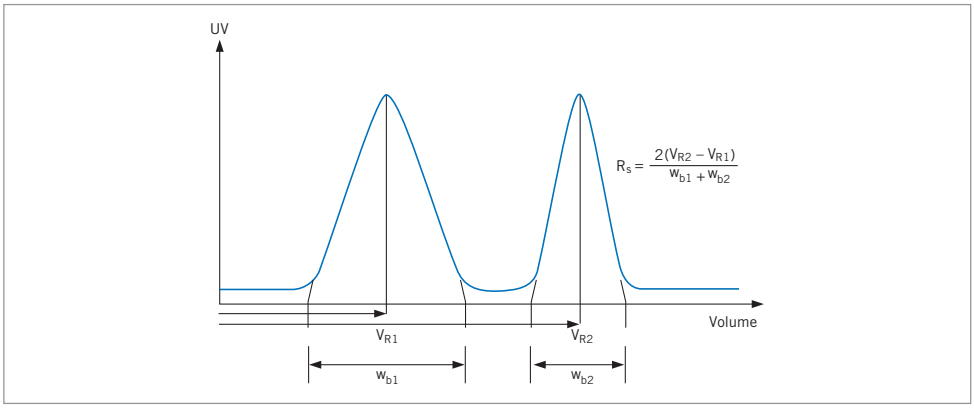


Fig. 4. Determination of the resolution (R_s) between two peaks.

Elution volumes and peak widths are measured with the same units to give a dimensionless resolution value. R_s gives a measure of the relative separation between two peaks and can be used to determine if further optimization of the chromatographic procedure is necessary. If R_s = 1.0 (Figure 5) then 98% purity has been achieved at 98% of peak recovery, provided the peaks are symmetrical and approximately equal in size. Baseline resolution requires that $R_s \geq 1.5$. At this value, peak purity is 100%.

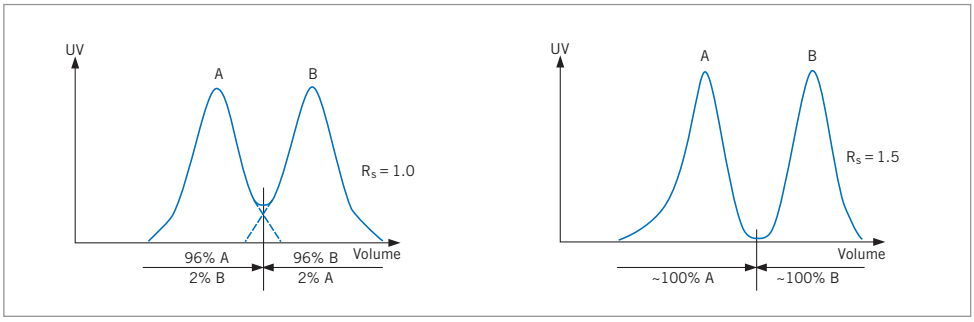


Fig. 5. Separation results with different resolutions.

A single, well resolved peak is not necessarily a pure substance, but may represent a series of components which could not be separated under the chosen elution conditions.

Efficiency

Column efficiency (the ability to elute narrow, symmetrical peaks from a packed bed) relates to the *zone broadening* which occurs on the column and is frequently stated in terms of the number of theoretical plates (see Appendix 3 for determination of column efficiency). One of the main causes of zone broadening is longitudinal diffusion of the solute molecules i.e. proteins, peptides or oligonucleotides. Zone broadening can be minimized if the distances available for diffusion are minimized. In all situations, a well-packed column will contribute significantly to resolution. Columns that are packed unevenly, too tightly, too loosely or that contain air bubbles will lead to channeling (uneven passage of buffer through the column), zone broadening and hence loss of resolution. Figure 6 illustrates the parameters that contribute to good column efficiency. Obviously particle size is a significant factor in resolution and, in general, the smallest particles will produce the narrowest peaks under the correct elution conditions and in a well-packed column.

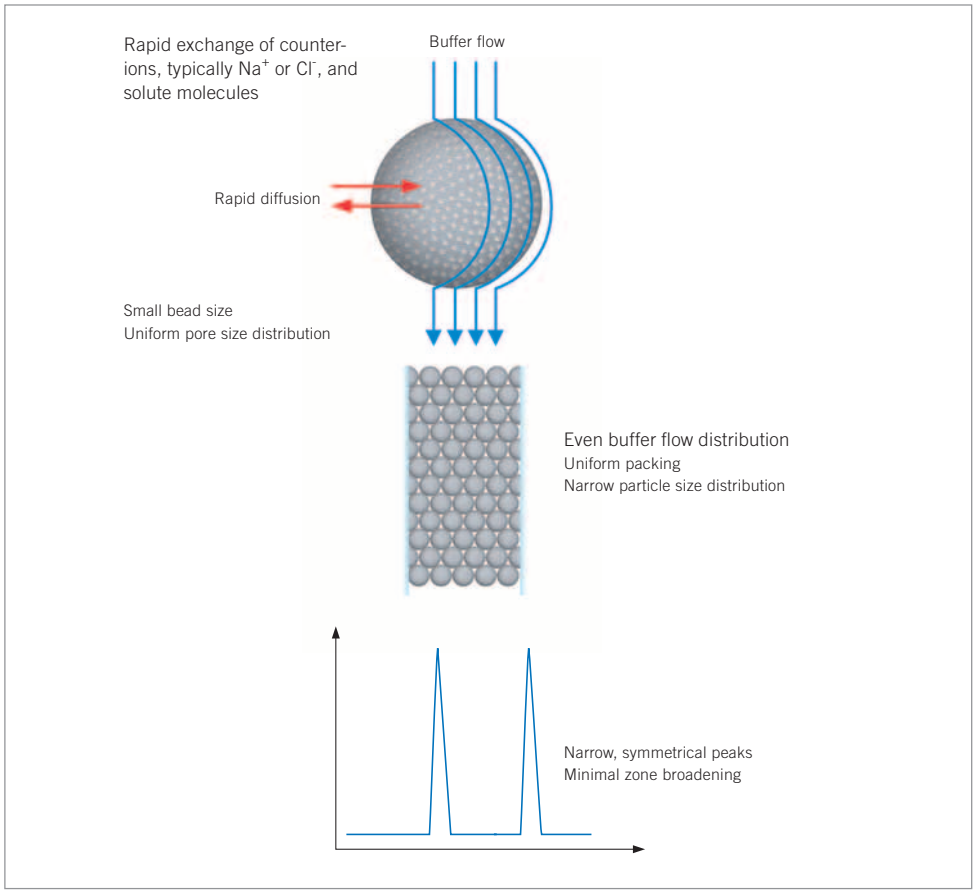


Fig. 6. Factors that effect column efficiency.

Figure 7 demonstrates the influence of particle size on efficiency by comparing several different IEX media under exactly the same running conditions. Note that different media selectivities also influence the final resolution.

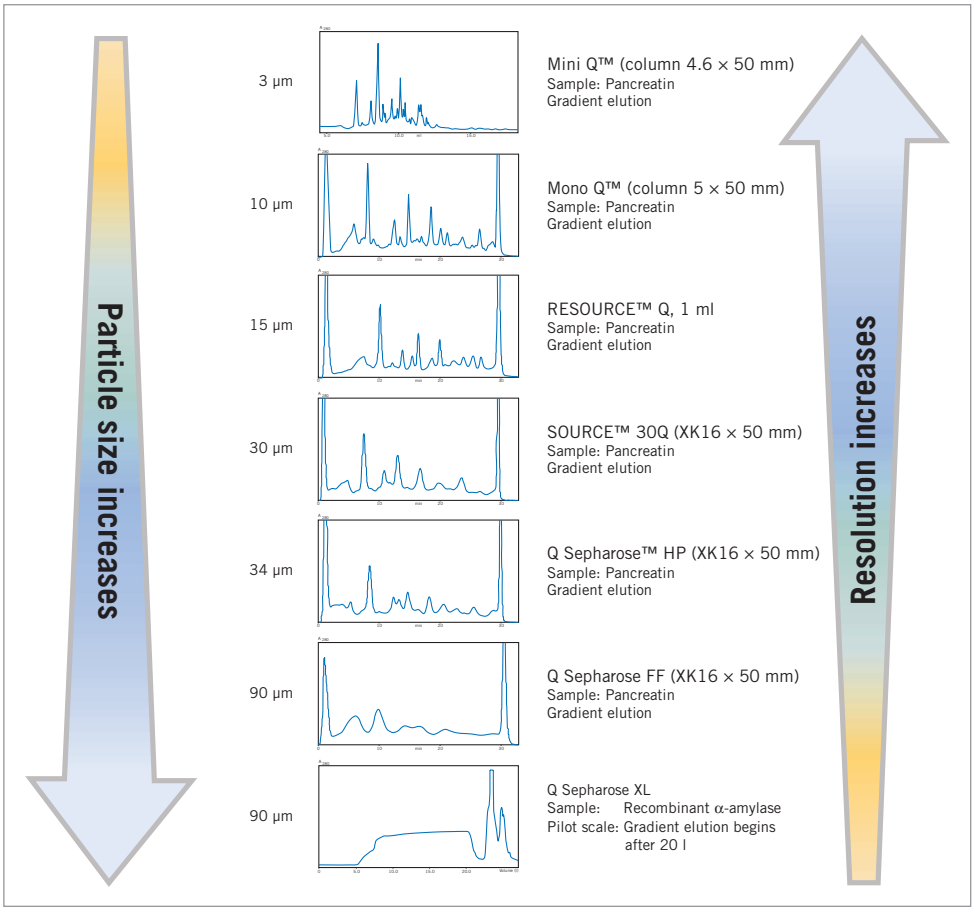


Fig. 7. The influence of particle size and selectivity on final resolution.

Although resolution in terms of efficiency can be improved by decreasing the particle size of the matrix, using a smaller particle size often creates an increase in back pressure so that flow rates need to be decreased, lengthening the run time. Hence the need to match the medium with the requirements for the purification (speed, resolution, purity etc).

The viscosity of highly concentrated samples may reduce resolution if large sample volumes are loaded onto columns packed with small particles. Samples may need to be diluted or, alternatively, a larger particle size needs to be used.

Selectivity

Good selectivity (the degree of separation between peaks) is a more important factor than high efficiency in determining resolution (Figure 8) and depends not only on the nature and number of the functional groups on the matrix, but also on the experimental conditions, such as pH (influencing the protein charge), ionic strength and elution conditions. It is the ease and predictability with which these experimental conditions can be manipulated, when using a suitably designed chromatography medium, that gives IEX the potential of extremely high resolution.

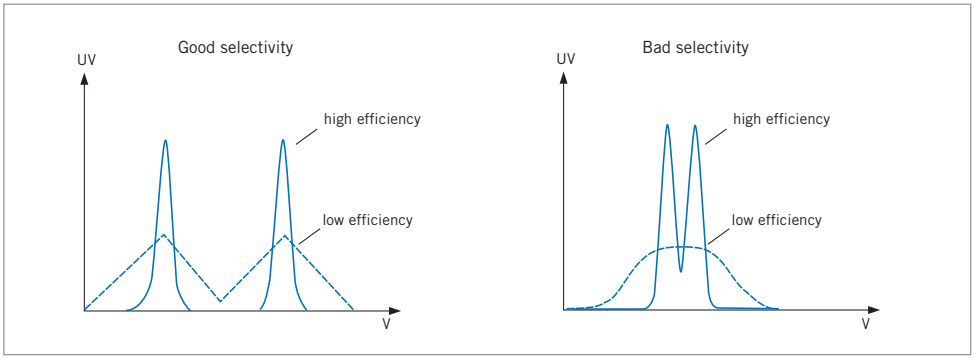


Fig. 8. Effect of selectivity and efficiency on resolution.

Selectivity and pH

Good selectivity is achieved by performing IEX separations at pH values carefully selected to maximize the differences in net charge of the components of interest. Figure 9 (overleaf) emphasizes the significance of pH.

Optimum selectivity can be expected at a pH where there is maximum separation between the titration curves for the individual proteins (i.e. the difference in net charges between the species is greatest) and when using an ion exchanger with a charge opposite to the charge of the proteins at the particular pH.

The order in which proteins are eluted cannot always be predicted with absolute certainty since a titration curve (produced in practice by measuring electrophoretic mobility in a gel) reflects the total net charge on a protein and IEX chromatography depends on the net charge on the surface of the protein.

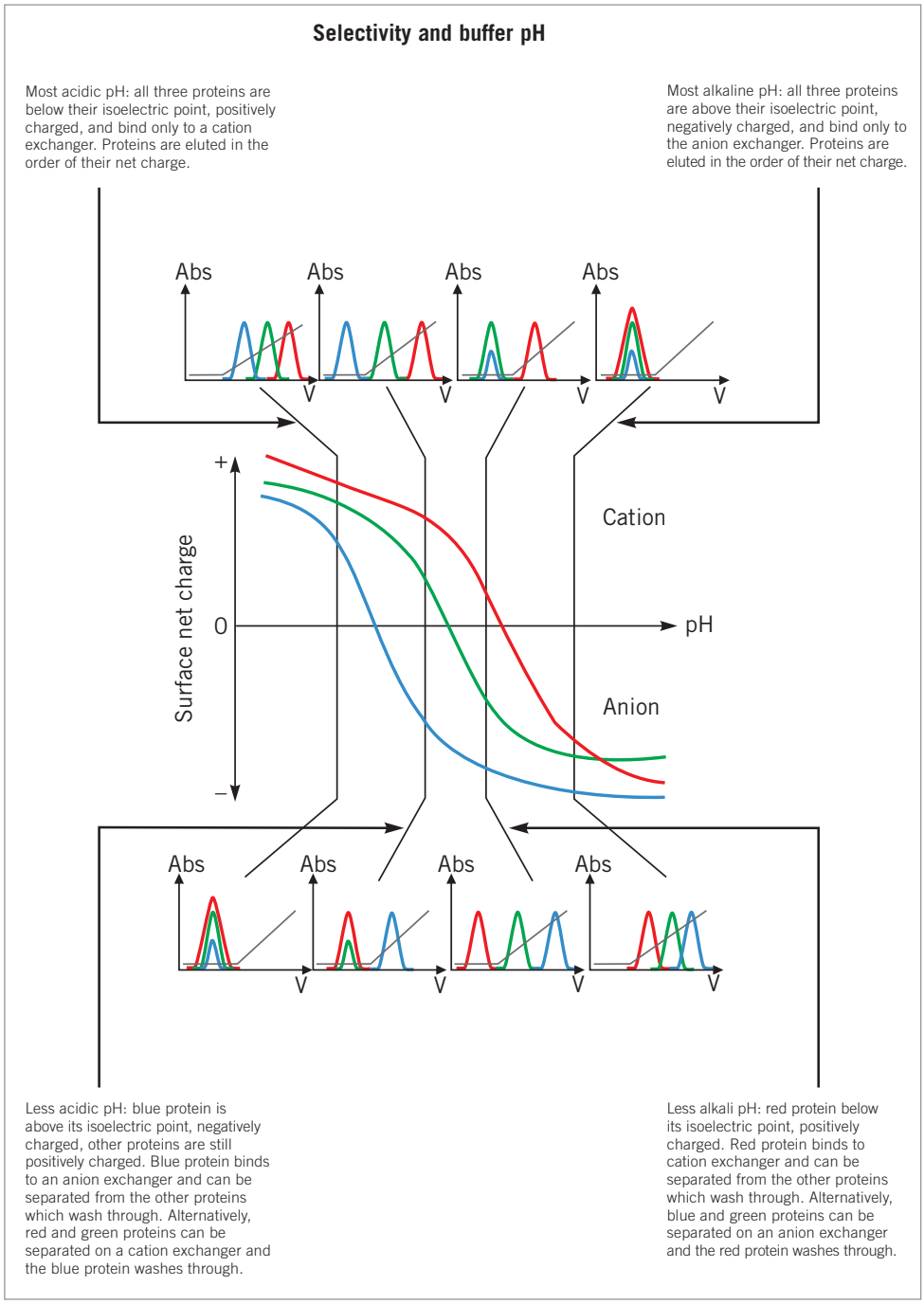


Fig. 9. Effect of pH on protein binding and elution patterns.

Selectivity and elution

The figures below illustrate the most common forms of IEX separation in which proteins are eluted by increasing the ionic strength of a buffer (typically with NaCl) using *linear gradient or step elution*. The UV absorbance and conductivity traces show the elution of protein peaks and the changes in salt concentration, respectively, during elution.

Buffer volumes used during sample application, elution, washing and re-equilibration are expressed in *column volumes*, for example 5 CV=5 ml for a column with a 1 ml bed volume. Using column volumes to describe a separation profile facilitates method development and transfer of methods to columns of different dimensions when scaling-up.

Gradient elution (Figure 10a) is often used when starting with an unknown sample (as many components as possible are bound to the column and eluted differentially to see a total protein profile) and for high resolution separation or analysis.

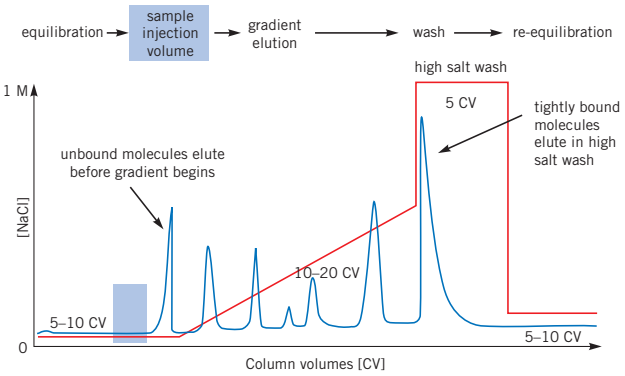


Fig. 10a. Typical high resolution IEX separation using linear gradient elution (25–45 column volumes).

Step elution is used in several ways. When an IEX separation has been optimized using gradient elution, changing to a step elution *speeds up separation times and reduces buffer consumption* while retaining the required purity level (Figure 10b).

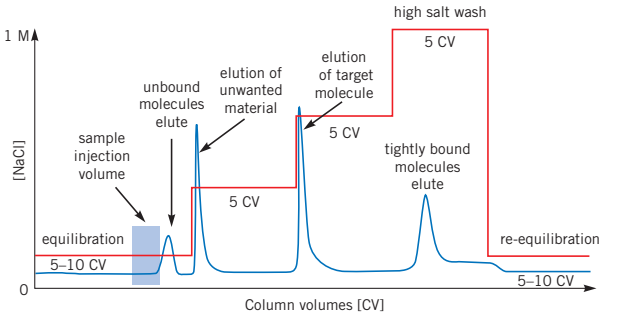


Fig. 10b. Typical IEX separation using step elution (25–30 column volumes).

Step elution can also be used for *group separation* in order to concentrate the proteins of interest and rapidly remove them from unwanted substances (Figure 10c). The target protein(s) is eluted in an enriched, concentrated form.

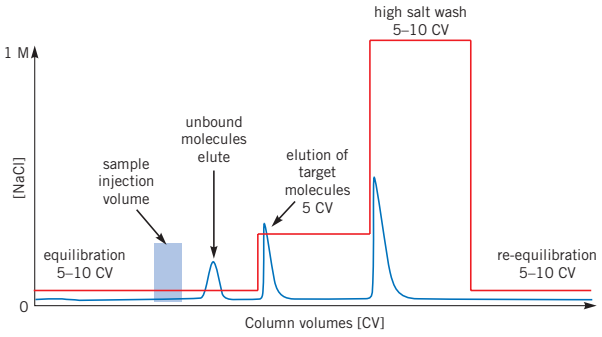


Fig. 10c. Typical IEX separation using a step elution to separate groups of proteins with very different charge properties (20–35 column volumes).

Occasionally, step elution is used to *remove contaminants* by choosing conditions that maximize binding of the contaminants and allow the target protein(s) to pass through the column (Figure 10d). Care must be taken to ensure that the binding capacity of the column is sufficient to bind all contaminants.

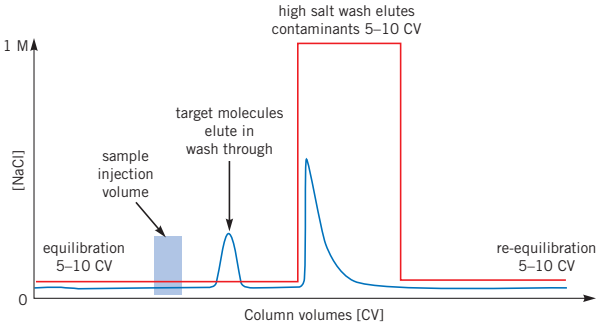


Fig. 10d. Contaminant removal: target protein(s) elute in the wash through, contaminants bind to the column.

Components of ion exchange media

Chromatography media for ion exchange are made from porous or non-porous matrices, chosen for their physical stability, their chemical resistance to stringent cleaning conditions and their low level of non-specific interaction. The matrices are substituted with functional groups that determine the charge of the medium.

Matrix

- High porosity offers a large surface area covered by charged groups and so ensures a high binding capacity. High porosity is also an advantage when separating large biomolecules. Non-porous matrices are preferable for extremely high resolution separations when diffusion effects must be avoided.
- An inert matrix minimizes non-specific interactions with sample components.
- High physical stability ensures that the volume of the packed medium remains constant despite extreme changes in salt concentration or pH thus improving reproducibility and avoiding the need to repack columns.
- High physical stability and uniformity of particle size facilitate high flow rates, particularly during cleaning or re-equilibration steps, to improve throughput and productivity.
- High chemical stability ensures that the matrix can be cleaned using stringent cleaning solutions if required.
- Modern IEX media use either polymer or agarose-based matrices to fulfil not only the requirements for high binding capacity, chemical and physical stability, but to generate media with suitable particle sizes for a range of applications (Table 1).

Table 1. Ion exchange matrices.

	Form	Mean particle size
MiniBeads™	Polystyrene/divinyl benzene	3 µm
MonoBeads™	Polystyrene/divinyl benzene	10 µm
SOURCE 15	Polystyrene/divinyl benzene	15 µm
SOURCE 30	Polystyrene/divinyl benzene	30 µm
Sepharose High Performance	Agarose 6%	34 µm
Sepharose Fast Flow	Agarose 6%	90 µm
Sepharose 4 Fast Flow	Agarose 4%	90 µm
Sepharose XL	Agarose 6%, dextran chains coupled to agarose	90 µm
Sepharose Big Beads	Agarose 6%	200 µm

MiniBeads is a matrix made from polystyrene, with divinyl benzene as cross-linker, to produce highly spherical (monodispersed), very small (3 µm), non-porous particles that facilitate micropreparative or analytical separations when *extremely high resolution* is more important than high binding capacity or high flow rates.

MonoBeads and SOURCE are matrices made from polystyrene with divinyl benzene to produce highly spherical (monodispersed), small (10, 15 or 30 µm), porous particles (Figure 11) that facilitate *high resolution separations at high flow rates*.

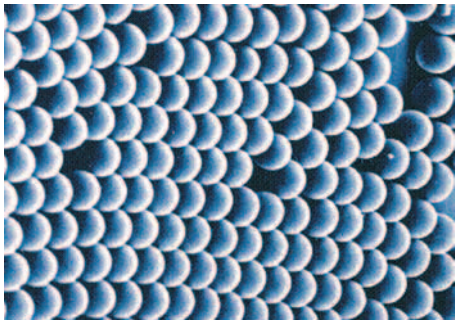


Fig. 11. Electron micrograph of MonoBeads showing spherical, monodispersed particles.

Sepharose media are based on chains of agarose, arranged in bundles and with different degrees of intra-chain cross-linking (Figure 12), to give a range of rigid, macroporous matrices with good capacity and low non-specific adsorption. The most suitable matrix can be selected according to the degree of resolution, binding capacity and flow rates desired for the separation. For example, gradient elution on Sepharose High Performance (34 µm) will give a high resolution separation whereas the larger particles of Sepharose Fast Flow (90 µm) or Sepharose Big Beads (200 µm) would be best suited for high capacity, step elution at high flow rate.

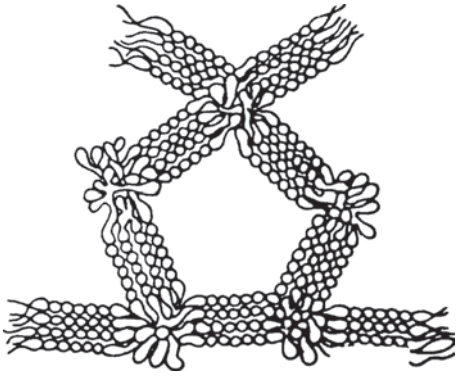


Fig. 12. Structure of cross-linked agarose media (Sepharose).

Many different matrices have been used over the years and references to these will still be found in scientific literature, for example, Sephadex™, Sephacel™ and Sepharose CL-4B. In most cases, more recently developed matrices offer improved capacity with greater physical and chemical stability. To benefit from significantly faster separations and improved performance, transfer and optimize older protocols onto modern media.

Functional groups

The functional groups substituted onto a chromatographic matrix (Table 2) determine the charge of an IEX medium i.e. a positively-charged anion exchanger or a negatively-charged cation exchanger.

Table 2. Functional groups used on ion exchangers.

Anion exchangers		**Functional group**
Quaternary ammonium (Q)	strong	$-O-CH_2N^+(CH_3)_3$
Diethylaminoethyl (DEAE)*	weak	$-O-CH_2CH_2N^+H(CH_2CH_3)_2$
Diethylaminopropyl (ANX)*	weak	$-O-CH_2CHOHCH_2N^+H(CH_2CH_3)_2$
Cation exchangers		**Functional group**
Sulfopropyl (SP)	strong	$-O-CH_2CHOHCH_2OCH_2CH_2CH_2SO_3^-$
Methyl sulfonate (S)	strong	$-O-CH_2CHOHCH_2OCH_2CHOHCH_2SO_3^-$
Carboxymethyl (CM)	weak	$-O-CH_2COO^-$

** The active end of the charged group is the same for DEAE and ANX. The difference between them is in the length of the carbon chain of the charged group. DEAE has a diethylaminoethyl-group bound to the agarose. ANX has a diethylaminopropyl-group attached which prevents the formation of quaternary groups, giving a different selectivity compared to DEAE.*

The terms strong and weak refer to the *extent that the ionization state of the functional groups varies with pH*. The terms strong and weak do not refer to the strength with which the functional groups bind to proteins. Strong ion exchangers show no variation in ion exchange capacity with change in pH (Figure 13). These exchangers do not take up or lose protons with changing pH and so have no buffering capacity, remaining fully charged over a broad pH range. Strong ion exchangers include Q (anionic), S and SP (cationic).

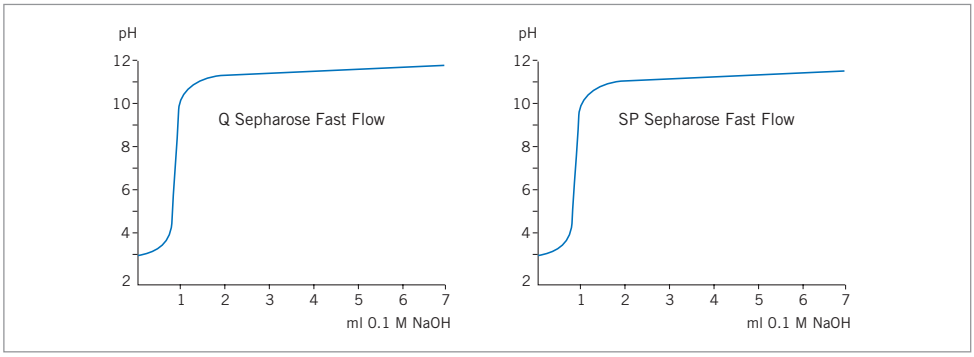


Fig. 13. Titration curves show the ion exchange capacity of strong ion exchangers Q and S. Approximately 5 ml of Q or S Sepharose Fast Flow are equilibrated in 1 M KCl and titrated with 0. 1 M NaOH.

There are several advantages to working with strong ion exchangers:

- development and optimization of separations is fast and easy since the charge characteristics of the medium do not change with pH.
- the mechanism of interaction is simple since there are no intermediate forms of charge interaction.
- sample loading (binding) capacity is maintained at high or low pH since there is no loss of charge from the ion exchanger.

The majority of proteins have isoelectric points within the range 5.5 to 7.5 and can be separated on either strong or weak ion exchangers. An advantage of a weak ion exchanger, such as DEAE (anionic), ANX (anionic) and CM (cationic), is that they can offer a different selectivity compared to strong ion exchangers. A disadvantage is that, because weak ion exchangers can take up or lose protons with changing pH, their ion exchange capacity varies with pH (Figure 14).

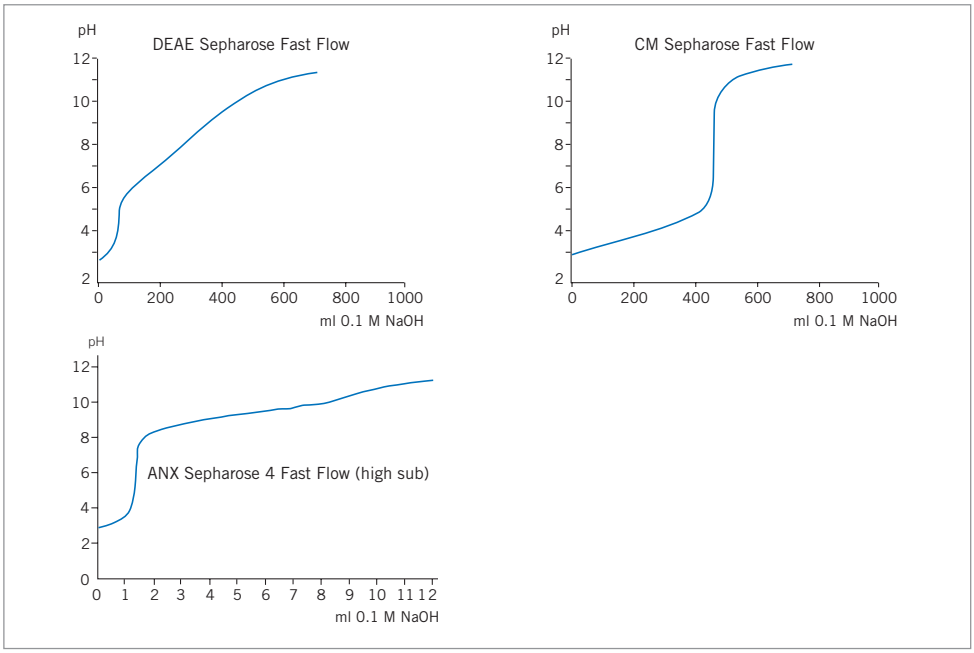


Fig. 14. Titration curves show how the ion exchange capacity of weak ion exchangers varies with pH.

Try a weak ion exchanger such as DEAE, CM or ANX Sepharose Fast Flow, if a strong ion exchanger (substituted with Q, S or SP) does not give the required selectivity.

Binding capacity and recovery

The capacity of an IEX medium is a quantitative measure of its ability to take up counter-ions (proteins or other charged molecules). The *total ionic capacity* is the number of charged functional groups per ml medium, a fixed parameter of each medium. Of more practical relevance is the actual amount of protein which can bind to an IEX medium, under defined experimental conditions. This is referred to as the *available capacity* of a medium for a specific protein. If the defined conditions include the flow rate at which the medium was operated, the amount bound is referred to as the *dynamic capacity* for the medium. Figures for binding capacity in this handbook refer to the dynamic capacity.

The available and dynamic capacities depend upon the properties of the protein, the IEX medium and the experimental conditions. The capacity of an IEX medium will vary according to the molecular size of the specific protein (which affects its ability to enter all the pores of the matrix) and its charge/pH relationship (the protein must carry the correct net charge at a sufficient surface density at the chosen pH). With earlier ion exchange media, larger biomolecules had limited access to the functional groups, significantly reducing the binding capacity. Nowadays, ion exchange matrices such as MonoBeads, SOURCE and Sepharose media all have exclusion limits for globular proteins in excess of 1×10^6 and are therefore suitable for the majority of biomolecule separations. Binding capacities will still vary according to the molecular size of the biomolecules. For example, a matrix with a high degree of small pores will exhibit a higher binding capacity for smaller molecules. Experimental conditions such as pH, ionic strength, counter-ion, flow rate and temperature should all be considered when comparing binding capacities of different IEX medium.

Modern IEX medium show very low levels of non-specific adsorption so that sample recovery under suitable separation conditions is very high, typically between 90–100%.

Chapter 2
Ion exchange in practice

Introduction

This chapter includes practical advice on how to control experimental conditions to achieve a successful separation and guidelines for selection of the most appropriate medium or prepacked column for each application. The final resolution of an ion exchange (IEX) separation is determined by selectivity and column efficiency. These parameters are influenced in turn by factors such as particle size, porosity and column packing. The separation is influenced by a number of factors, for example the way in which the net surface charge of each protein in the sample varies with pH, the pH and ionic strength of buffers and the elution conditions. Understanding the role and importance of each parameter ensures that every separation can be performed with the required resolution, throughput and speed. Additional application examples and product-related information are found in Chapter 3.

Media selection

The origin and differences between modern IEX matrices are explained in Chapter 1. Choice of a suitable matrix depends on factors such as the scale of the final purification, the purpose of the separation (for example to concentrate sample in a capture step or to achieve high resolution in a final polishing step) and the throughput required. Refer to Chapter 4 for more details on the use of *capture, intermediate purification and polishing steps* in a purification strategy.

Capture

Media for primary capture steps, where the aim is to isolate, concentrate and stabilize the target products, should offer high speed and high capacity. Select from the following:

- Sepharose Fast Flow (90 µm particle size) – capture or intermediate purification steps that require good resolution (flows up to 300 cm/h).

Use Sepharose Big Beads (200 µm particle size) for capture steps when viscosity and back-pressure may limit the throughput attainable with IEX media of smaller particle size (flows up to 1000 cm/h).

Use a weak ion exchanger such as DEAE, CM or ANX Sepharose Fast Flow, if a strong ion exchanger (substituted with Q, S or SP) does not give the required selectivity.

Use Sepharose XL (90 µm particle size) for capture when a high binding capacity and rapid separation is required for a selected protein from clarified samples.

If only milligram quantities of product are needed and the capture step will not be scaled up, use high performance media such as Sepharose High Performance, MonoBeads or MiniBeads according to the capacity required. Note that, when using MonoBeads or MiniBeads, it is especially important to remove particulate matter to avoid column blockage.

Intermediate purification

Media for intermediate purification, where the aim is to remove most of the bulk impurities, should offer high capacity and high resolution. Select from the following:

- SOURCE 15 (15 µm) – intermediate purification in laboratory or large-scale applications that require high resolution and high throughput (flows up to 1800 cm/h).
- Sepharose High Performance (34 µm) – for intermediate purification steps that require high capacity and high resolution (flows up to 150 cm/h).
- Sepharose Fast Flow (90 µm) – intermediate purification steps that require good resolution (flows up to 300 cm/h).

If only microgram – milligram quantities are required and the intermediate purification step will not be scaled-up, use MonoBeads or MiniBeads according to the capacity required.

Use SOURCE 30 (30 µm) as an alternative to SOURCE 15 for large scale applications or large sample volumes when speed rather than resolution is a priority (flows up to 2000 cm/h).

Polishing

Media for polishing steps, where the aim is to achieve final purity by removing trace impurities or closely related substances, should offer the highest possible resolution. Select from the following:

- MiniBeads (3 µm) – polishing at microscale when highest resolution is essential.
- MonoBeads (10 µm) – polishing at laboratory scale when highest resolution is essential and a higher capacity than MiniBeads is required.
- SOURCE 15 (15 µm) – polishing in laboratory or large-scale applications that require high resolution and high throughput (flows up to 1800 cm/h).

Use SOURCE 30 (30 µm) as an alternative to SOURCE 15 for large scale applications or large sample volumes when speed rather than resolution is a priority (flows up to 2000 cm/h).

Figure 15 gives a comprehensive selection guide for IEX media and prepacked columns currently available.

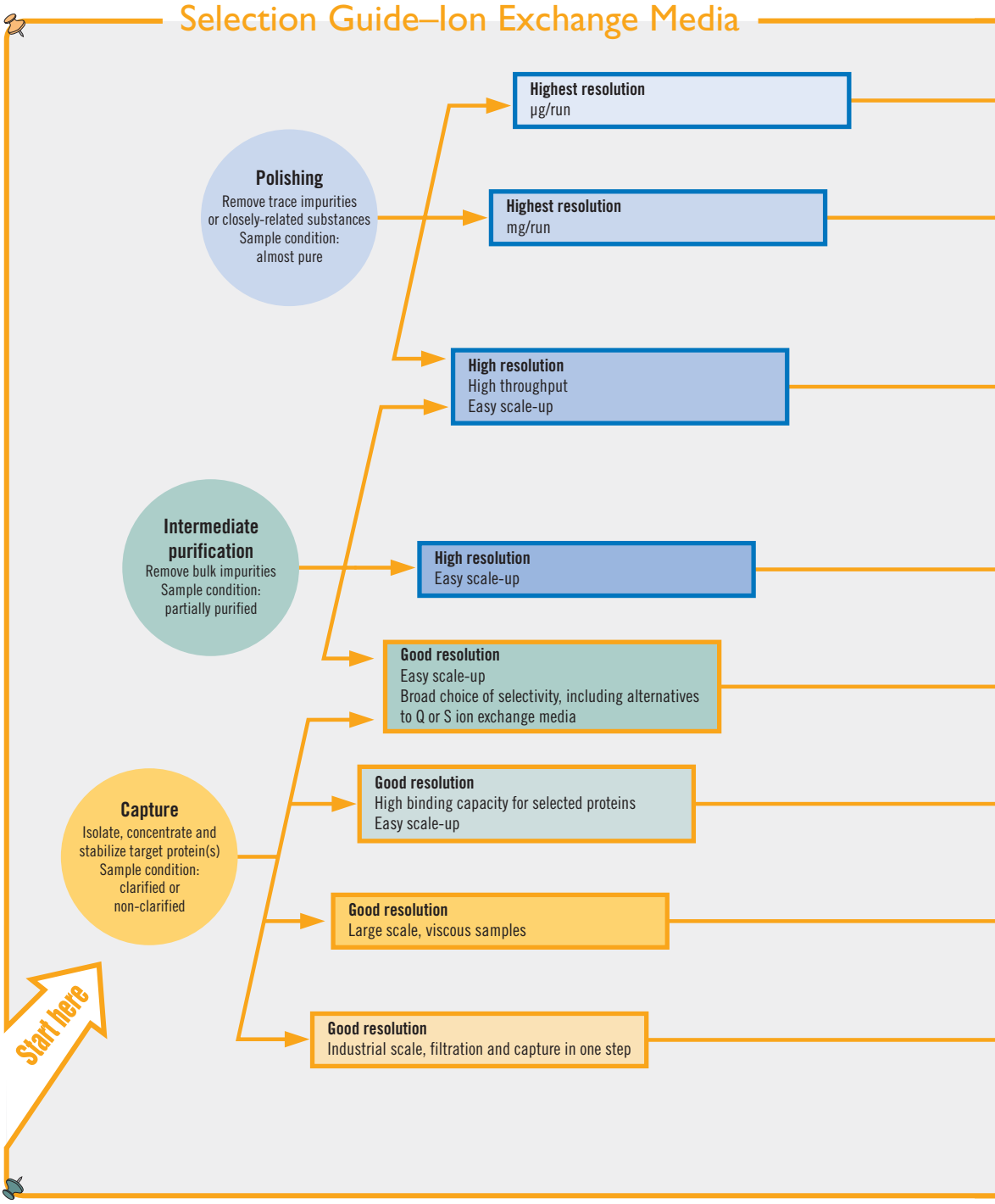


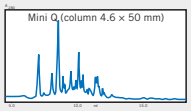
Fig. 15.

A typical purification strategy has three phases: Capture, Intermediate Purification and Polishing (CIPP)

Each phase has a specific objective, dependent largely on the properties of the starting material. Select the appropriate ion exchange medium according to the objective of your purification step and the condition of your starting material.

MiniBeads (Q or S)

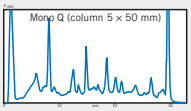
Use for intermediate purification if column capacity is sufficient and no scale-up is required.



Sample: Pancreatin
Gradient elution

MonoBeads (Q or S)

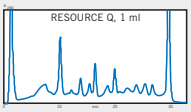
Use for intermediate purification if column capacity is sufficient and no scale-up is required. Can be used for capture steps if sample is free from particulate matter.



Sample: Pancreatin
Gradient elution

SOURCE 15 (Q or S)

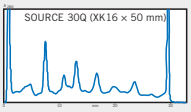
Use SOURCE 15 when resolution is top priority.



Sample: Pancreatin
Gradient elution

SOURCE 30 (Q or S)

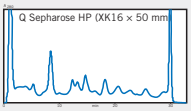
Use SOURCE 30 when speed is top priority.



Sample: Pancreatin
Gradient elution

Sepharose High Performance (Q or SP)

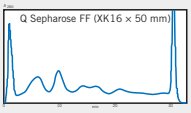
Use HiTrap™ columns prepacked with Sepharose High Performance, Sepharose XL and Sepharose Fast Flow for media selection and pH scouting.



Sample: Pancreatin
Gradient elution

Sepharose Fast Flow (Q, SP, DEAE, CM, ANX)

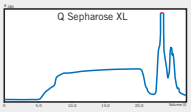
Try weak ion exchangers such as DEAE, CM or ANX if the selectivity of Q or S is unsatisfactory.



Sample: Pancreatin
Gradient elution

Sepharose XL (Q or SP)

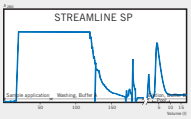
Use Sepharose Q XL virus licensed as an alternative to cesium chloride gradients for purification of viruses, including adenovirus, or viral vectors.



Sample: Recombinant α-amylase
Pilot scale: Gradient elution begins after 20 l

Sepharose Big Beads (Q or SP)

Use with step elution.



Sample: Recombinant antigen binding fragment
Pilot scale: Step elution

STREAMLINE™ (Q XL, SP XL, SP, DEAE, CST 1)

Use STREAMLINE for direct capture from unclarified feed-stock. Use CST 1, a salt tolerant adsorbent, to minimize dilution and reduce process time.

Resolution

Note: STREAMLINE products, based on expanded bed adsorption technology, enable proteins to be purified from crude, particulate feedstock without the need for separate clarification, concentration or initial purification. STREAMLINE products are designed for use in industrial scale processes and for producing gram quantities of product. For more information go to *www.bioprocess.amershambiosciences.com* or ask for the *Expanded Bed Adsorption Handbook*.

Fast media selection and method development

Fig. 16. IEX Selection Kit.

Time and sample can be saved in the early stages of development by using small, prepacked HiTrap columns such as those in the HiTrap IEX Selection Kit to quickly and efficiently screen for the most suitable charge group and to develop the basic separation method. This approach is particularly helpful if the properties of the target protein(s) are unknown. Even if the preferred matrix is not available in a HiTrap format, the method can be easily transferred for optimization at the required scale of operation on the preferred matrix.

HiTrap columns are prepacked with Sepharose Fast Flow media (strong or weak ion exchangers) or Sepharose XL (strong exchangers) and can be run with a syringe, a peristaltic pump or any ÄKTAdesign system. These columns can be used for small scale purification as well as fast method development and are supplied with detailed protocols for use.

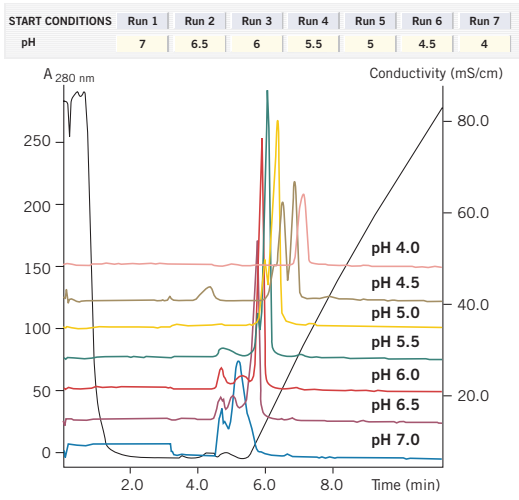
Automated media selection, method development and optimization

Users of ÄKTAdesign systems with BufferPrep functionality can select from a range of buffer recipes to test different media over a range of pH values and other elution conditions (see page 18 for an explanation of the effect of pH on selectivity). BufferPrep automatically calculates and mixes the correct proportions of stock solutions in order to maintain a constant pH during the separation (increasing ionic strength normally causes fluctuations in the actual pH of the running buffer, BufferPrep compensates for these fluctuations and maintains a constant pH throughout the run). When pH and medium have been selected, methods are programmed to vary flow rate and gradient slope in order to optimize the separation.

Note that the condition of the sample is very important in order to achieve the most effective separations. Ideally, samples should be in the same conditions as the start buffer (see Appendix 1, Sample preparation and, in particular, *Buffer exchange and desalting*, page 156 for details). When working with small volumes during screening and scouting, it may be sufficient to dilute the sample in start buffer in order to lower the ionic strength and adjust the pH to a value similar to that of the start buffer.

1. Scout for optimum pH by testing a range of pH values within which the proteins of interest are known to be stable. If the isoelectric point of the target protein is known, then begin with a narrower pH range, for example, 0.5–1 pH unit away from the isoelectric point. Typical results from an automatic pH scouting run are shown in Figure 17.
2. If required, scout for optimum selectivity (testing strong or weak exchangers) using automatic media scouting.
3. Scout for the steepest gradient that gives acceptable resolution at the selected pH.
4. Scout for the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
5. Scout for the maximum sample load that can be applied while maintaining satisfactory resolution. In general, loading 20–30% of the total binding capacity of the column gives optimal resolution with gradient elution.

Reduce separation time and buffer consumption by transfering to a step elution when optimized separation conditions have been established. Sample loads can often be increased when using a step elution.



Column: RESOURCE S 6 ml
Sample: Fab fraction from HIC separation 20 ml
Eluents: Automatic BufferPrep with 0.06 M sodium acetate, 0.03 M sodium phosphate, 0.03 M sodium formate 0.1 M HCl and 2 M NaCl
Gradient: 20 column volumes, to I M NaCl
Flow: 60 ml/min
Curves: A_{280nm}, from top: pH 4.0; 4.5; 5.0; 5.5; 6.0; 6.5; 7.0

Fig. 17. Automatic pH scouting on ÄKTAexplorer™.

Manual media selection, method development and optimization

HiTrap columns are well-suited for manual media screening, method development and method optimization since they can be used with a syringe or peristaltic pump as well as an automated chromatography system.

Note that the condition of the sample is very important in order to achieve the most effective separations. Ideally, samples should be in the same conditions as the start buffer (see Appendix 1, Sample preparation and, in particular, *Buffer exchange and desalting*, page 156 for details). When working with small volumes during screening, it may be sufficient to dilute the sample in start buffer in order to lower the ionic strength and adjust the pH to a value similar to that of the start buffer.

Scout for optimum pH by testing a range of pH values within which the proteins of interest are known to be stable. If the isoelectric point of the target protein is known, then begin with a narrower pH range, for example, 0.5–1 pH unit away from the isoelectric point. The methods here are optimized for use with 1 ml HiTrap columns and should be adjusted if other column volumes are used.

Screening for IEX medium and pH conditions

1. Start buffers: set up a series of buffers with pH values in the range 4–8 (SP, CM) or 5–9 (Q, DEAE, ANX) and with 0.5–1 pH unit intervals between each buffer. See Appendix 2 for recommended buffers.
2. Elution buffers: set up a second series of buffers with the same pH values, but including 1 M NaCl.
3. Equilibrate the column (s) with 5 ml start buffer at 1 ml/min. Wash with 5 ml elution buffer.
4. Re-equilibrate with 5–10 ml start buffer.
5. Adjust the sample to the pH of the start buffer and apply a known amount of the sample at 1 ml/min. Collect eluate.
6. Wash with at least 5 ml of start buffer or until no material appears in eluent. Collect eluate.
7. Elute bound material with elution buffer (3–5 ml is usually sufficient, but other volumes may be required dependent on the exact experimental conditions). Collect eluate.
8. Analyze all eluates (for example by an activity assay) and determine purity and the amount bound to the column.
9. Perform steps 3–8 for the next buffer pH.
10. Select medium and pH: the most suitable pH should allow the protein(s) of interest to bind, but should be as close to their point of release as possible.

Screening for ionic strength conditions

1. Using the selected medium, start buffer and pH from the previous protocol, set up a series of elution buffers at the same pH, but vary the salt concentration from 0–0.5 M with intervals of 0.05–0.1 M salt between each buffer.
2. Repeat steps 3–8 from the previous protocol for each salt concentration.
3. Determine the maximum ionic strength which permits binding of the protein(s) of interest and the minimum ionic strength required for complete elution.

Further optimization

1. If gradient making equipment is available, determine the steepest gradient that gives acceptable resolution at the selected pH. Begin with a gradient of 10 column volumes over an ionic strength range based on the maximum and minimum values determined when screening. Alternatively, begin with a gradient of 0–50% elution buffer that contains 1 M NaCl and a gradient volume of 10–20 column volumes.
2. Determine the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
3. Determine the maximum sample load that can be applied while maintaining satisfactory resolution. In general, loading 20–30% of the total binding capacity of the column gives optimal resolution with gradient elution. Sample loads can often be increased if resolution is satisfactory or when using a step elution.

Using PD-10 columns for media selection and method development

If an assay is available to detect the target protein(s) and the media of interest are available, PD-10 columns can be packed with alternative media and used to select the medium and establish some idea of the most suitable separation conditions. With basic information on the requirements for pH and ionic strength, a suitable column can be packed in order to begin optimization.

Note that the condition of the sample is very important in order to achieve the most effective separations. Ideally, samples should be in the same conditions as the start buffer (see Appendix 1, Sample preparation and, in particular, *Buffer exchange and desalting*, page 156 for details). When working with small volumes during screening and scouting, it may be sufficient to dilute the sample in start buffer in order to lower the ionic strength and adjust the pH to a value similar to that of the start buffer.

pH selection

1. Set up a series of 10 PD-10 columns for each medium to be tested and thoroughly resuspend the medium in its storage solution.
2. Pour approximately 25 mls of slurry into the PD-10 column, allowing the medium to settle as the column fills. Do not allow the column to dry out.
3. Equilibrate each column to a different pH by washing (5 × 5 ml) with buffer (0.5 M) using buffers between pH 5–9 for anion exchangers or pH 4–8 for cation exchangers and with 0.5 pH unit intervals between columns (see Appendix 2 for buffer recommendations).
4. Equilibrate each column at a lower ionic strength: wash with 5 × 5 ml of buffer (0.02–0.05 M) at the same pH.
5. Load a known constant amount of sample to each column while collecting the eluent.
8. Assay the eluent for the protein of interest. The most suitable medium and pH should allow the protein to bind (protein is absent from the eluent), but should be as close to the point of release as possible (the first pH at which the protein appears in the eluent).

Ionic strength selection

1. Set up a series of 10 PD-10 columns, each containing 5 ml of the chosen IEX medium.
2. Equilibrate the column by washing (5 × 5 ml) with buffer (0.5 M) at the selected starting pH.
3. Equilibrate the columns at different ionic strengths, but constant pH, ranging from 0.01 M to 0.3 M NaCl by washing (5 × 5 ml). Intervals of 0.05 M NaCl are sufficient.
4. Apply sample while collecting the eluent.
5. Assay the eluent to determine the maximum ionic strength which permits binding of the target protein and the minimum ionic strength required for complete elution. The highest ionic strength which permits binding and the lowest ionic strength for elution are used as start and elution buffers, respectively, during subsequent gradient elution.

Practical considerations for IEX separation

This section covers detailed aspects of each step in an IEX separation, together with practical hints and tips to improve resolution and overall performance. In practice a separation can be summarized as follows:

1. **Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.**
2. **Adjust the sample to the chosen starting pH and ionic strength and apply to the column.**
3. **Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.**
4. **Begin elution using a gradient volume of 10–20 column volumes with an increasing ionic strength up to 0.5 M NaCl (50%B).**

   Alternatively (if gradient-making equipment is not available) elute bound proteins with 5 column volumes of start buffer + NaCl at chosen ionic strength. Repeat at higher ionic strengths until the target protein(s) has been eluted.
5. **Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically-bound material.**
6. **Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.**

These steps are highlighted throughout this section.

Buffer volumes referred to are expressed in *column volumes*, for example 3 CV=3 ml for a column with a 1 ml bed volume. Using column volumes to describe a separation profile facilitates method development and transfer of methods to columns of different dimensions.

The number of column volumes used at each stage of the separation can often be reduced by optimization. For example, less buffer is required to equilibrate a strong ion exchanger, the gradient volume can be reduced if resolution can be maintained and less buffer may be required for washing when separating less complex and reasonably clean samples.

pH and ionic strength

Buffer pH and ionic strength must be compatible with protein stability and activity. The most suitable pH should allow the proteins of interest to bind, but should be as close to the point of release (elution) as possible. If the pH is too low or too high, elution becomes more difficult and high salt concentrations may be needed. This should be avoided since some proteins begin to precipitate at high ionic strength and high salt concentrations may interfere with assays or subsequent chromatographic steps.

Avoid extreme changes in pH or other conditions that may cause inactivation or even precipitation.

The pH and ionic strength of the sample are extremely important in order to achieve the most effective high resolution or group separations and to make the most of the high loading capacity. Ideally, samples should be in the same conditions as the start buffer

(see Appendix 1, Sample preparation and, in particular, *Buffer exchange and desalting*, page 156 for details). When working with small volumes during screening and scouting, it may be sufficient to dilute the sample in start buffer in order to lower the ionic strength and adjust the pH to a value similar to that of the start buffer.

Proteins often begin to dissociate from IEX media about 0.5 pH units from their isoelectric points at an ionic strength around 0.1 M. The pH of the start buffer should be at least 0.5–1 pH unit above the pI of the target substance when using an anion exchanger (Q, DEAE or ANX) or 0.5–1 pH unit below the pI of the target substance when using a cation exchanger (SP or CM).

For samples with unknown charge properties, try the following:

– anion exchange (Q, DEAE or ANX)
start buffer: pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

– cation exchange (S, SP, CM)
start buffer: pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

See Appendix 2 for recommendations on volatile and non-volatile buffer systems for anion and cation exchangers.

Whenever possible, check for stability at the pH and ionic strength values selected, especially if recovery of biological activity is a priority.

Anion or cation exchanger

For molecules such as nucleic acids which carry only negatively-charged groups, an anion exchanger is the obvious choice. However, since the net charge of molecules such as proteins (carrying positively and negatively charged groups) depends on pH, the choice is based on which type of exchanger and pH give the desired resolution within the constraints of sample stability. For example, Figure 18 shows a theoretical protein which has a net positive charge below its isoelectric point and can bind to a cation exchanger. Above its isoelectric point the protein has a net negative charge and can bind to an anion exchanger. However, the protein is only stable in the range pH 5–8 and so an anion exchanger has to be used.

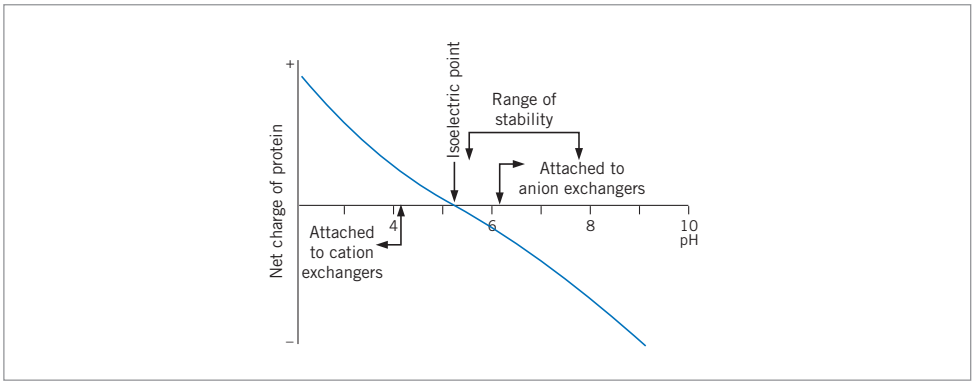


Fig. 18. Considerations when selecting a suitable IEX medium.

If sample components are most stable below their isoelectric points, use a cation exchanger.

If sample components are most stable above their isoelectric points, use an anion exchanger.

If stability is high over a wide pH range on both sides of the isoelectric point, use either type of ion exchanger.

Strong or weak ion exchangers

Table 3 shows the functional groups used on IEX media. The terms strong and weak refer to the *extent that the ionization state of the functional groups varies with pH*. The terms strong and weak do not refer to the strength with which the functional groups bind to proteins.

Table 3. Functional groups used on ion exchangers.

Anion exchangers		**Functional group**
Quaternary ammonium (Q)	strong	$-O-CH_2N^+(CH_3)_3$
Diethylaminoethyl (DEAE)*	weak	$-O-CH_2CH_2N^+H(CH_2CH_3)_2$
Diethylaminopropyl (ANX)*	weak	$-O-CH_2CHOHCH_2N^+H(CH_2CH_3)_2$
Cation exchangers		**Functional group**
Sulfopropyl (SP)	strong	$-O-CH_2CHOHCH_2OCH_2CH_2CH_2SO_3^-$
Methyl sulfonate (S)	strong	$-O-CH_2CHOHCH_2OCH_2CHOHCH_2SO_3^-$
Carboxymethyl (CM)	weak	$-O-CH_2COO^-$

** The active end of the charged group is the same for DEAE and ANX. The difference between them is in the length of the carbon chain of the charged group. DEAE has a diethylaminoethyl-group bound to the agarose. ANX has a diethylaminopropyl-group attached which prevents the formation of quaternary groups, giving a different selectivity compared to DEAE.*

Begin with a strong exchanger to enable development work to be performed over a broad pH range. Use a strong anion exchanger (Q) to bind the protein(s) of interest if their isoelectric point is below pH 7.0 or unknown.

Use a strong exchanger in those cases where maximum resolution occurs at an extreme pH and the proteins of interest are stable at that pH.

Consider using a weak exchanger if the selectivity of the strong ion exchanger is unsatisfactory, but remember that the ion exchange capacity of a weak ion exchanger varies with pH. As a result:

- sample loading (binding) capacity can vary with increasing pH due to loss of charge from the exchanger.
- resolution is more readily affected by changes in flow rate or sample load due to the intermediate forms of charge interaction which can occur.
- predicted results (based on known information about the sample components such as their isoelectric points and how their net surface charge changes with pH) may not correlate with actual results since the number of charged groups on weak ion exchangers can vary with pH.
- longer equilibration times may be required in order to titrate the weak ion exchange functional groups.

When using a weak exchanger, work within the pH values given below to minimize variations in performance:

DEAE: pH 2–9
ANX: pH 2–9
CM: pH 6–10

Buffer selection and preparation

Buffer ions

Buffering ions should have the same charge as the functional groups on the IEX medium (buffering ions that carry a charge opposite to that of the functional groups will take part in the ion exchange process and can cause significant pH fluctuations during elution) and, preferably, a pKa value within 0.6 pH units of the working pH. An exception to this rule is seen in the frequent use of phosphate buffers with anion exchange separations. However, phosphate buffers must be very carefully prepared to ensure reproducibility between batches.

Use a buffer concentration that is sufficient to maintain buffering capacity and constant pH, typically 20–50 mM.

Use volatile buffers if the purified product is to be lyophilized.

See Appendix 2 for recommendations on volatile and non-volatile buffer systems for anion and cation exchangers.

Filter buffers after all salts and additives have been included. Use high quality water and chemicals. Filter solutions through 1 µm filters for particle sizes above 90 µm, 0.45 µm filters for 34 µm particles or 0.22 µm filters for particles sizes below 15 µm or when sterile or extra clean samples are required. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

Effect of temperature on buffer pH

Select buffers that have appropriate pKa values for the working temperature. The pKa of a buffering substance varies with temperature. For example Tris™ has a pKa of 8.85 at 0 °C, 8.06 at 25 °C and 7.72 at 27 °C. Using Tris at 4 °C at a pH 7.9 would give a very low buffering capacity and the working pH would be outside the useful pH range (pKa $\pm$ 0.5) of the buffer.

Prepare buffers at the same temperature at which they will be used.

Temperatures <10 °C can minimize aggregation caused by hydrophobic interactions between sample components. Working at these lower temperatures may be an alternative solution to using a detergent to improve solubility.

Counter-ions

The counter-ions (salt ions) used in IEX are almost always Na^+ for cation exchange and Cl^- for anion exchange.

Salts such as NaCl have a chaotropic character (i.e. an ability to make water less polar) and therefore a lower 'salting-out' effect on hydrophobic molecules. This ensures maximum solubility during elution and improves recovery. Chaotropic salts can also be used in the presence of organic solvents if required. Salts such as $(NH_4)_2SO_4$ or K_3PO_4 should be avoided as they are most likely to cause precipitation at high concentrations.

In certain applications alternative counter-ions such as Li^+, Br^-, I^-, SO_4^{2-}, CH_3COO^- or $HCOO^-$ may improve and even alter, selectivity since they exhibit different elution strengths, but it should be noted that using these ions may affect the binding capacity of the medium. Figure 19 shows how selectivity and resolution can vary when using different counter-ions.

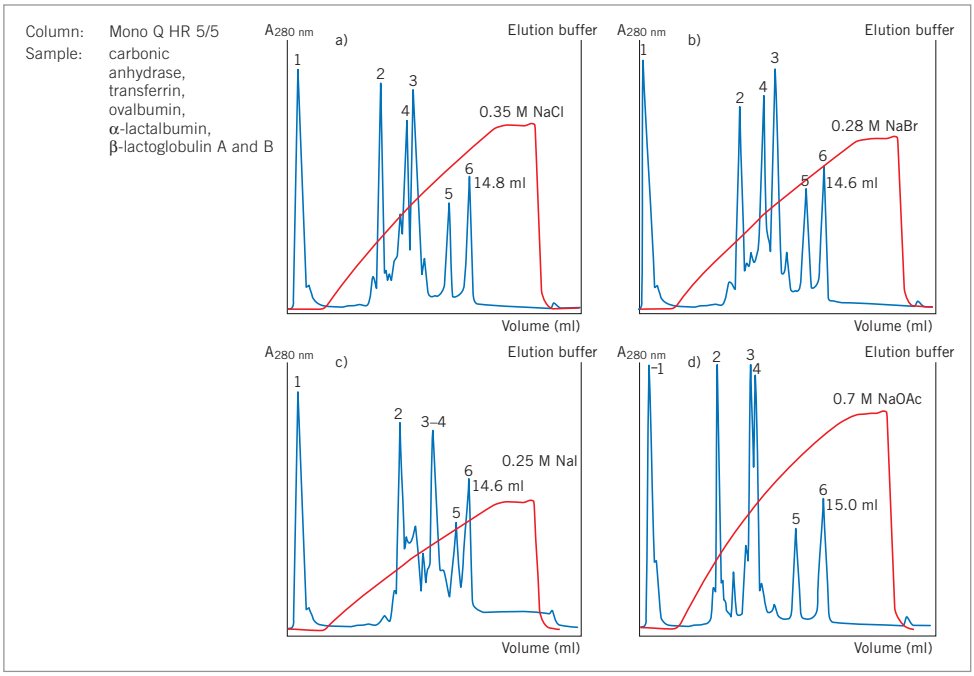


Fig. 19. Effect of counter-ions on selectivity and resolution (Mono Q HR 5/5 now available as Mono Q 5/50 GL). Note the variation in elution order of peaks 3 and 4.

Use the following procedure if a medium is to be used with counter-ions other than sodium or chloride:

1. Wash the packed column with 10 column volumes 0.5–1 M salt solution containing the new counter-ion. Flow rate: see relevant media section in Chapter 3.
2. Wash with 10 column volumes of start buffer at the same flow rate as in step 1.
3. Repeat steps 1 and 2 several times.

Perform a blank run to check conductivity and pH.

Column and media preparation

Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.

Using prepacked columns is highly recommended to ensure the best performance and reproducible results. An evenly packed column ensures that component peaks are not unnecessarily broadened as sample passes down the column so that the best resolution can be achieved.

Allow buffers, media or prepacked columns to reach the same temperature before use. Rapid changes in temperature, for example removing packed columns from a cold room and then applying buffer at room temperature, can cause air bubbles in the packing and affect the separation.

Wash away storage solutions and preservatives before using any IEX medium.

Increase the volumes used for column equilibration before the first run if using buffers containing detergents or a different counter-ion to the one in which the medium has been stored.

Appendix 3 gives details on column packing. The volume required for the packed bed is determined by the amount of sample to be purified and the binding capacity of the medium. Pack a column that will have approximately 5-fold excess of the binding capacity required with a bed height up to 20 cm.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3. Note that this does not apply to HiTrap or HiPrep™ columns.

Sample preparation

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Simple steps to clarify a sample before application to a column will avoid the risk of blockage and reduce the need for stringent washing procedures. Appendix 1 contains a detailed overview of sample preparation techniques.

Desalt samples and transfer into the chosen start buffer (refer to page 156 for details of buffer exchange and desalting). The pH and ionic strength of the sample are extremely important in order to achieve the most effective high resolution or group separations and to make the most of the high loading capacity.

For small sample volumes in a high salt concentration and with no major contaminants such as lipids or ionic detergents, it may be sufficient to dilute the sample with start buffer in order to lower the salt concentration to a level that does not interfere with binding to the medium. However, buffer exchange and desalting is the only way to guarantee the correct pH and ionic strength conditions of a sample.

Samples must be clear and free from particulate matter, particularly when working with particle sizes of 34 µm or less. For small sample volumes, a syringe-tip filter of cellulose acetate or PVDF can be sufficient for sample filtration.

Concentration and viscosity

The solubility or viscosity of the sample may limit the quantity that can be applied to a column. High sample viscosity can cause instability of the separation and an irregular flow pattern resulting in broad, distorted peaks and problems with back pressure. The critical parameter is the viscosity of the sample *relative to* the viscosity of the eluent.

Dilute viscous samples with start buffer. If high viscosity is caused by the presence of nucleic acid contaminants, see Appendix 1 for advice on their removal. Remember that viscosity varies with temperature. If dilution is not an option, using a medium with a larger particle size may help to overcome viscosity problems.

Samples should *generally* not exceed 50–70 mg/ml protein, but may vary according to the type of sample and the type of chromatographic medium.

Sample application

Adjust the sample to the chosen starting pH and ionic strength (see sample preparation) and apply to the column.

Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.

Starting conditions should maximize binding of the target proteins near the top of the column and, when possible, minimize binding of contaminants so that they pass through the column.

For efficient binding the sample should be at the same pH and ionic strength as the start buffer. The sample volume can be relatively large without affecting the separation since the sample will bind at the top of the column as long as equilibration and sample conditions are correct.

Apply samples directly to the column via a chromatography system, a peristaltic pump or a syringe. The choice of equipment depends largely on the sample volume, the size of column, the type of IEX medium and the requirements for accuracy in gradient elution. Ensure that the top of the column bed is not disturbed during sample application

Do not change buffer conditions until all unbound material has been washed through the column (monitored by UV absorbance) and until UV and conductivity values have returned to starting conditions.

Sample load

Sample load (mass) is of greater importance than sample volume. The amount of sample which can be applied to a column depends on the dynamic binding capacity of the IEX medium and the degree of resolution required. Sample load has a major influence on resolution since the width of the peaks is directly related to the amount of substance present, as shown in Figure 20. Consequently, in order to achieve satisfactory resolution, the total amount of protein applied and bound to the medium should not exceed the total binding capacity of the packed column.

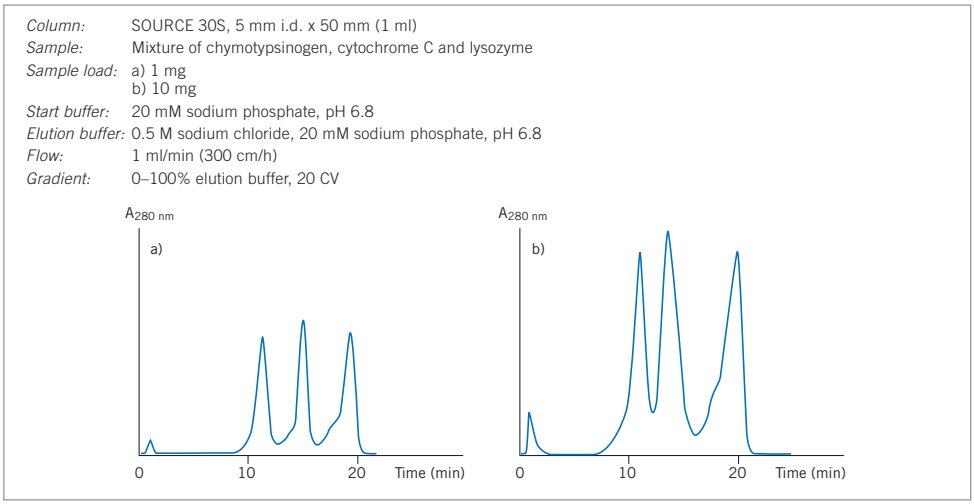


Fig. 20. The influence of increasing sample load on resolution.

Apply up to 30% of the total binding capacity of the column for optimal resolution with gradient elution. Sample loads can be increased if resolution is satisfactory or when using a step elution.

If sample volumes are large compared to the total column volume, the sample buffer composition, in particular the ionic strength, should be the same as that of the start buffer to ensure adequate binding conditions.

Chapter 3 gives typical binding capacities for each medium as a guideline for total binding capacity.The actual (dynamic) binding capacity is also affected by factors such as size and shape of the molecules, the pore size of the matrix, flow rate, sample concentration, pH/protein charge and ionic strength. Capacity will decrease for molecules of very large diameter or length, e.g. protein complexes >M_r 400 000, assymmetric proteins and DNA. These molecules are unable to penetrate the matrix pores, limiting their binding primarily to the charged groups on the surface of the matrix. Since the exact distribution of pore sizes in some matrices can vary and the apparent size of a molecule can vary according to the buffer conditions, there is no distinct molecular weight cut-off point when molecules can or cannot penetrate the matrix pores.

The binding step and the dynamic binding capacity can be increased by applying sample at a pH where the target protein has a higher charge than if the optimal pH for separation was used.

Sample volume

As a binding technique, IEX is independent of sample volume as long as the ionic strength of the sample is the same or as low as the start buffer and the target proteins are sufficiently charged at the selected pH. Large volumes of dilute solutions, such as fractions from a desalting step or a cell culture supernatant, can be applied directly to an IEX medium without prior concentration.

Elution

Bound proteins are eluted by controlled changes in ionic strength or pH. The way in which these changes take place, by using a linear or step elution, is selected according to the aim of the separation:

- Linear gradient elution
  - high resolution separation or analysis
  - optimized gradient elution at increased speed while retaining required resolution
- Step elution
  - faster separation time, reduced buffer consumption
  - group separation

Linear gradient elution

Aim: high resolution separation or analysis, screening

Begin elution using a linear gradient volume of 10–20 column volumes with an increasing ionic strength up to 0.5 M NaCl (50%B).

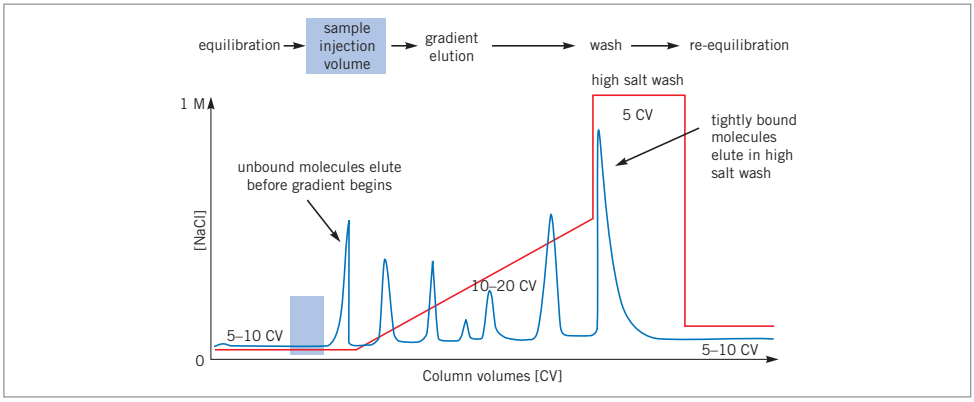


Fig. 21. Typical IEX separation using linear gradient elution. The UV (protein) and conductivity (salt) traces show the elution of protein peaks and the changes in salt concentration during elution.

Linear ionic strength gradients, as shown in Figure 21, are the most frequently used type of elution and should always be used when starting with an unknown sample (when as many components as possible are bound to the column and eluted differentially to see a total protein profile). At low ionic strengths, competition for charged groups on the IEX medium is at a minimum. Increasing the ionic strength increases competition and reduces the interaction between the medium and the bound substances which begin to elute. The elution buffer is usually the same buffer salt and pH as the start buffer, but contains additional salt, most often sodium chloride.

It is strongly recommended to use linear gradient elution during method development. Linear ionic strength gradients are easy to prepare and very reproducible when generated by a suitable chromatography system. The results obtained can then serve as a base from which to optimize the separation.

The retention of charged proteins on the medium is related to the volume of the column and the concentration difference across it:

- long, shallow gradients give maximum separation between peaks, but separation times will be longer and there will be greater peak broadening.
- short, steep gradients give faster separations and sharper peaks, but peaks will be eluted closer together.
- peaks eluted later in the gradient tend to be slightly broader than those eluted early on.

Select the steepest gradient to give acceptable resolution at the selected pH.

The effects of gradient slope are shown in Figure 22.

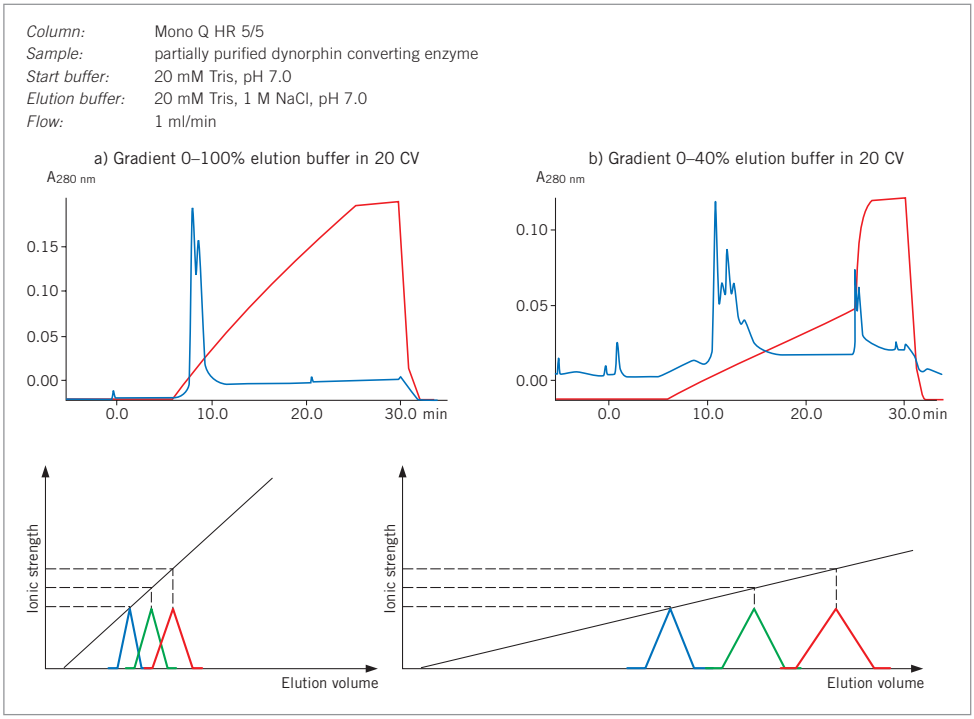


Fig. 22. Effect of gradient slope on resolution, in theory and in practice.

If gradient elution volumes are decreased, it may be necessary to decrease the sample load proportionally in order to maintain the same resolution. Similarly, if sample load is increased (within the total capacity of the column), gradient volumes may need to be increased to maintain resolution.

Gradients are best formed using purpose-designed equipment, such as ÄKTAdesign systems with preprogrammed method templates, that automatically controls the mixing of solutions being supplied to a column. Users of ÄKTAdesign systems with BufferPrep functionality can select from a range of buffer recipes to run salt gradient elutions at constant pH.

BufferPrep automatically calculates and mixes the correct proportions of stock solutions in order to maintain a constant pH throughout the run. Alternatively, systems may use two separate pumps for start and elution buffers or a single pump in combination with a switch valve to mix the buffers.

Accurate buffer preparation, efficient mixing and the shortest possible flow path between a mixer and the top of a column will help to ensure accurate gradient formation.

Aim: reduced separation time, maintained resolution

For certain separations, when conditions for a high resolution separation using a linear gradient have been established, it may be possible to reduce the total separation time by using a more complex elution profile, as illustrated in Figure 23. Shallow gradients can be used where maximum resolution is required while steeper gradients can be used in areas where resolution is satisfactory.

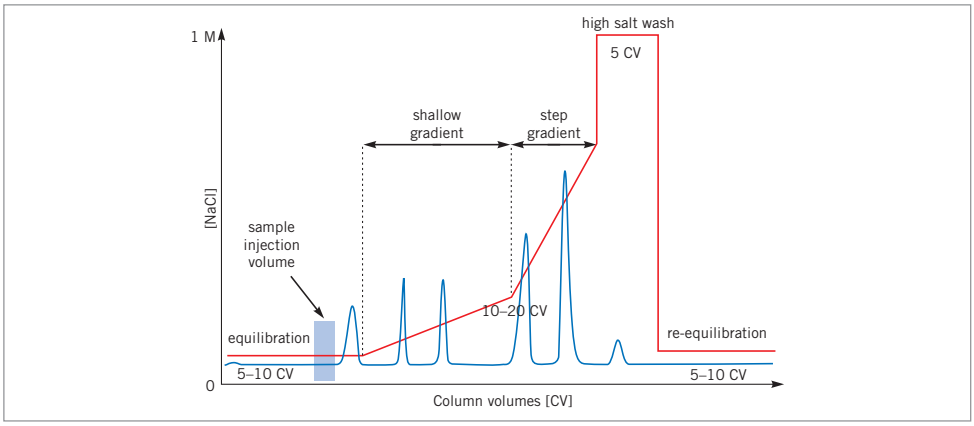


Fig. 23. Complex gradient profiles can reduce total separation time for certain separations.

Step elution

Elute bound proteins with 5 column volumes of start buffer + NaCl at chosen ionic strength.

Repeat at higher ionic strengths until the target protein(s) has been eluted.

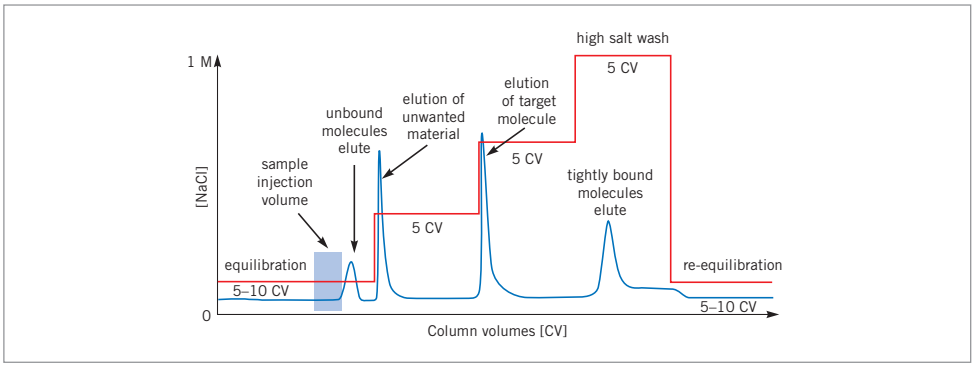


Fig. 24. Typical IEX separation using step elution. The UV (protein) and conductivity (salt) traces show the elution of protein peaks and the changes in salt concentration during elution.

As shown in Figure 24, step elutions are performed by sequential addition of the same buffer at increasing ionic strengths. Step elution is technically simple, but care must be taken in the design of the steps and the interpretation of results since substances eluted by a sharp change in ionic strength elute close together, giving a false peak that may contain several components. Peaks tend to have sharp fronts and pronounced tailing since they frequently contain more than one component. Tailing may lead to the appearance of false peaks if a change in ionic strength is introduced too early. For these reasons it is recommended to use a linear ionic strength gradient when developing a new method.

Aim: faster separation time, reduced buffer consumption

When an IEX separation has been optimized using gradient elution, changing to a step elution reduces the total number of column volumes used for a separation. This *speeds up separation times and reduces buffer consumption* while retaining the required purity level. Step elutions of this type are often used for routine, large scale separation. An added advantage of a step elution when used at larger scale is that it is often possible to apply a greater amount of sample, since the molecules which would elute early in a gradient separation no longer take up binding capacity on the column.

Aim: group separation

In a group separation the molecules of interest are concentrated and rapidly removed from unwanted substances. When binding and elution conditions for a target protein(s) and contaminants have been determined, usually during preliminary gradient elution separations, conditions are chosen to maximize binding of the target protein(s) and minimize binding of contaminants during sample application. The target protein(s) is then eluted by a single buffer change in an enriched, concentrated form. Figure 25 shows an example of such a separation in which a HiTrap Q HP column is used to separate human serum proteins from the unwanted IgG fraction which passes directly through the column.

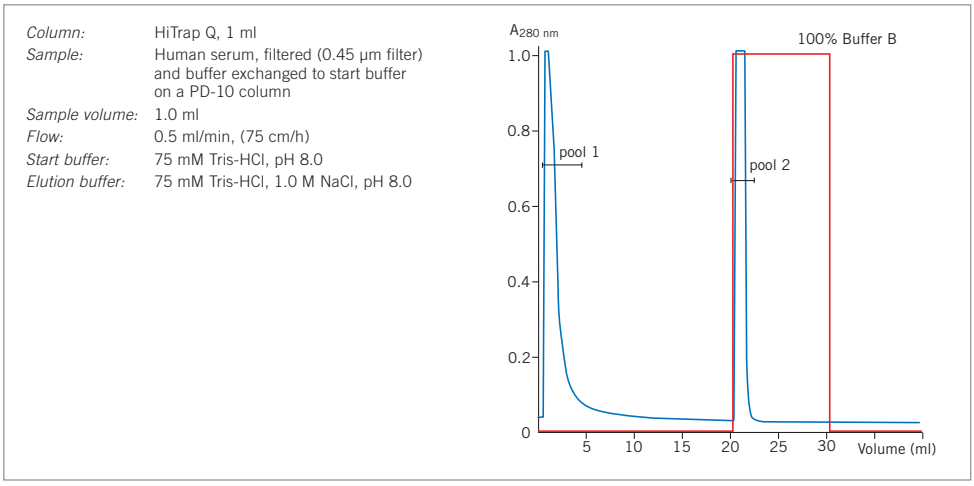


Fig. 25. Group separation of serum proteins on HiTrap Q HP.

Aim: removal of contaminants

If starting conditions have been chosen to maximize the binding of contaminants then no change in elution conditions is required since the target protein(s) will pass through the column. For many applications it is preferable to discard the column rather than spend time and effort removing unwanted bound substances.

pH elution

Since the net charge on a protein is pH dependent, samples can also be eluted from an IEX medium by altering the pH of the elution buffer. As there is no salt gradient, samples are simply retained on the column at one pH and eluted by increasing or decreasing the pH. The various charged groups in the sample or on the column are titrated until they are neutral or of opposite charge to the medium and the sample elutes.

- Proteins bound to an anion exchanger (Q, DEAE, ANX) will elute as pH is decreased.
- Proteins bound to a cation exchanger (SP, S, CM) will elute as pH is increased.

Since pH elution will involve working at pH values close to the isoelectric point of a protein and since many proteins show minimum solubility close to their isoelectric points, precautions must be taken to avoid precipitation on the column (see page 48 for information on the use of additives to avoid precipitation).

Always test in advance the solubility of sample components at the pH and salt concentrations to be used during separation.

For any type of pH elution, care must be taken in the selection and mixing of buffer systems in order to achieve reproducibility. Stepwise pH elution is easier to produce and more reproducible than using a linear pH gradient. Note that for weak ion exchangers the buffer may have to titrate the charged groups on the medium and there will be a short period of re-equilibration before the new pH is reached.

Linear pH gradients are very difficult to produce at constant ionic strength, since simultaneous changes in ionic strength, although small, also occur. These gradients cannot be obtained simply by mixing buffers of different pH in linear volume ratios since the buffering capacities of the systems produced are pH dependent. A relatively linear gradient can be produced over a narrow pH interval (maximum 2 pH units) by mixing two solutions of the same buffer salt adjusted to 1 pH unit above and 1 pH unit below the pKa for the buffer.

In general, separation of proteins according to their isoelectric points, using chromatofocusing (Chapter 5), is likely to provide a more reliable and higher resolution result than attempting to elute proteins from an IEX column using a pH gradient.

Flow rates

The maximum flow rate applied during a separation can vary according to the stage of the separation. For example, during sample application and elution, lower flow rates allow time for sample components to diffuse in and out of the pores as they to bind to or dissociate from the functional groups. Figure 26 shows an example of the influence of flow rate on resolution. Higher flow rates can be used for equilibration, washing and re-equilibration, limited primarily by the rigidity of the media and by pressure specifications of the equipment.

Recommended flow rates for each chromatography medium are given in Chapter 3. Working from these recommendations, *select the highest flow rate that maintains resolution and minimizes separation time*. For example, if peaks are well separated at a low flow rate, increase the flow rate or, alternatively, increase the sample volume to benefit from a higher capacity without significant loss of resolution.

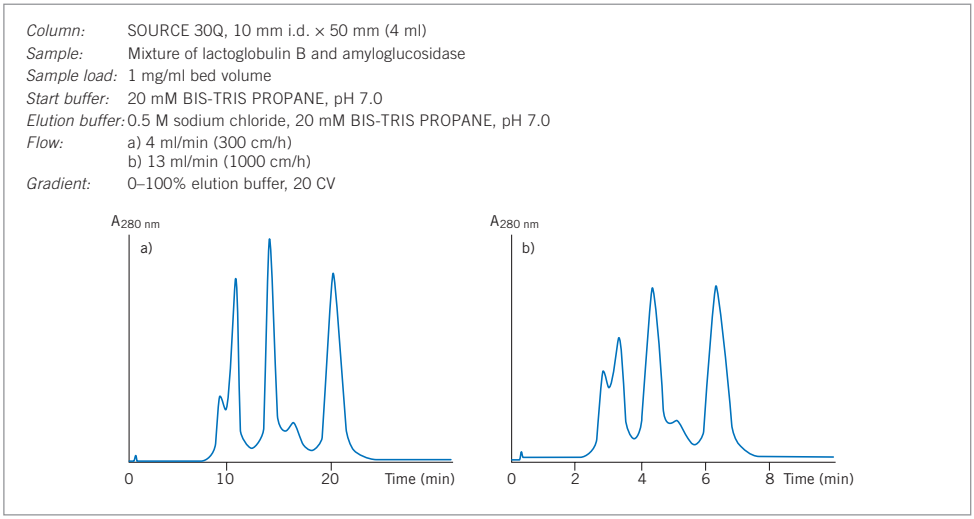


Fig. 26. Influence of increasing flow rate on resolution.

Flow rate is measured in simple volume terms, e.g. ml/min, but when comparing results between columns of different sizes or when scaling-up, it is useful to use 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.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.

Higher flow rates and viscous buffers increase 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.

Flow control

Accurate, reproducible flow control is essential for good resolution and reproducibility.

Use a pump within a chromatography system (rather than a peristaltic pump) to fully utilize the high rigidity and excellent flow properties of media such as MiniBeads, MonoBeads, SOURCE or Sepharose High Performance.

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 in order to avoid shrinking of the column bed by pressure increases that may occur when running a sample.

Wash and re-equilibration

Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically-bound material.

Include a wash step at the end of every run in order to remove any molecules that are still bound to the medium. Monitor UV absorbance so that the wash step can be shortened or prolonged, as necessary.

Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

A re-equilibration step after washing returns the column to start conditions before applying further samples. Whenever possible, monitor pH and conductivity to check when start conditions have been reached. The re-equilibration step can then be shortened or prolonged as necessary.

Increase flow rates during wash and re-equilibration steps to save time between runs.

If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Detergents, denaturing agents and other additives

Any additives used for dissociation, solubilization, metal chelation, enzyme inhibition etc. should always be checked for their charge characteristics at the working pH. Run blank gradients with additives included in order to check their effect on the chromatographic profile.

Additives used during sample preparation will be separated from the sample components during IEX. If proteins are seen to precipitate, elute later than expected or are poorly resolved, add a suitable concentration of the additives used for initial solubilization to the start and elution buffers.

Zwitterionic additives such as betaine can prevent precipitation and can be used at high concentrations without interfering with the gradient elution.

Detergents are useful as solubilizing agents for proteins with low aqueous solubility such as membrane components. Anionic, cationic, zwitterionic and non-ionic (neutral) detergents can be used during IEX chromatography.

Denaturing agents such as guanidine hydrochloride or urea can be used for initial solubilization of a sample and during separation. However, they should be avoided unless denaturation is a requirement. Note that, at the pH values used for separation, guanidine is a charged molecule with a counter-ion and will therefore participate in the ion exchange process in the same way as NaCl.

Examples of commonly used detergents and denaturing agents are given in Table 4.

Table 4. Commonly used detergents and denaturing agents.

Detergent	Type	Typical conditions for use	Compatibility
Urea		2 M–8 M	anion or cation ion exchangers
Guanidine hydrochloride		3 M–6 M	anion or cation ion exchangers
Triton™ X-100	non-ionic	2%	anion or cation ion exchangers
N-octylglucoside	non-ionic	2%	anion or cation ion exchangers
Sodium dodecyl sulfate	ionic	0.1%–0.5%	exchange for non-ionic detergent during first chromatography step, avoid anion exchangers
Sarcosyl	non-ionic	1.5%	anion or cation ion exchangers
Nonidet P40	non-ionic		anion or cation ion exchangers
Polyoxyethylene ethers (e.g. Brij 35)	non-ionic		anion or cation ion exchangers
Polyoxyethylene sorbitans (e.g. Tween™ 80)	non-ionic		anion or cation ion exchangers
CHAPS	zwitterionic, derivative of cholic acid		anion or cation ion exchangers (pH dependent)
CHAPSO	zwitterionic, derivative of cholic acid		anion or cation ion exchangers (pH dependent)
Deoxycholate	cation		anion ion exchangers

Temperatures <10 °C can minimize aggregation caused by hydrophobic interactions between sample components. Working at these lower temperatures may be an alternative to using a detergent to improve solubility.

Developing or optimizing a separation using buffers that contain detergents

1. Select detergents that are compatible with the sample. A detergent must be neutral, zwitterionic or have the same charge as the IEX medium. Detergents that bind to the medium can be difficult to remove and may affect protein loading capacity, pH, conductivity and resolution.
2. Determine the minimum concentration that is likely to keep the sample in solution during the separation. Note that different detergents will have different solubilization properties resulting in different peak profiles.
3. Equilibrate the column thoroughly with the detergent solution, using a concentration that is below the critical micelle concentration for the specific detergent.
4. Run blank salt gradients to determine the UV absorbance profile of the detergent and to detect any effect pH. Micelle formation causes light scattering and the appearance of a peak during UV monitoring. If micelle formation is a problem try the following:
   - decrease detergent concentration as far as possible without impairing sample solubility
   - increase detergent concentration to run the gradient above the critical micelle concentration (this creates a gradual rather than abrupt UV increase)
   - change the salt gradient so that the sudden change in UV absorption does not occur during the run
   - change to highly chaotropic salts such as $LiClO_4$ or sodium trichloroacetate that can be used at higher concentrations without causing micelle formation
5. Perform test runs with sample to find the detergent that gives the best solubilization and resolution.

A single peak obtained from a 'detergent run' often contains more than one component and should be analyzed carefully. Selecting a different detergent may improve the separation.

Detergent concentrations that are too high will increase buffer viscosity so that flow rates must be reduced to avoid over-pressure of the column.The concentration of detergent required for solubilization can often be reduced during the separation.

Use detergents of the highest quality that are free from salts. Filter buffers that contain detergents under weak suction and ultrasonication for degassing in order to avoid foaming.

Wash previously used columns thoroughly using recommended procedures before working with buffers that contain detergents.

Reagents to reduce polarity

Monoethylene glycol, glycerol and similar mild reagents that reduce polarity can be included in buffers. Avoid high concentrations (>40% w/w) as buffer viscosity will increase and may over-pressure the column.

Metal chelators: EDTA, EGTA

EDTA (ethylenediaminetetracetic acid) and EGTA (ethylene glycol-bis-(2-aminoethyl)-N,N,N',N'-tetraacetic acid) are often used in buffers as metal chelators and can be used with IEX chromatography. EDTA and EGTA contain several carboxylic acid groups that may interact with anion exchangers. During anion exchange separations EDTA and EGTA can concentrate as a band on the column and elute during a salt gradient. Both molecules absorb UV and will appear as a peak or as background noise in the chromatogram.

Analysis of results and further steps

The analysis of results from the first separation will indicate if conditions can be improved to increase the yield, achieve higher purity, speed up the separation or increase the amount of sample that can be processed in a single run.

Samples eluted using a salt gradient will contain a range of salt concentrations. Dilute or desalt fractions before analysis, if the assay is sensitive to changes in salt concentration.

Commonly used analytical assays are outlined in Appendix 8.

Scaling-up

For fast separations it may be easier to repeat a separation several times on a small column and pool the fractions of interest, rather than scale-up to a larger column. However, a larger column may be preferred for routine processing of large sample volumes. General guidelines for scaling-up are shown in Table 5.

Table 5. Guidelines for scaling-up.

Maintain	Increase
Column bed height	Column volume i.e. column diameter
Linear flow (cm/h)	Volumetric flow rate (ml/min)
Sample concentration	Sample load
Gradient elution volume i.e. number of column volumes used for the gradient	

When scaling-up an IEX separation, follow the points below to ensure the same cycle time for small scale and larger scale separations.

1. Optimize the separation at small scale.
2. Maintain bed height, sample concentration and the ratio of sample volume: volume of medium.
3. Increase the column volume by increasing the cross-sectional area (diameter) of the column.
4. Run the separation at the same linear flow (see Appendix 5) as used on the smaller column with the same ratio of gradient volume: column volume.

During method development a small particle size may have been used to improve resolution. However, smaller particles can also result in increased back pressure and this factor may become restrictive when scaling-up. Consider moving to a larger particle size, preferably of the same medium, to take advantage of lower back pressures and higher flow rates.

When scaling-up, the salt concentrations at which peaks elute may decrease with increased sample loads. As sample is applied to the column, components with a low net charge are displaced by components with a higher net charge. Molecules will elute in the same order, but at a different point in the elution profile.

When method scouting, develop the method, whenever possible, on the medium that will be used at the larger scale.

For production scale separations which must satisfy throughput and cleaning-in-place (CIP) requirements of industry, transfer an optimized method, as early as possible, to a matrix designed for bioprocessing such as SOURCE, Sepharose High Performance, Sepharose Fast Flow or Sepharose Big Beads.

See Appendix 3 for column selection and column packing.

Equipment selection

Appendix 4 provides a guide to the selection of systems for IEX chromatography.

Care of ion exchange media

When IEX media have been in use for some time, it may become necessary to remove precipitated proteins or other contaminants that have built up in the column. The need for cleaning may be indicated by the appearance of a colored band at top of the column, a space between the upper adaptor and the bed surface, a loss in resolution or a significant increase in back pressure. A general cleaning procedure for each IEX medium is given in Chapter 3 and Appendix 10 also contains recommended procedures for severe contamination by precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins. In all cases, prevention is better than cure and routine cleaning is recommended.

Always use filtered buffers and samples to reduce the need for additional column maintenance. See Appendix 1 for further details on sample preparation.

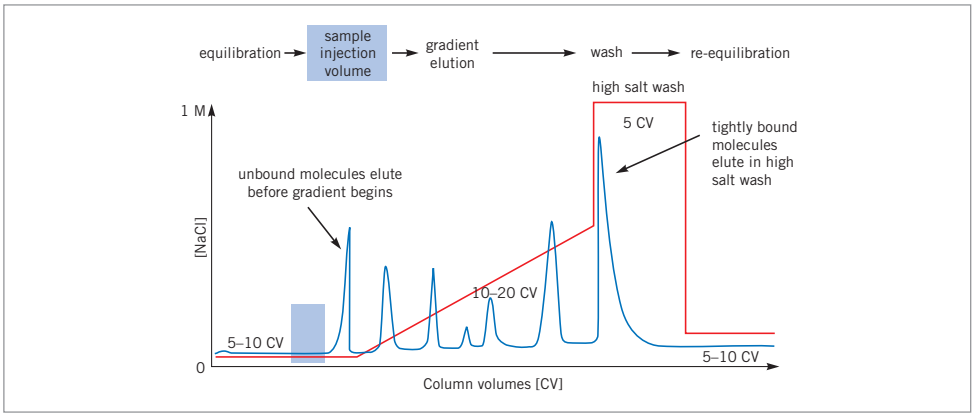
Always degas buffers and keep buffers, columns and samples at the same temperature to avoid the formation of air bubbles in the column.

Filter cleaning solutions before use and always re-equilibrate the column with start buffer before the next separation.

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.

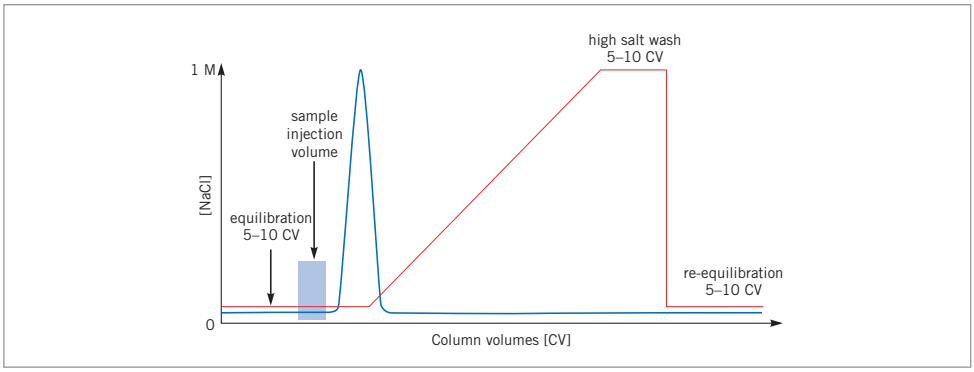
Troubleshooting

The ideal IEX separation: target proteins well resolved by gradient elution



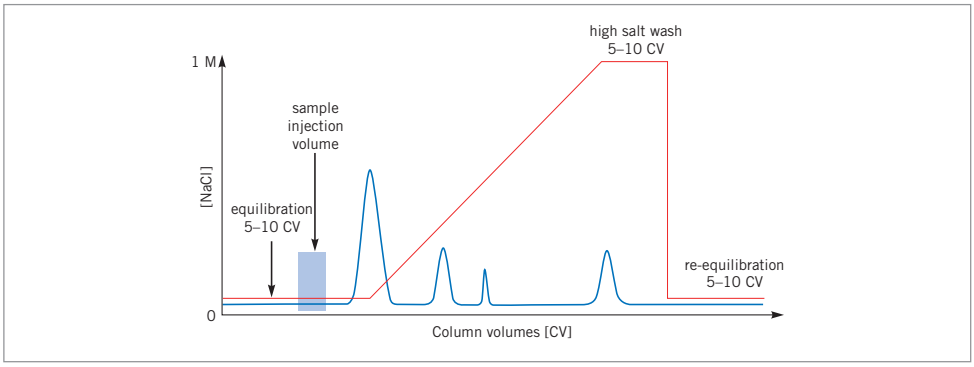
If only certain peaks are of interest in this well-resolved separation, it may be advantageous to transfer to a step elution in order to save time and buffer. The rest of this section focuses on practical problems that may lead to a non-ideal IEX separation.

Sample elutes before salt gradient begins



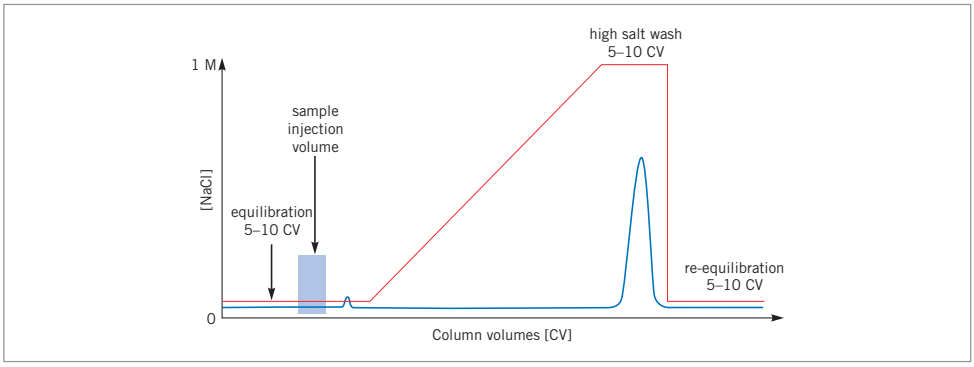
Ensure that buffers are in the correct containers. Reduce ionic strength of sample by desalting, page 156, or dilution with start buffer. For an anion exchanger, increase buffer pH, for a cation exchanger, decrease buffer pH. If proteins still do not bind at any pH, it is possible that the column has been contaminated by detergent.

Sample still eluting when gradient begins



After sample application the UV trace must return to baseline before elution begins, otherwise proteins that do not bind to the column interfere with the separation. Increase the volume of start buffer (equilibration step) before starting the gradient elution.

Sample elutes during high salt wash



Proteins are binding too strongly. Ensure that buffers are in the correct containers. If using an anion exchanger, decrease buffer pH, if using a cation exchanger, increase buffer pH.

Protein(s) of interest eluting late in gradient

Proteins are binding too strongly. Increase ionic strength of gradient. It is preferable to alter pH if a very high salt concentration is required for elution. For an anion exchanger, decrease buffer pH and for a cation exchanger, increase buffer pH. Refer also to Table 6.

Protein(s) of interest eluting too early in gradient:

Proteins are not binding strongly. Check ionic strength of gradient. Alter pH, for an anion exchanger, increase buffer pH and for a cation exchanger, decrease buffer pH. Refer also to Table 6.

Proteins(s) of interest not sufficiently resolved

Refer to the contents of this chapter to review key parameters for improving resolution. Refer also to Table 6.

Table 6. Troubleshooting.

Situation	Cause	Remedy
Reduced or no flow through the column.	Outlet closed or pumps not working.	Open outlet. Check pumps for signs of leakage (if using a peristaltic pump, check tubing also).
	Blocked filter, end-piece, adaptor or tubing.	Remove and clean or replace if possible. Always filter samples and buffer before use.
	Lipoproteins or protein aggregates have precipitated.	Remove lipoproteins and aggregates during sample preparation, (see Appendix 1). Follow cleaning procedures, Appendix 10.
	Protein precipitation in the column.	Modify buffer, pH and/or salt conditions during the run to maintain stability. Follow cleaning procedures, Appendix 10.
	Protein precipitation in the column caused by removal of stabilizing agents during separation.	Modify eluent to maintain stability.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10.
Peak of interest is poorly resolved from other major peaks.	Sample applied incorrectly.	Check bed surface and top filter for possible contamination.
	Large mixing spaces at top of or after column.	Adjust top adaptor to surface of medium if necessary. Reduce all post-column volumes.
	Incorrect buffer pH and/or ionic strength.	Check pH and ionic strength to ensure that column was re-equilibrated after previous run. Check conditions required. Prepare new solutions.
	Sub-optimal elution conditions e.g. incorrect pH, gradient too steep, flow rate too high.	Alter elution conditions: alter pH, use shallower gradient, reduce flow rate (listed in priority order).
	Sample is too viscous.	Dilute with buffer. Maintain protein concentration below 50 mg/ml.
	Column is poorly packed.	Check column efficiency (see Appendix 3). Repack if needed. Use prepacked columns.
	Column overloaded.	Decrease sample load.
	Lipoproteins or protein aggregates have precipitated.	Remove lipoproteins and aggregates during sample preparation (see Appendix 1).
	Precipitation of proteins in the column.	Modify buffer, pH and/or salt conditions during the run to maintain stability.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol to prevent microbial growth. Always filter buffers. Follow cleaning procedures, Appendix 10.
Proteins do not bind or elute as expected.	Proteins or lipids have precipitated on the column or column filter.	Clean the column and exchange or clean the filter. Check pH and salt stability of sample.
	Sample not filtered properly.	Clean the column, filter the sample and repeat.
	Sample has changed during storage.	Prepare fresh samples.
	Protein may be unstable or inactive in the elution buffer.	Determine the pH and salt stability of the protein.
	Column equilibration incomplete.	Repeat or prolong the equilibration step until conductivity and pH are constant.
	Incorrect buffer pH and/or ionic strength.	Check conditions required. Prepare new solutions.
	Proteins are forming aggregates and binding strongly to the medium.	Use urea or zwitterions, betaine up to 10%, taurine up to 4%.
	Sample or buffer conditions are different from previous runs.	Check sample and buffer conditions.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10.

continues on following page

Situation	Cause	Remedy
Protein elutes later than expected or not at all.	Incorrect buffer pH.	Check pH meter calibration. Use a buffer pH closer to the pI of the protein.
	Ionic strength too low.	Increase salt concentration in elution buffer.
	Ionic interactions between protein and matrix.	Maintain ionic strength of buffers above 0.05 M.
	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.
Protein elutes earlier than expected (during the wash phase).	Ionic strength of sample or buffer is too high.	Decrease ionic strength of sample or buffer.
	Incorrect pH conditions.	Increase pH (anion exchanger). Decrease pH (cation exchanger).
	Column equilibration incomplete.	Repeat or prolong the equilibration step until conductivity and pH are constant
Leading or very rounded peaks in chromatogram.	Channeling in the column.	Repack column using a thinner slurry of medium. Check column packing (see Appendix 3).
	Column overloaded.	Decrease sample load and repeat.
	Column contaminated.	Clean using recommended procedures.
Peaks are tailing.	Incorrect start buffer conditions, sample is not binding to column.	Adjust pH. Check salt concentration in start buffer.
	Sample too viscous.	Dilute in application buffer.
	Column packing too loose.	Check column efficiency (see Appendix 3). Repack using a higher flow rate. Use prepacked columns.
Peaks have a leading edge.	Column packing compressed.	Check column efficiency (see Appendix 3). Repack using a lower flow rate. Use prepacked columns.
Medium/beads appears in eluent.	Column packing compressed.	Check column efficiency (see Appendix 3). Repack using a slower flow rate. Use prepacked columns.
	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.
	Medium has been damaged during column packing.	Do not use magnetic stirrers when equilibrating loose medium
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.
Protein yield lower 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.	Check pH and salt conditions, adjust to improve sample solubility.
	Hydrophobic proteins.	Add denaturing agents, polarity reducing agents or detergents. Add 10% ethylene glycol to running buffer to prevent hydrophobic interactions.
	Non-specific adsorption.	Reduce salt concentration to minimize hydrophobic interaction. Add suitable detergent or organic solvent e.g. 5% isopropanol. If necessary, add 10% ethylene glycol to running buffer to prevent hydrophobic interactions.

continues on following page

Situation	Cause	Remedy
Peaks too small.	Sample absorbs poorly at chosen wavelength.	If appropriate, check absorbance range on monitor. If satisfactory, use a different wavelength, e.g. 214 nm instead of 280 nm.
	Different assay conditions have been used before and after the chromatographic step.	Use same assay conditions for all assays.
	Excessive band broadening.	Check column packing. Repack if necessary.
More sample is recovered than expected.	Protein 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 same assay conditions for all assays.
	Removal of inhibitors during separation.	
Back pressure increases during a run or during successive runs.	Bed compressed.	If possible repack the column or use a new column. Check sample preparation.
	Microbial growth.	Store in the presence of 20% ethanol to prevent microbial growth. Always filter buffers. Follow cleaning procedures, Appendix 10.
	Turbid sample.	Improve sample preparation (see Appendix 1). Improve sample solubility: add betaine (max. 10% w/v at 25°C), taurine (max. 4% w/v at 25°C, below pH 8.5) or glycerol (1–2 %). For hydrophobic samples, add ethylene glycol, urea, 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.
	Incorrect pH is causing precipitation.	Calibrate pH meter, prepare new solutions and try again. Change pH.
	Precipitation of lipoproteins at increased ionic strength.	Lipoproteins may be removed prior to chromatography by the addition of 10% dextran sulfate (final 0.2%) and 1 M calcium chloride (final 0.5 M).
Air bubbles in the bed.	Buffers not properly degassed.	Degas buffers thoroughly.
	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 3).
Cracks in the bed.	Large air leak in column.	Check all connections for leaks. Repack the column if possible (see Appendix 3).
Negative peaks at solvent front.	Refractive index effects.	Exchange the sample into start buffer.
Unexpected peaks in chromatogram.	Buffer impurities.	Clean the buffer by running it through a precolumn. Use high quality reagents.
Peaks appear on gradients.	Incomplete elution of previous sample.	Wash the column according to recommended blank methods.
Spikes in chromatogram.	Air bubble trapped in UV monitor flow cell.	Always use degassed buffers.
UV baseline rises with gradient.	Micelle formation as salt concentration changes.	Work below or above the critical micelle concentration of any detergents being used or change the gradient so that the increase in UV absorption does not occur while the samples are eluting.
	Buffer impurities.	Use high quality reagents.

**Polar organic solvents such as methanol, ethanol, isopropanol and acetonitrile can be used at concentrations from 0–20%, but remember that some proteins may irreversibly lose their biological activity in the presence of organic solvents. Check sample and buffer solubility, buffer pH and chemical stability of the medium before running a column. Note that back pressure may increase when working with organic solutions.*

BioProcess Media – made for bioprocessing

Specific BioProcess™ Media have been designed for each chromatographic stage in a production process from Capture to Polishing. Large capacity production integrated with efficient ordering and delivery routines ensure that BioProcess Media are available in the right quantity, at the right place, 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 fulfil high performance specifications. A certificate of analysis is available with each order.

Regulatory Support Files 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 productivity 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. For the latest information about BioProcess Products, visit *www.bioprocess.amershambiosciences.com*

Custom Designed Media

Custom Designed Media (CDM) is a collaborative service for industrial customers to develop tailor-made chromatography 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 large scale purification.

Custom Packed Laboratory Columns

Prepacked columns can be supplied by the Custom Products Group at Amersham Biosciences. A wide range of columns ensures the highest performance from all our purification media and meets the demands of modern pharmaceutical manufacturing. Each column is packed, tested and certified under stringent ISO 9001 standards.

Please ask your local representative for further details.

Chapter 3
Ion exchange media

Introduction

Historically several different types of material have been used as a base matrix to which positively or negatively charged groups are covalently attached to form an ion exchange (IEX) medium. Chapter 1 describes how the matrix characteristics determine chromatographic properties such as efficiency, capacity and recovery as well as chemical and physical stability and flow properties.

The first synthetic ion exchange matrices were unsuitable for use with biological samples. They had very strong binding properties, but very low binding capacity for proteins. These hydrophobic matrices tended to denature labile, biological materials. Cellulose matrices, although less harsh than synthetic matrices, had low binding capacity and poor flow properties due to their irregular shape. Dextran matrices had a tendency to shrink as the ionic strength of a buffer increased or pH changed, interfering with resolution and reproducibility between runs.

Many different matrices have been used over the years, including Sephadex, Sephacel and Sepharose CL-4B, and references to these will still be found in older scientific literature. More recently developed matrices offer improved capacity with greater physical and chemical stability.

Modern ion exchange media have much improved flow properties compared to earlier media and show no change in bed volume under conditions of changing ionic strength or pH. Stringent conditions can be used for cleaning the media when required and there is no need for frequent column repacking. Most of these media are also designed to meet the throughput and cleaning-in-place requirements for large-scale industrial chromatography.

To benefit from faster separations and improved performance, transfer and optimize older protocols onto one of the newer media described in this chapter.

MiniBeads: purification or analysis of microgram-milligram quantities with highest resolution

Use MiniBeads for purification and analysis of proteins, peptides or oligonucleotides.

Use MiniBeads for polishing steps at microscale when highest resolution is essential and the capacity of the prepacked column is sufficient.

Use MiniBeads for intermediate purification if only microgram – milligram quantities are required, if there is no requirement for scale-up and if the capacity of the prepacked column is sufficient. Note that, to avoid column blockage, it is especially important to remove particulate matter before using MiniBeads.

Use MiniBeads for faster, higher resolution separations, when compared to MonoBeads, if the capacity of the prepacked column is sufficient.

Run MiniBeads on systems such as ÄKTAdesign, FPLC™ System and HPLC. Appendix 4 gives guidance on how to select the most suitable ÄKTAdesign system.

MiniBeads are based on a non-porous, monodispersed matrix of rigid, hydrophilic polymer particles, substituted with quaternary amino (Q) or methyl sulfonate (S) groups. The very small size (3 µm), uniformity and physical rigidity of the particles create ideal conditions for extremely high resolution ion exchange separations at relatively high flow rates and low back pressures (non-uniform, porous particles would create higher back pressures, reduce flow rate and impair achievable resolution). Such high resolution is essential for successful separation of complex samples in the pg to µg scale. The strong ion exchange groups (Q and S) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application.

Reference lists highlighting the use of MiniBeads are available at *www.chromatography.amershambiosciences.com*

Purification options

Fig. 27. Mini Q and Mini S™ media are available prepacked in Tricorn™ (4.6/50 PE) and Precision (PC 3.2/3) columns.

Product, column volume	Binding capacity per column	Maximum flow	Recommended working flow	Working pH range*	Maximum operating back pressure ** (MPa/psi) *1 MPa=10 bar*
Strong anion exchangers					
Mini Q PC 3.2/3, 0.24 ml***	1.44 mg (α-amylase, M_r 49 000) 1.44 mg (trypsin inhibitor, M_r 20 100)	1 ml/min	0.1–1.0 ml/min	3–11	10/1450
Mini Q 4.6/50 PE, 0.8 ml	4.8 mg (α-amylase, M_r 49 000) 4.8 mg (trypsin inhibitor, M_r 20 100)	2 ml/min	0.5–2.0 ml/min	3–11	18/2600
Strong cation exchangers					
Mini S PC 3.2/3, 0.24 ml***	1.2 mg (ribonuclease, M_r 13 700) 1.2 mg (lysozyme, M_r 14 300)	1 ml/min	0.1–1.0 ml/min	3–11	10/1450
Mini S 4.6/50 PE, 0.8 ml	4 mg (ribonuclease, M_r 13 700) 4 mg (lysozyme, M_r 14 300)	2 ml/min	0.5–2.0 ml/min	3–11	18/2600

**Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

***Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

****Requires a Precision Column Holder for attachment to ÄKTApurifier™ and other HPLC systems.*

Purification examples

Fast separations at high resolution

Column: Mini S 4.6/50 PE
Sample: α-chymotrypsinogen A (25 µg/ml)
ribonuclease A (75 µg/ml)
lysozyme (25 µg/ml)
Sample volume: 200 µl
Start buffer: 20 mM sodium acetate, pH 5.0
Elution buffer: 20 mM sodium acetate, 0.4 M NaCl, pH 5.0
Flow: 0.83 ml/min
Gradient: 0–100% elution buffer in 12 CV

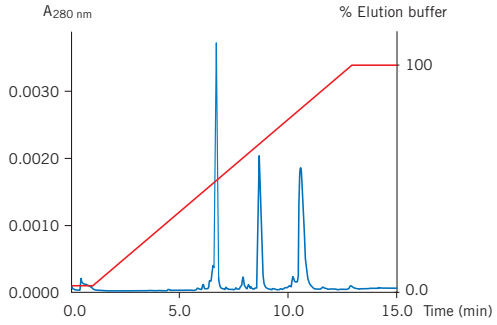


Fig. 28. Separation of a protein mixture on Mini S 4.6/50.

Column: Mini S PC 3.2/3
Sample: chymotrypsinogen A, ribonuclease A, cytochrome C, lysozyme (6:10:6:5), 25 µg/ml
Sample load: 6 µg
Start buffer: 20 mM acetic acid, pH 5.0
Elution buffer: 20 mM acetic acid, 0.5 M LiCl, pH 5.0
Flow: 800 µl/min, 10 cm/min
Gradient: 0–100% elution buffer in 6 min. (20 CV)

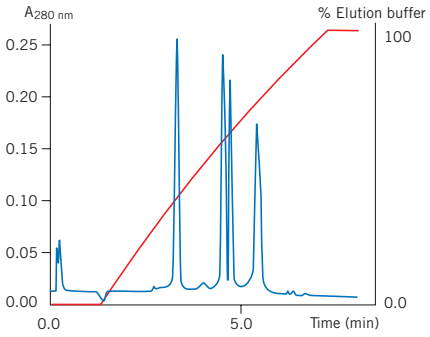


Fig. 29. Mini S PC 3.2/3 gives fast, high resolution separation.

Purity check

Column: Mini Q 4.6/50 PE
Start buffer: 10 mM NaOH
Elution buffer: 10 mM NaOH, 2 M NaCl
Flow: 1.0 ml/min

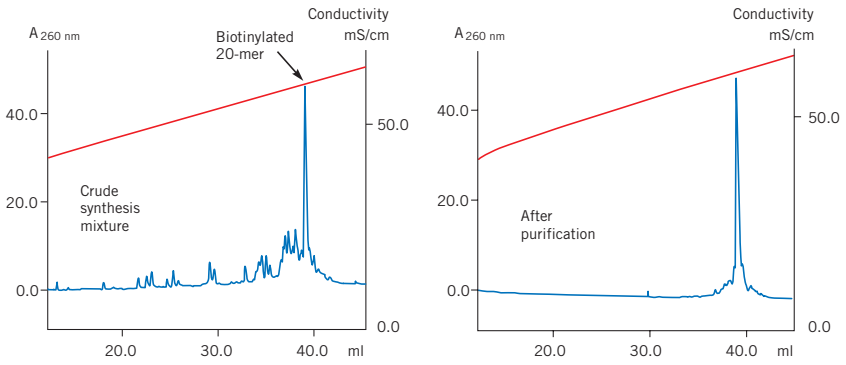


Fig. 30. Purity check of 5'-biotinylated synthetic oligonucleotide 20-mer on Mini Q 4.6/50 PE before and after purification on a RESOURCE RPC column.

Long term reproducibility

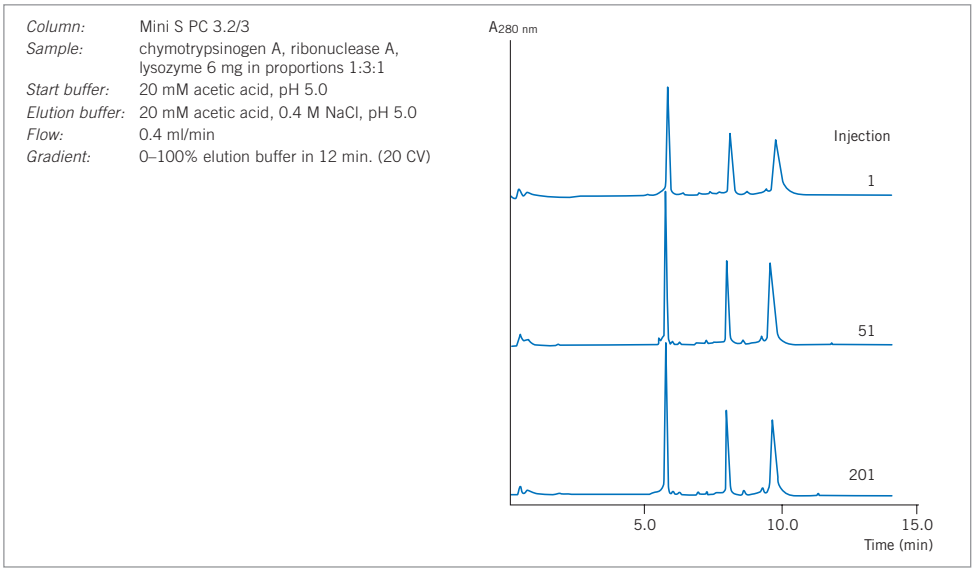


Fig. 31. Chromatograms from the 1st, 5th and 201st separation of a series run on the same Mini S PC 3.2/3 column. The same consistent reproducibility has been confirmed on Mini Q PC 3.2/3 (data not shown).

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance, especially when using small particles such as MiniBeads. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions through 0.22 µm filters. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

The pH of the start buffer should be at least 0.5–1 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5–1 pH unit below the pI of the target substance when using a cation exchanger (S). See Appendix 2 for recommendations on volatile and non-volatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

– **anion exchange (Q)**
start buffer: pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

– **cation exchange (S)**
start buffer: pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

Users of ÄKTAdesign systems with BufferPrep functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8 or cation exchange chromatography at pH 6.

First time use or after long term storage

1. To remove ethanol, wash with 4 column volumes of distilled water at 0.5 ml/min. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 4 column volumes of start buffer at 0.8 ml/min.
3. Wash with 4 column volumes of elution buffer at 0.8 ml/min.
4. Wash with 4 column volumes of start buffer at 0.8 ml/min.
5. Run a blank elution before applying sample.

Separation by gradient elution

Flow: 0.4 ml/min (PC columns) or 0.8 ml/min (PE columns). Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically-bound material.
6. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Although separations by step elution (see Chapter 2, page 19) can be performed using MiniBeads, gradient elution is recommended in order to achieve the highest possible resolution.

If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes of 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Refer to Chapter 2 for advice on optimizing the separation. Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination.

The following procedure should be satisfactory to remove common contaminants:

1. Wash with 2 column volumes of 2 M NaCl at 0.2 ml/min.
2. Wash with 4 column volumes of 1 M NaOH at 0.2 ml/min.
3. Wash with 2 column volumes of 2 M NaCl at 0.2 ml/min.
4. Rinse with at least 2 column volumes of distilled water at 0.2 ml/min until the UV-baseline and elutent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer at 0.2 ml/min until pH and conductivity values have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins, refer to Appendix 1.

Media characteristics

Composition: rigid, non-porous matrix of monodisperse, hydrophilic polymer particles (3 µm) substituted with quaternary amino (Q) or methyl sulfonate (S) groups.

Product	Functional group	pH stability*	Mean particle size
Mini Q	$-CH_2N^+(CH_3)_3$	Long term: 3–11 Short term: 1–14	3 µm (monosized)
Mini S	$-CH_2SO_3^-$	Long term: 3–11 Short term: 1–14	3 µm (monosized)

**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 the 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

For daily use, MiniBeads are stable in all common aqueous buffers in the range pH 3–11 and in the presence of additives such as denaturing agents (8 M urea or 6 M guanidine hydrochloride), non-ionic or ionic detergents and up to 30% acetonitrile in aqueous buffers. Note that aqueous solutions of urea, ethylene glycol and similar compounds will increase the back-pressure due to increased viscosity.

MiniBeads can be used with organic solutions such as dimethylsulfoxide, dimethylformamide or formic acid, but the separation properties of the media will change.

Avoid anionic detergents with Mini Q. Avoid cationic detergents with Mini S. Avoid oxidizing agents.

Storage

For column storage, wash with 4 column volumes of distilled water followed by 4 column volumes of 20% ethanol. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Ensure that the column is sealed well to avoid drying out. Do not freeze.

MonoBeads: purification of milligram quantities with highest resolution

Use MonoBeads for purification of proteins, peptides or oligonucleotides.

Use MonoBeads for polishing steps at laboratory scale when highest resolution is essential and a higher capacity than MiniBeads is required.

Use MonoBeads for capture or intermediate purification when milligram quantities are required, when there is no requirement for scale-up and/or when prepacked MiniBead columns do not offer sufficient capacity. Note that, to avoid column blockage, it is especially important to remove particulate matter before using MonoBeads.

Run MonoBeads on systems such as ÄKTAdesign, FPLC System and HPLC. Appendix 4 gives guidance on how to select the most suitable ÄKTAdesign system.

Mono Q and Mono S ion exchange media are based on a hydrophilic matrix made from monodispersed, rigid, polystyrene/divinyl benzene particles, substituted with quaternary ammonium (Q) or methyl sulfonate (S) groups (Figure 32). This combination confers extreme chemical and physical stability to the media. The small particle sizes (10 µm) allow fast binding and dissociation to facilitate high resolution while the uniformity of the particles ensures high flow rates at low back pressures. The strong ion exchange groups (Q and S) maintain their charge over a broad pH range Figure 33, allowing selection of the most suitable pH for each application.

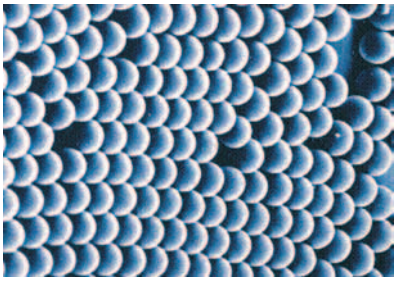


Fig. 32. Electron micrograph of MonoBeads showing their distinct monodispersity.

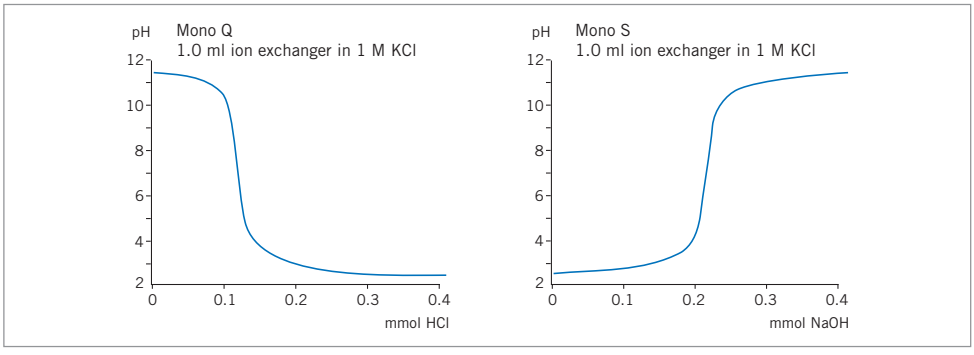


Fig. 33. Titration curves for Mono Q and Mono S™. Binding capacity remains constant over a broad pH working range.

Reference lists highlighting the use of MonoBeads are available at *www.chromatography.amershambiosciences.com*

Purification options

Fig. 34. MonoBeads (Q and S) are available prepacked in Tricorn PE (PEEK) and Tricorn GL (glass) columns.

Product, column volume	Binding capacity per column	Recommended working flow	Maximum flow	Working pH range*	Maximum operating back pressure ** (MPa/psi) *1 MPa=10 bar*
Strong anion exchangers					
Mono Q 5/50 GL, 1 ml	25 mg (thyroglobulin, M_r 669 000) 65 mg (HSA, M_r 68 000) 80 mg (α-lactalbumin, M_r 14 300)	0.5–3.0 ml/min	3 ml/min	2–12	4/580
Mono Q 4.6/100 PE, 1.7 ml	40 mg (thyroglobulin, M_r 669 000) 110 mg (HSA, M_r 68 000) 140 mg (α-lactalbumin, M_r 14 300)	0.5–3.0 ml/min	3 ml/min	2–12	4/580
Mono Q 10/100 GL, 8 ml	200 mg (thyroglobulin, M_r 669 000) 520 mg (HSA, M_r 68 000) 640 mg (α-lactalbumin, M_r 14 300)	2.0–6.0 ml/min	10 ml/min	2–12	4/580
Mono Q HR 16/10, 20 ml	500 mg (thyroglobulin, M_r 669 000) 1300 mg (HSA, M_r 68 000) 1600 mg (α-lactalbumin, M_r 14 300)	up to 10 ml/min	10 ml/min	2–12	3/435
Strong cation exchangers					
Mono S 5/50 GL, 1 ml	75 mg (human IgG, M_r 160 000) 75 mg (ribonuclease, M_r 13 700)	0.5–3.0 ml/min	3 ml/min	2–12	4/580
Mono S 4.6/100 PE, 1.7 ml	130 mg (human IgG, M_r 160 000) 130 mg (ribonuclease, M_r 13 700)	0.5–3.0 ml/min	3 ml/min	2–12	4/580
Mono S 10/100 GL, 8 ml	600 mg (human IgG, M_r 160 000) 600 mg (ribonuclease, M_r 13 700)	2.0–6.0 ml/min	10 ml/min	2–12	4/580
Mono S HR 16/10, 20 ml	1500 mg (human IgG, M_r 160 000) 1500 mg (ribonuclease, M_r 13 700)	up to 10 ml/min	10 ml/min	2–12	3/435

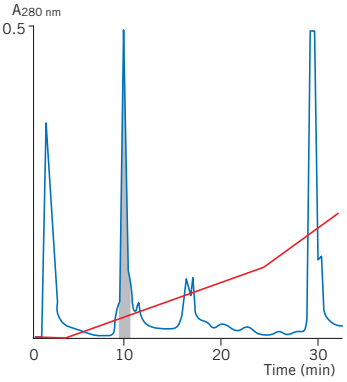
**Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

***Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

Purification examples

Two step purification using complementary selectivities

Column: Mono Q HR 5/5
Sample: 500 ml of *T. reesei* crude cellulases in buffer start buffer, 2.5 mg
Start buffer: 20 mM Tris-HCl, pH 7.6
Elution buffer: 20 mM Tris-HCl, 0.5 M NaCl, pH 7.6
Flow: 1.0 ml/min
Gradient: 0% elution buffer (4 CV), 0–40% elution buffer (21 CV), 40–100% elution buffer (15 CV)



Column: Mono S HR 5/5
Sample: Peak 3 from Mono Q HR 5/5
Start buffer: 20 mM acetate, pH 3.6
Elution buffer: 20 mM acetate, 0.2 M NaCl, pH 3.6
Flow: 1.0 ml/min
Gradient: 0–100% elution buffer (26 CV)

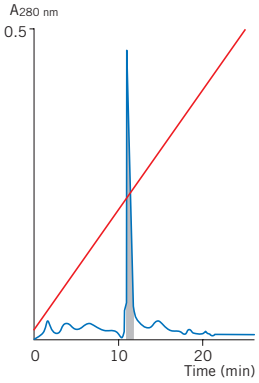
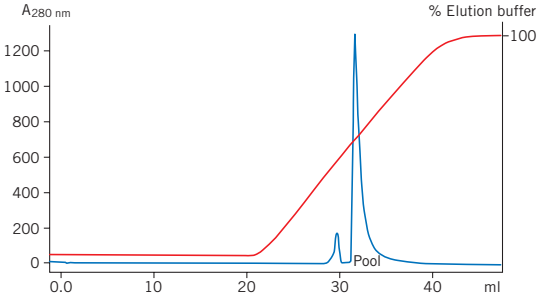
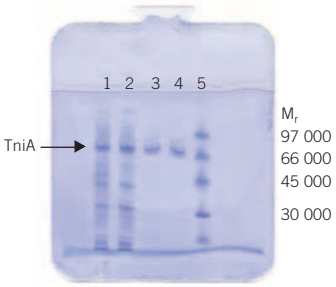


Fig. 35. Purification of cellulose on Mono Q and Mono S HR 5/5 columns (now available as Mono Q 5/50 GL and Mono S 5/50 GL).

High resolution, polishing step

Column: Mono S 5/50 GL
Sample: Recombinant transposase TniA partially purified on SOURCE 15Q 4.6/100 and HiTrap Heparin HP, 5 ml
Sample load: 14.5 ml
Start buffer: 20 mM MES, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, pH 6.5
Elution buffer: 20 mM MES, 1 mM EDTA, 2 mM $MgCl_2$, 1 mM DTT, 1M NaCl, pH 6.5
Flow: 1 ml/min
Gradient: 0–100% elution buffer in 20 CV





Lane 1. Sample, clarified extract diluted 5-fold
Lane 2. Pooled from SOURCE 15Q 4.6/100 PE
Lane 3. Pooled from HiTrap Heparin
Lane 4. Pooled from Mono S 5/50 GL
Lane 5. LMW-SDS Marker Kit

Fig. 36. Final polishing step in purification of a DNA binding protein, transposase TniA. Two well-resolved peaks after separation on Mono S 5/50 GL (a). SDS-PAGE analysis shows fractions from each of the three steps used in this protocol (b), PhastSystem™ using SDS-PAGE PhastGel™ Homogenous – 12.5 and Coomassie™ staining.

Long term reproducibility

Column: Mono Q 5/50 GL
Sample: conalbumin (3.0 mg/ml), α-lactalbumin (4 mg/ml), STI (2.5 mg/ml)
Sample load: 200 µl
Start buffer: 20 mM Tris, pH 7.0
Elution buffer: 20 mM Tris, 0.5 M NaCl, pH 7.0
Flow: 1.0 ml/min
Gradient: 0–100% elution buffer in 20 CV

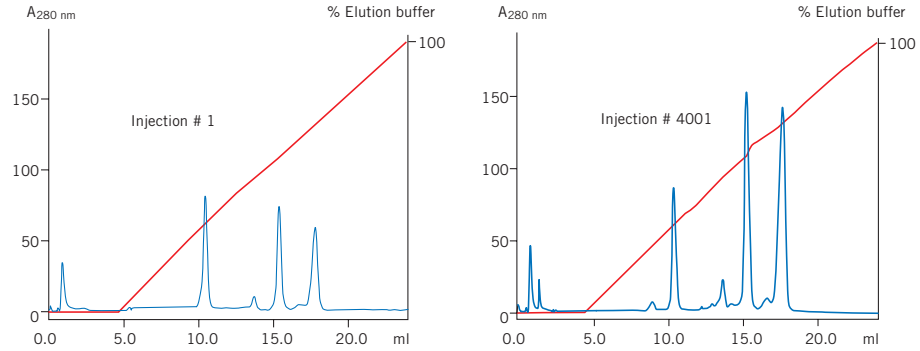


Fig. 37. Chromatograms illustrating run to run reproducibility for Mono Q 5/50 GL (Tricorn). Runs 1, 1000 and 2000 are shown.

Separation in organic solvents

Column: Mono S HR 5/5
Sample: bacitracin 4 mg/ml in start buffer
Sample load: 200 µl
Start buffer: 90% methanol, 50 mM formic acid/LiOH, pH 3.8
Elution buffer: 90% methanol, 50 mM formic acid/LiOH, 0.35 M $LiClO_4$, pH 3.8
Flow: 1 ml/min
Gradient: 0–100% elution buffer in 20 CV

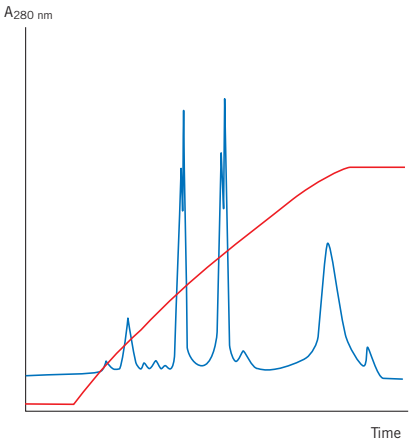


Fig. 38. Separation of the peptide bacitracin on Mono S HR 5/5 (now available as Mono S 5/50 GL).

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. Use these instructions as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance, especially when using small particles such as MonoBeads. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions through 0.22 µm filters. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

The pH of the start buffer should be at least 0.5–1 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5–1 pH unit below the pI of the target substance when using a cation exchanger (S). See Appendix 2 for recommendations on volatile and non-volatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

– **anion exchange (Q)**
start buffer: pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

– **cation exchange (S)**
start buffer: pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

Users of ÄKTAdesign systems with BufferPrep functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8 or cation exchange chromatography at pH 6.

First time use or after long term storage

1. To remove ethanol, wash with 5 column volumes of distilled water at 1 ml/min (1.7 ml and 1 ml columns), 2 ml/min (8 ml column) or 4 ml/min (20 ml column). This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 column volumes of start buffer at 2 ml/min (1.7 ml and 1 ml columns), 4 ml/min (8 ml column) or 8 ml/min (20 ml column).
3. Wash with 5 column volumes of elution buffer, same flow as step 2.
4. Wash with 5 column volumes of start buffer, same flow as step 2.
5. Run a blank elution before applying sample.

Separation by gradient elution

Flow: 2 ml/min (1.7 ml column), 2 ml/min (1 ml column), 4 ml/min (8 ml column) or 8 ml/min (20 ml column). Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically-bound material.
6. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Although separations by step elution (see Chapter 1, page 19) can be performed using MonoBeads, gradient elution is recommended in order to achieve the highest possible resolution.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the column.

If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3. Refer to Chapter 2 for advice on optimizing the separation.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl at 0.2 ml/min (1.7 ml column), 0.5 ml/min (1 ml column), 2 ml/min (8 ml column) or 5 ml/min (20 ml column).
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until pH and c onductivity values have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins, refer to Appendix 1.

Media characteristics

Composition: rigid, monodisperse, polystyrene/divinyl benzene particles (10 µm) with an optimized pore size distribution. The base matrix is substituted with quaternary amino (Q) or methyl sulfonate groups (S).

Product	Functional group	pH stability*	Mean particle size
Mono Q	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 2–14	10 µm (monosized)
Mono S	$-CH_2SO_3^-$	Long term: 2–12 Short term: 2–14	10 µm (monosized)

**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 the 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

For daily use, MonoBeads are stable in all common, aqueous buffers in the range pH 2–12, and in the presence of additives such as denaturing agents (8 M urea or 6 M guanidine hydrochloride), non-ionic or ionic detergents and up to 20% acetonitrile in aqueous buffers. Note that aqueous solutions of urea, ethylene glycol and similar compounds will increase the back-pressure due to increased viscosity.

MonoBeads can be used with organic solutions such as dimethylsulfoxide, dimethylformamide or formic acid, but the separation properties of the media will change.

Avoid anionic detergents with Mono Q. Avoid cationic detergents with Mono S. Avoid oxidizing agents.

Storage

For column storage, wash with 5 column volumes of distilled water followed by 5 column volumes of 20% ethanol. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Do not freeze.

SOURCE: purification at high throughput with high resolution and easy scale-up

Use SOURCE media for purification of proteins, peptides or oligonucleotides.

Use SOURCE 15 for intermediate purification or polishing steps in laboratory or large-scale applications that require high resolution and high throughput (flows up to 1800 cm/h).

Use SOURCE 30 as an alternative to SOURCE 15 for intermediate purification or polishing steps in large-scale applications where speed rather than resolution is a priority (flows up to 2000 cm/h).

Use SOURCE 30 as an alternative to SOURCE 15 for large sample volumes where speed rather than resolution is a priority. The larger particle size slightly reduces resolution, but separations can be performed at higher flow rates.

Run SOURCE columns on systems such as ÄKTAdesign, FPLC System and HPLC or systems using peristaltic pumps. Appendix 4 gives guidance on how to select the most suitable ÄKTAdesign system.

SOURCE media are based on a hydrophilic matrix made from monodispersed, rigid, polystyrene/divinyl benzene and substituted with quaternary ammonium (Q) or methyl sulfonate (S) groups (Figure 39). This combination confers extreme chemical and physical stability to the media. The small particle sizes allow fast binding and dissociation to facilitate high resolution while the uniformity and stability of the particles ensures high flow rates at low back pressure. The strong ion exchange groups (Q and S) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application. The high flow rates that can be used with SOURCE media are more likely to be limited by the equipment available rather than the physical properties of the media.

Separation methods can be easily scaled up from columns such as RESOURCE Q or S, 1 ml prepacked with SOURCE 15 through to large scale columns such as FineLINE™.

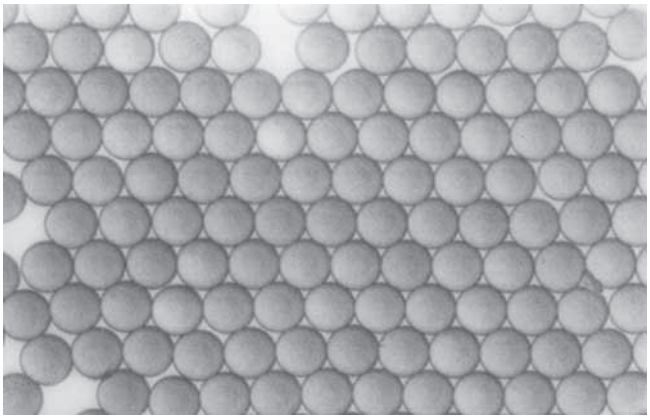


Fig. 39. Uniform size distribution of SOURCE monodispersed particles.

Purification options

Fig. 40. SOURCE is available in media packs and prepacked in Tricorn or RESOURCE columns.

Product, column volume	Binding capacity per column or per ml medium	Recommended working flow*	Maximum flow*	Working pH range**	Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar*
Strong anion exchangers					
RESOURCE Q 1 ml	45 mg (BSA, M_r 67 000)	1.0–10 ml/min	10 ml/min	2–12	1.5/220
RESOURCE Q 6 ml	270 mg (BSA, M_r 67 000)	1.0–60 ml/min	60 ml/min	2–12	0.6/87
SOURCE 15Q 4.6/100 PE, 1.7 ml	75 mg (BSA, M_r 67 000)	0.5–2.5 ml/min	5 ml/min	2–12	4/580
SOURCE 15Q	45 mg/ml (BSA, M_r 67 000)	150–900 cm/h	1800 cm/h	2–12	0.5/72
SOURCE 30Q	40 mg/ml (BSA, M_r 67 000)	300–1000 cm/h	2000 cm/h	2–12	0.5/72
Strong cation exchangers					
RESOURCE S 1 ml	80 mg (lysozyme, M_r 14 500)	1.0–10 ml/min	10 ml/min	2–13	1.5/220
RESOURCE S 6 ml	480 mg (lysozyme, M_r 14 500)	1.0–60 ml/min	60 ml/min	2–13	0.6/87
SOURCE 15S 4.6/100 PE, 1.7 ml	140 mg (lysozyme, M_r 14 500)	0.5–2.5 ml/min	5 ml/min	2–13	4/580
SOURCE 15S	80 mg/ml (lysozyme, M_r 14 500)	150–900 cm/h	1800 cm/h	2–13	0.5/72
SOURCE 30S	80 mg/ml (lysozyme, M_r 14 500)	300–1000 cm/h	2000 cm/h	2–13	0.5/72

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

***Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

****Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

Use prepacked RESOURCE columns (1 ml or 6 ml) for fast media selection, method scouting, group separations, sample concentration or clean-up.

Use SOURCE 15Q PE 4.6/100 to improve resolution by increasing column length with further optimization and as the first step towards scaling up.

For column packing:

	Volume	Bed height
SOURCE 15		
Tricorn 10/100	up to 8 ml	up to 10 cm
Tricorn 10/150	up to 12 ml	up to 15 cm
Tricorn 10/200	up to 16 ml	up to 20 cm
SOURCE 30		
XK 16/20	up to 30 ml	up to 15 cm
XK 26/20	up to 80 ml	up to 15 cm
XK 26/40	up to 196 ml	> 15 cm

Select a production column such as FineLINE for larger volumes.

Purification examples

Fast, high resolution separations

Column: RESOURCE Q 1 ml
Sample: pancreatin 5 mg/ml
Sample volume: 200 µl
Start buffer: 20 mM bis-Tris-propane, pH 7.5
Elution buffer: 20 mM bis-Tris-propane, 0.5 M NaCl, pH 7.5
Flow: 9.6 ml/min, 1800 cm/h
Gradient: 0–80% elution buffer in 20 CV

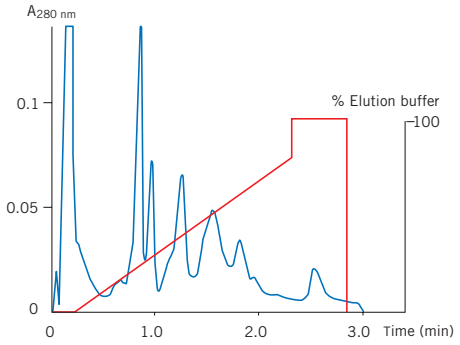


Fig. 41. Separation of pancreatin on RESOURCE Q, 1 ml within 3 minutes.

Column: RESOURCE S 1 ml
Sample: snake venom, 4 mg/ml
Sample volume: 100 µl
Start buffer: 20 mM sodium phosphate, pH 6.8
Elution buffer: 20 mM sodium phosphate, 0.4 M NaCl, pH 6.8
Flow: 1 ml/min, 180 cm/h
Gradient: 0–100% elution buffer in 20 CV

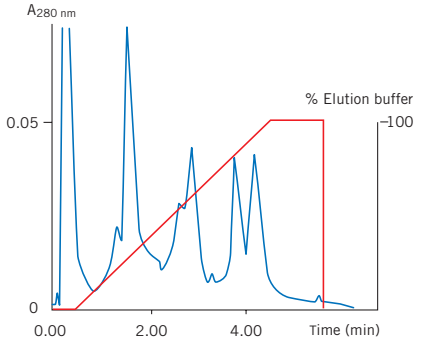


Fig. 42. Separation of snake venom on RESOURCE S, 1 ml within 4 minutes.

Scaling up: resolution maintained

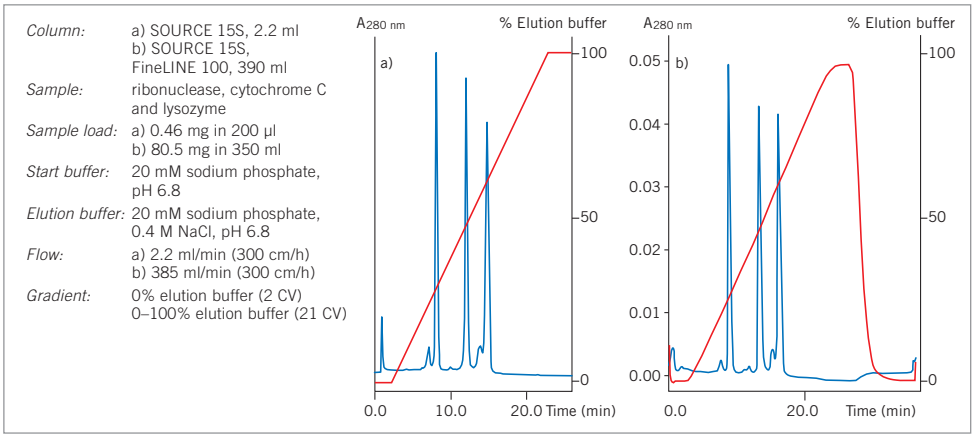


Fig. 43. Separation of proteins scaled up from a 2.2 ml column to a 390 ml column.

Intermediate purification

Figure 44 shows an example of SOURCE 30Q used for an intermediate purification step in a large scale process. Recombinant *P. aeruginosa* exotoxin A, produced as a periplasmic protein in *E. coli*, was initially purified with STREAMLINE DEAE expanded bed adsorption, followed by hydrophobic interaction chromatography (HIC) on Phenyl Sepharose 6 Fast Flow (high sub). The fraction of interest was then further purified on SOURCE 30Q before a final HIC polishing step on SOURCE 15PHE to remove the final contaminants.

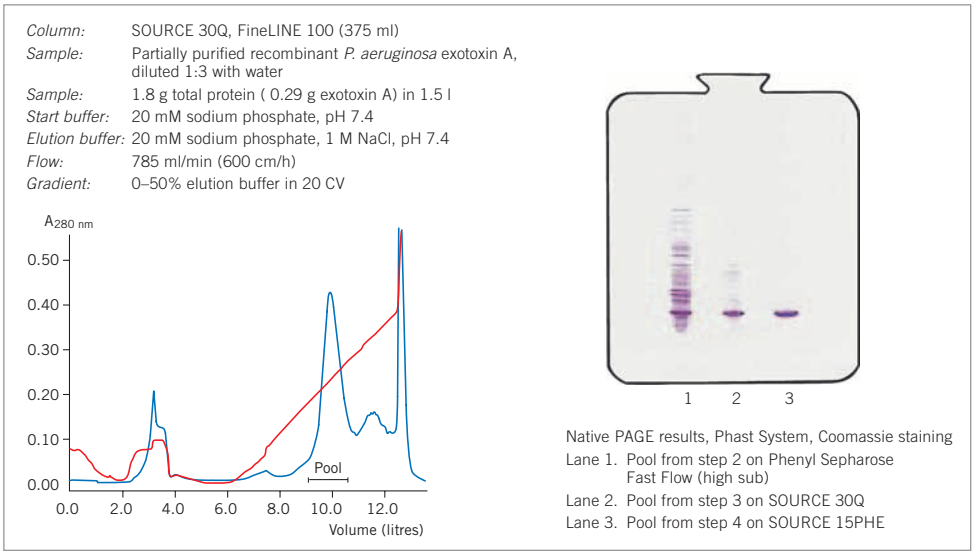


Fig. 44. Intermediate purification of recombinant *P. aeruginosa* exotoxin A on SOURCE 30Q.

Separations under extreme pH conditions

The high pH stability of SOURCE media makes them well-suited for applications requiring conditions of extreme pH such as purification of certain peptides and synthetic oligonucleotides, as shown in Figures 45 and 46.

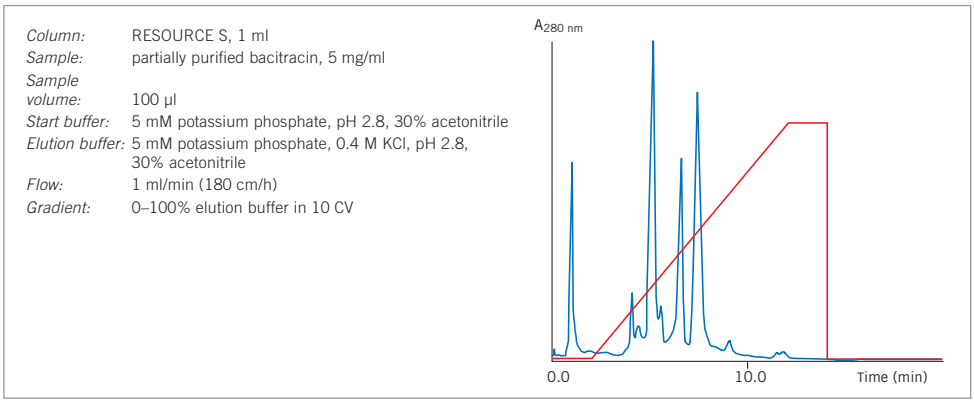


Fig. 45. Intermediate purification of the peptide bacitracin from *Bacillus subtilis* on RESOURCE Q, 1 ml.

Method optimization

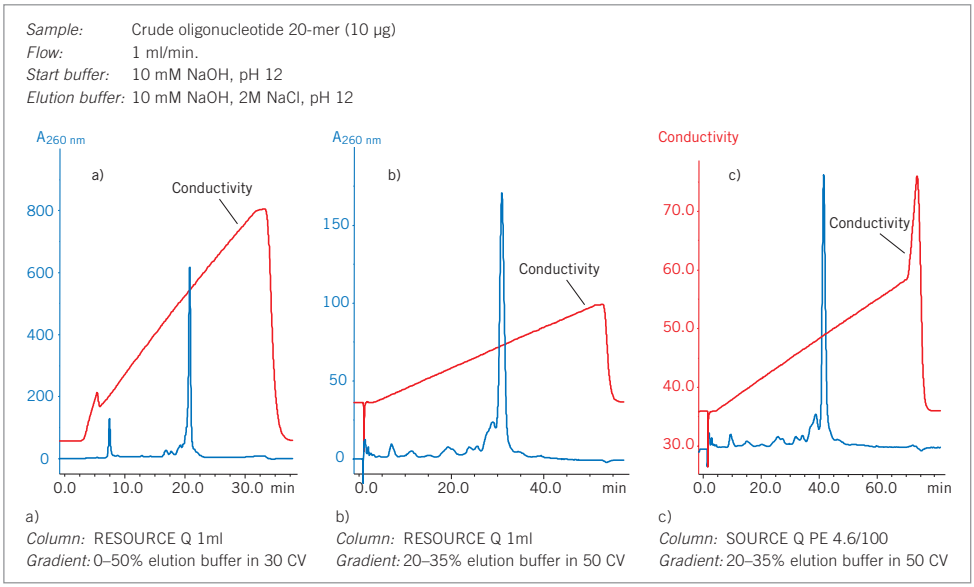


Fig. 46. Manipulation of gradient slope and shape to maximize resolution. Initial purification of a 20 mer oligonucleotide was optimized on RESOURCE Q 1 ml and transferred to SOURCE Q PE 4.6/100 to further increase resolution by increasing bed height to 10 cm.

Batch-to-batch reproducibility

Batch-to-batch reproducibility is particularly important for media used for scaling-up and large scale industrial applications which are under strict regulatory control. Figures 47 and 48 demonstrate the high batch-to-batch reproducibility of SOURCE 15 and SOURCE 30 media.

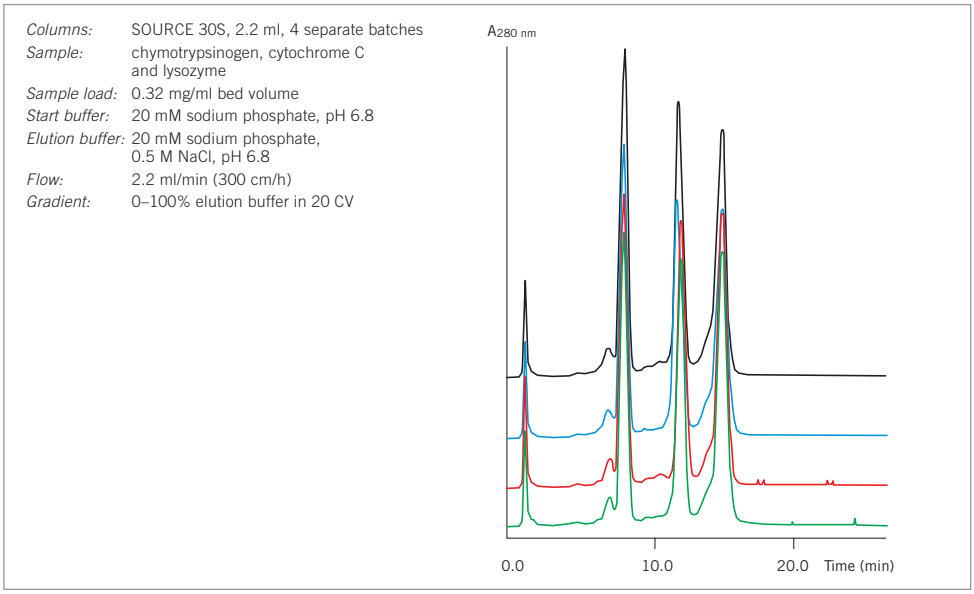


Fig. 47. Selectivity tests on 4 production batches of SOURCE 30S.

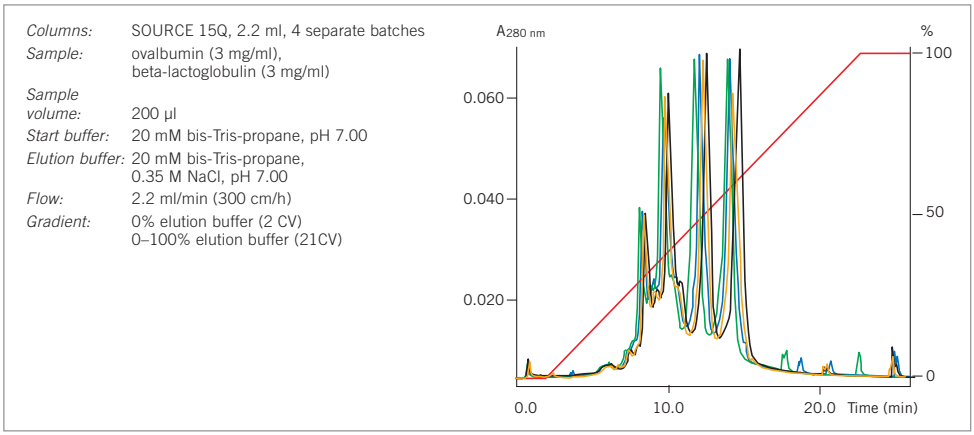


Fig. 48. Selectivity tests on 4 production batches of SOURCE 15Q.

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions using filters of 0.45 µm or 0.22 µm for 30 µm particles and 0.22 µm filters for 15 µm particles. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

The pH of the start buffer should be at least 0.5–1 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5–1 pH unit below the pI of the target substance when using a cation exchanger (S). See Appendix 2 for recommendations on volatile and non-volatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

– anion exchange (Q)
start buffer: pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

– cation exchange (S)
start buffer: pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

Users of ÄKTAdesign systems with BufferPrep functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8 or cation exchange chromatography at pH 6.

First time use or after long term storage:

1. To remove ethanol, wash with 5 column volumes of distilled water at 2 ml/min (SOURCE 15 4.6/100 PE), 4 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml) or 200 cm/h for SOURCE packed in larger columns. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 column volumes of start buffer, at 2 ml/min (SOURCE 15 4.6/100 PE), 4 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml) or 200 cm/h for SOURCE packed in larger columns.
3. Wash with 5 column volumes of elution buffer, same flow as step 2.
4. Wash with 5 column volumes of start buffer, same flow as step 2.

Perform a blank run to check conductivity and pH.

Separation by gradient elution

Flow: 2 ml/min (SOURCE 15 4.6/100 PE), 4 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml) or 200 cm/h for SOURCE packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically-bound material.
6. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Flow: 2 ml/min (SOURCE 15 4.6/100 PE), 4 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml) or 200 cm/h for SOURCE packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Elute with 5 column volumes of start buffer + NaCl at chosen ionic strength.
5. Repeat step 4 at higher ionic strengths until the target protein(s) has been eluted.
6. Wash with 5 column volumes of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically-bound material.
7. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.

If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

It is recommended to reverse the direction of flow during column cleaning so that contaminatants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl at 0.2 ml/min (SOURCE 15 4.6/100 PE), 1 ml/min (RESOURCE 1 ml), 6 ml/min (RESOURCE 6 ml) or 40 cm/h with a contact time of 1–2 h for SOURCE packed in larger columns.
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as in step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as in step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as in step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as in step 1) until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins, refer to Appendix 1.

Media characteristics

Composition: rigid, monodisperse, polystyrene/divinyl benzene particles (15 mm) with an optimized pore size distribution. The base matrix is substituted with quaternary amino groups (Q) or methyl sulfonate groups (S).

Product	Functional group	pH stability*	Mean particle size
SOURCE 15Q	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 1–14	15 µm (monosized)
SOURCE 30Q	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 1–14	30 µm (monosized)
SOURCE 15S	$-CH_2SO_3^-$	Long term: 2–13 Short term: 1–14	15 µm (monosized)
SOURCE 30S	$-CH_2SO_3^-$	Long term: 2–13 Short term: 1–14	30 µm (monosized)

**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 the 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

For daily use, SOURCE media are stable in all common, aqueous buffers pH 2–12, denaturing agents (8 M urea, 6 M guanidine hydrochloride), 75% acetic acid, 1 M NaOH, 1 M HCl, 70% ethanol, 30% acetonitrile and with additives such as non-ionic detergents.

Avoid cationic detergents with SOURCE S. Avoid anionic detergents with SOURCE Q. Avoid oxidizing agents.

Storage

For column storage, wash with 5 column volumes of distilled water followed by 5 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the 20% ethanol solution for SOURCE S. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at +4° C +30° C in 20% ethanol. Do not freeze.

Sepharose High Performance: purification with high resolution

Use Sepharose High Performance media for purification of proteins, peptides or oligonucleotides.

Use Sepharose High Performance for intermediate purification steps that require high capacity and high resolution (flows up to 150 cm/h).

Run Sepharose High Performance columns on systems such as ÄKTAdesign, FPLC System and HPLC. Appendix 4 gives guidance on how to select the most suitable ÄKTAdesign system.

Sepharose High Performance media are based on a matrix of 34 µm particles made from 6% agarose and highly cross-linked for chemical and physical stability. The small particle size ensures fast binding and dissociation even at high sample loads and flow rates which, in combination with high selectivity, give high resolution separations. Particle size and bed volumes remain stable, despite changes in ionic strength or pH, to ensure fast separations at high flow rates. The strong ion exchange groups (Q and S) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application.

Sepharose High Performance media, as other ion exchangers, can be used for group separations or sample concentration. However, these separations should be limited to reasonably clean samples to avoid the risk of blocking the column filter (34 µm particle size requires the use of finer column filters).

Reference lists highlighting the use HiLoad™ Sepharose High Performance columns are available at *www.chromatography.amershambiosciences.com*

Purification options

Fig. 49. Q and S Sepharose High Performance media are available prepacked in HiTrap and HiLoad columns or in media packs.

Product	Binding capacity per column or per ml medium	Recommended working flow*	Maximum flow*	Working pH range**	Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar*
Strong anion exchangers					
HiTrap Q HP, 1 ml	50 mg (HSA, M_r 68 000)	up to 1 ml/min	4 ml/min	2–12	0.3/43
HiTrap Q HP, 5 ml	250 mg (HSA, M_r 68 000)	up to 5 ml/min	20 ml/min	2–12	0.3/43
HiLoad 16/10 Q Sepharose High Performance, 20 ml	<1200 mg (BSA, M_r 67 000)	up to 5 ml/min	5 ml/min	2–12	0.3/43
HiLoad 26/10 Q Sepharose High Performance, 53 ml	<3000 mg (BSA, M_r 67 000)	up to 13 ml/min	13 ml/min	2–12	0.3/43
Q Sepharose High Performance	70 mg/ml (HSA, M_r 68 000)	30–150 cm/h	150 cm/h	2–12	0.5/72
Strong cation exchangers					
HiTrap SP HP, 1 ml	55 mg (ribonuclease, M_r 13 700)	up to 1 ml/min	4 ml/min	4–13	0.3/43
HiTrap SP HP, 5 ml	275 mg (ribonuclease, M_r 13 700)	up to 5 ml/min	20 ml/min	4–13	0.3/43
HiLoad 16/10 SP Sepharose High Performance, 20 ml	<1000 mg (ribonuclease, M_r 13 700)	up to 5 ml/min	5 ml/min	4–13	0.3/43
HiLoad 26/10 SP Sepharose High Performance, 53 ml	<3000 mg (ribonuclease, M_r 13 700)	up to 13 ml/min	13 ml/min	4–13	0.3/43
SP Sepharose High Performance	55 mg/ml (ribonuclease, M_r 13 700)	30–150 cm/h	150 cm/h	4–13	0.5/72

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

***Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

****Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

Use prepacked HiTrap columns (1 ml or 5 ml) for media selection, method scouting, group separations, small scale purification, sample concentration or clean-up. Connect up to 3 HiTrap columns in series to scale-up.

Use prepacked HiLoad columns (20 ml or 53 ml) for method development, group separations, larger scale purification or sample concentration.

For column packing:

Column	Volume	Bed height
Tricorn 10/100	up to 8 ml	up to 10 cm
Tricorn 10/150	up to 12 ml	up to 15 cm
Tricorn 10/200	up to 16 ml	up to 20 cm
XK 16/20	up to 30 ml	up to 15 cm
XK 26/20	up to 80 ml	up to 15 cm
XK 26/40	up to 196 ml	>15 cm

Select a production column such as FineLINE for larger volumes.

Purification examples

Intermediate purification

Column:	HiLoad 26/10 Q Sepharose High Performance
Sample:	Flow through fraction from Blue Sepharose clean-up step
Sample load:	110 mg protein
Start buffer:	20 mM bis-Tris-propane, 35 mM Na_2SO_4, pH 7.2
Elution buffer:	20 mM bis-Tris-propane, 35 mM Na_2SO_4, 0.5 M NaCl, pH 7.2
Flow:	6.4 ml/min, 73 cm/h (sample application) 24.7 ml/min, 282 cm/h (during elution of non-binding material) 19.8 ml/min, 226 cm/h (gradient elution)

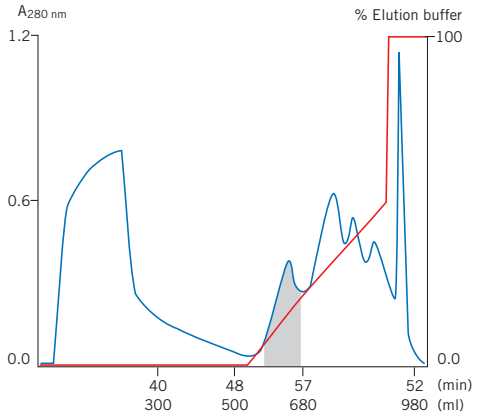


Fig. 50. Intermediate step from a purification protocol for α2-macroglobulin. An affinity step on Blue Sepharose was used to remove albumin, the major contaminant, before IEX.

Scaling-up

Using prepacked Sepharose High Performance columns facilitates quick scale-up and ensures reproducibility, as shown in Figures 51 and 52.

Column:	HiLoad 16/10 Q Sepharose High Performance
Column volume:	20 ml
Bed height:	10 cm
Flow:	150 cm/h, 5 ml/min
Start buffer:	0.02 M Tris-HCl, pH 7.6
Elution buffer:	0.02 M Tris-HCl, 0.5 M NaCl, pH 7.6
Sample:	1000 ml diluted pool from Phenyl Sepharose 6 Fast Flow (high sub), 0.021 mg EGF/ml
Load:	1.05 mg EGF/ml medium

Column:	BPG™ 200/500 Q Sepharose High Performance
Column volume:	3140 ml
Bed height:	10 cm
Flow:	150 cm/h, 48 l/h
Start buffer:	0.02 M Tris-HCl, pH 7.6
Elution buffer:	0.02 M Tris-HCl, 0.5 M NaCl, pH 7.6
Sample:	170 l diluted pool from Phenyl Sepharose 6 Fast Flow (high sub), 0.021 mg EGF/ml
Load:	1.09 mg EGF/ml medium

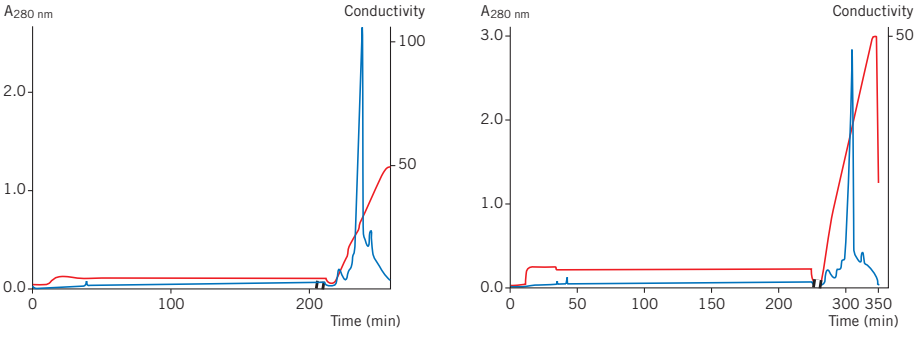
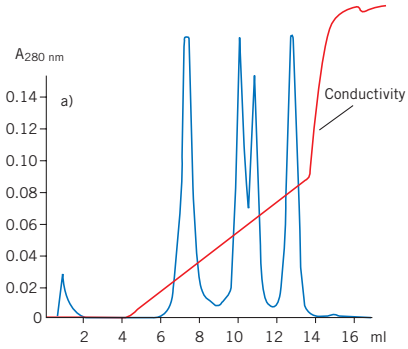
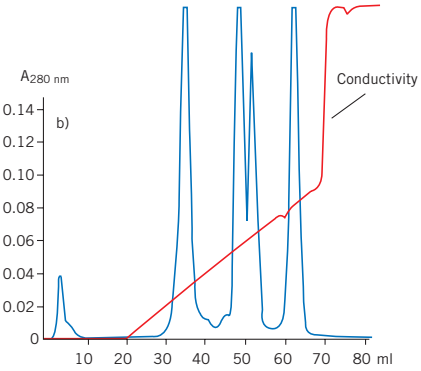


Fig. 51. Elution pattern, purity and yield are maintained when scaling-up from a HiLoad column to a BPG column.

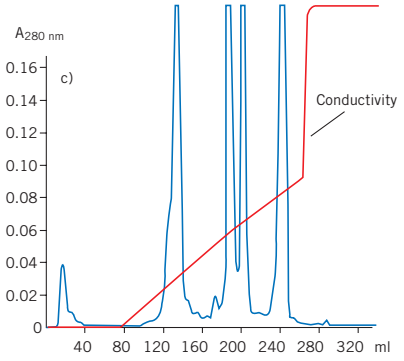
Column: HiTrap SP HP, 1 ml
Sample: Concanavalin A, ribonuclease A, α-chymotrypsinogen A, lysozyme, 4 mg protein/ml (3:3:1:1) in start buffer
Sample volume: 0.25 ml, 25% of column volume
Flow: 0.5 ml/min (75 cm/h)
Start buffer: 50 mM MES, pH 6.0
Elution buffer: 50 mM MES, 1.0 M NaCl, pH 6.0
Gradient: 0–43% elution buffer over 10 ml (10 CV)



Column: HiTrap SP HP, 5 ml
Sample: Concanavalin A, ribonuclease A, α-chymotrypsinogen A, lysozyme, 4 mg protein/ml (3:3:1:1) in start buffer
Sample volume: 1.25 m, 25% of column volume
Flow: 2.5 ml/min (75 cm/h)
Start buffer: 50 mM MES, pH 6.0
Elution buffer: 50 mM MES, 1.0 M NaCl, pH 6.0
Gradient: 0–43% elution buffer over 50 ml (10 CV)



Column: HiLoad 16/10 SP Sepharose High Performance
Sample: Concanavalin A, ribonuclease A, α-chymotrypsinogen A, lysozyme, 4 mg protein/ml (3:3:1:1) in start buffer
Sample volume: 5.0 ml, 25% of column volume
Column volume: 20 ml
Flow: 2.5 ml/min (75 cm/h)
Start buffer: 50 mM MES, pH 6.0
Elution buffer: 50 mM MES, 1.0 M NaCl, pH 6.0
Gradient: 0–43% elution buffer over 200 ml (10 CV)



Column: HiLoad 26/10 SP Sepharose High Performance
Sample: Concanavalin A, ribonuclease A, α-chymotrypsinogen A, lysozyme, 4 mg protein/ml (3:3:1:1) in start buffer
Sample volume: 13.25 ml, 25% of column volume
Column volume: 53 ml
Flow: 6.6 ml/min (75 cm/h)
Start buffer: 50 mM MES, pH 6.0
Elution buffer: 50 mM MES, 1.0 M NaCl, pH 6.0
Gradient: 0–43% elution buffer over 530 ml (10 CV)

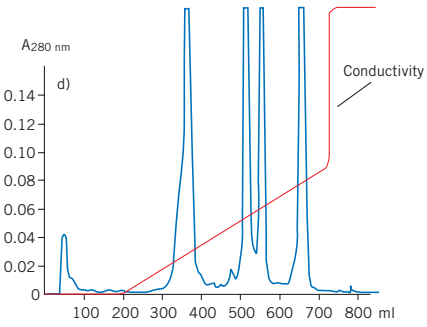


Fig. 52. Reproducibility maintained as purification is scaled up from a 1 ml HiTrap column through to a 53 ml HiLoad 26/10 SP Sepharose High Performance column.

Group separations

Figure 53 shows a group separation of human serum proteins on HiTrap Q HP using a one step elution that had been optimized to ensure that IgG flowed through the column leaving other serum components to be eluted separately. Figure 54 illustrates a multi-step elution to separate groups of human milk proteins.

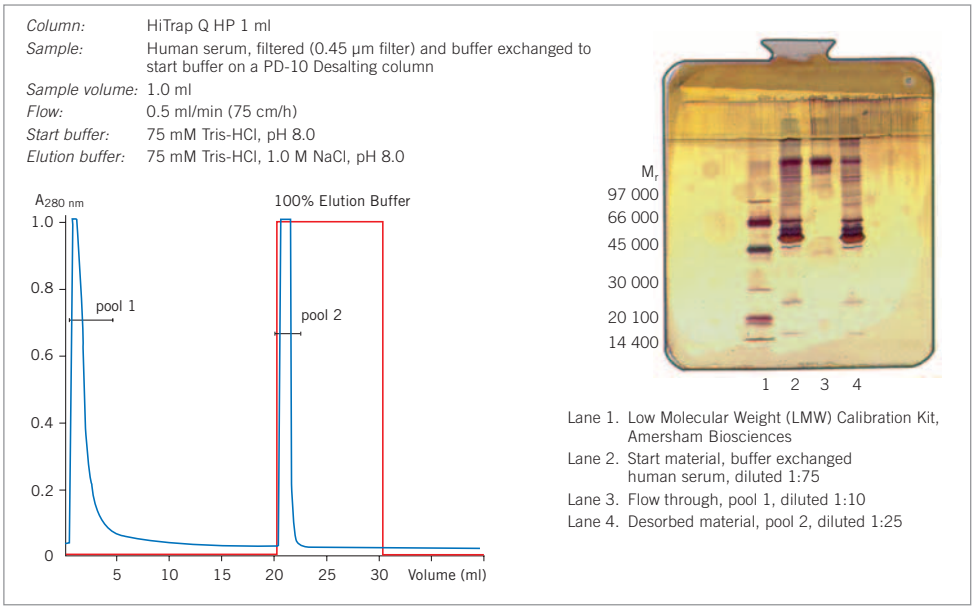


Fig. 53. Separation of IgG from human serum proteins on HiTrap Q HP, 1 ml, using one step elution. Analysis by SDS-PAGE (Phast System, PhastGel 10–15, silver staining).

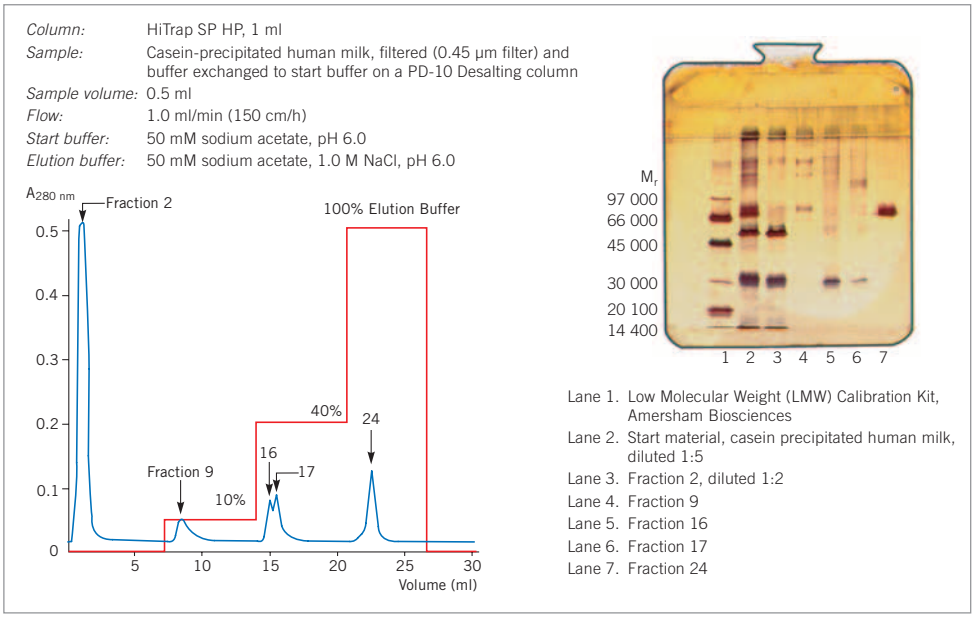


Fig. 54. Separation of human milk proteins on HiTrap SP HP, 1 ml, using one step elution. Analysis by SDS-PAGE (Phast System, PhastGel 10–15, silver staining).

Sample concentration

It can be an advantage to concentrate a sample prior to gel filtration in order to minimize sample volume and facilitate a rapid, high resolution size separation. HiTrap columns offer a convenient, ready to use solution for sample concentration. Table 7 gives examples of the high concentration factors achieved when concentrating proteins from very dilute starting material using HiTrap columns prepacked with Sepharose HP medium. Similar results can be achieved with HiTrap columns prepacked with Sepharose Fast Flow or Sepharose XL media.

Table 7. Sample concentration using 1 ml HiTrap ion exchange columns.

Column	Sample	Sample concentration µg/ml	Sample volume ml	Eluted concentration µg/ml	Volume eluted ml	Concentration factor (volume)	Yield %
HiTrap Q HP, 1 ml	Human IgG	23	450	3 180	3.0	150	92
		10	100	4 700	2.0	50	93
		1 010	10	3 370	3.0	3	100
HiTrap SP HP, 5 ml	Lysozyme	333	150	3 170	16.0	9	100
		33	1 500	3 720	13.2	114	98

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions through 0.45 µm or 0.22 µm filters. To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

The pH of the start buffer should be at least 0.5–1 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5–1 pH unit below the pI of the target substance when using a cation exchanger (S). See Appendix 2 for recommendations on volatile and non-volatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

– **anion exchange (Q)**
  start buffer: pH 8.0
  elution buffer: start buffer including 1 M NaCl, pH 8.0

– **cation exchange (S)**
  start buffer: pH 6.0
  elution buffer: start buffer including 1 M NaCl, pH 6.0

Users of ÄKTAdesign systems with BufferPrep functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8 or cation exchange chromatography at pH 6.

First time use or after long term storage:

1. To remove ethanol, wash with 1 column volume of distilled water at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 0.8 ml/min (HiLoad 20 ml), 2.2 ml/min (HiLoad 53 ml) or at 25 cm/h for Sepharose High Performance packed in larger columns. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 column volumes of start buffer, at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 3 ml/min (HiLoad 20 ml), 8 ml/min (HiLoad 53 ml) or at 50 cm/h for Sepharose High Performance packed in larger columns.
3. Wash with 5 column volumes of elution buffer, same flow as step 2.
4. Wash with 5 column volumes of start buffer, same flow as step 2.
5. Run a blank elution before applying sample.

Separation by gradient elution

Flow: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 3 ml/min (HiLoad 20 ml), 8 ml/min (HiLoad 53 ml) or at 50–100 cm/h for Sepharose High Performance packed in larger columns. Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically-bound material.
6. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Flow: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 3 ml/min (HiLoad 20 ml), 8 ml/min (HiLoad 53 ml) or at 50–100 cm/h for Sepharose High Performance packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Elute with 5 column volumes of start buffer + NaCl at chosen ionic strength.
5. Repeat step 4 at higher ionic strengths until the target protein(s) has been eluted.
6. Wash with 5 column volumes of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically-bound material.
7. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.

If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3. Note that this does not apply to HiTrap columns.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 3 ml/min (HiLoad 20 ml), 8 ml/min (HiLoad 53 ml) or at 40 cm/h with a contact time of 1–2 hours for Sepharose High Performance packed in larger columns.
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins, refer to Appendix 1.

Media characteristics

Composition: sulfopropyl (SP) or quaternary amino (Q) groups coupled to highly cross-linked 6% agarose via chemically stable ether bonds.

Product	Functional group	pH stability*	Mean particle size
Q Sepharose High Performance	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 1–14	34 µm
SP Sepharose High Performance	$-CH_2CH_2CH_2SO_{3-}$	Long term: 4–13 Short term: 3–14	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 the 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

For daily use, Sepharose High Performance media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), 70% ethanol, 1 M acetic acid, 30% acetonitrile and with additives such as non-ionic detergents.

Sepharose High Performance can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.

Avoid cationic detergents with SP Sepharose High Performance. Avoid anionic detergents with Q Sepharose High Performance. Avoid oxidizing agents.

Storage

For column storage, wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the 20% ethanol solution for columns packed with SP Sepharose High Performance. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at +4° C to +30° C in 20% ethanol. Do not freeze.

To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

Sepharose Fast Flow: purification with good resolution and easy scale-up

Use Sepharose Fast Flow media for purification of proteins.

Use Sepharose Fast Flow for capture or intermediate purification steps that require good resolution (flows up to 300 cm/h).

Use a weak ion exchanger such as DEAE, CM or ANX Sepharose Fast Flow, if a strong ion exchanger (substituted with Q, S or SP) does not give the required selectivity.

Run Sepharose Fast Flow columns on systems such as ÄKTAdesign, FPLC System and HPLC or systems using peristaltic pumps. Appendix 4 gives guidance on how to select the most suitable ÄKTAdesign system.

Sepharose Fast Flow media are based on a matrix of 90 µm particles made from 6% agarose and highly cross-linked for chemical and physical stability. ANX Sepharose 4 Fast Flow (high sub) is based on 4% agarose to form a medium that maintains a high binding capacity when separating large molecules such as thyroglobulin (M_r = 650 000), particularly suitable for large scale production when total binding capacity becomes economically significant.

Sepharose Fast Flow matrices are substituted with a range of ion exchange groups (Q, DEAE, ANX, SP and CM) giving the opportunity to test and use different selectivities (see Chapter 1 for an explanation of strong and weak ion exchangers). Ion exchangers containing strong ion exchange groups (Q and SP) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application.

Ion exchangers containing weak ion exchange groups (DEAE, CM and ANX) offer alternative selectivities, but over a narrower pH working range. Figure 55 illustrates how the selectivity of Sepharose Fast Flow media changes according to the anion exchange group.

Particle size and bed volumes remain stable, despite changes in ionic strength or pH, to ensure fast separations at high flow rates with good resolution. Methods can be easily scaled up from columns such as HiTrap Q FF (1 ml, prepacked with Q Sepharose Fast Flow) through to large scale columns such as FineLINE. The performance of Sepharose Fast Flow media is well documented and there are many examples of the smooth transfer from the laboratory to pilot scale and on to production.

Reference lists highlighting the use of Sepharose Fast Flow media are available at *www.chromatography.amershambiosciences.com*

Columns: a) HiTrap ANX Sepharose 4 Fast Flow (high sub) 1 ml
b) HiTrap Q XL 1 ml
c) HiTrap Q Sepharose Fast Flow 1 ml
d) HiTrap DEAE Sepharose Fast Flow 1 ml

Sample: 0.4 mg conalbumin (pI=6.3), 0.8 mg α-lactoglobulin (pI=5.8), 1.2 mg soya bean trypsin inhibitor (pI=4.5) dissolved in 2 ml start buffer

Sample volume: 2 ml

Start buffer: 20 mM Tris-HCl pH 7.4

Elution buffer: 20 mM Tris-HCl, 0.5 M NaCl pH 7.4

Flow: 1 ml/min (150 cm/h)

Gradient: 0% elution buffer (25 CV), 0-80% elution buffer (40 CV)

Wash: 5 ml start buffer
Elution: 40 ml, linear gradient, 0–80 % elution buffer

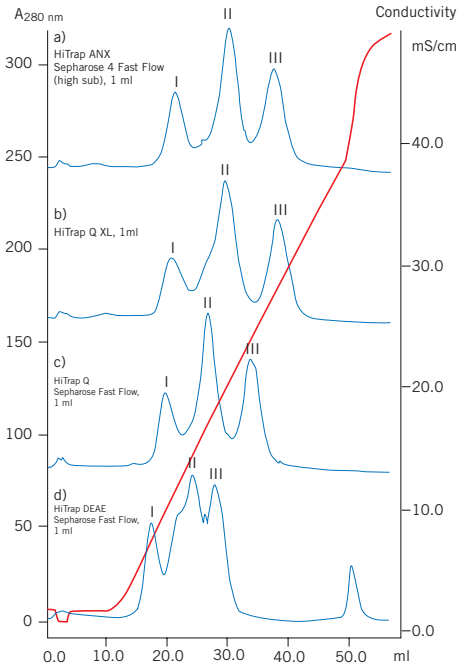


Fig. 55. Separation of conalbumin (I), α-lactalbumin (II) and soya bean trypsin inhibitor (III) on a range of anion exchange HiTrap columns demonstrates the difference in selectivity according to the anion exchange group.

Purification options

Fig. 56. Sepharose Fast Flow media, with a range of selectivities, are available prepacked in HiTrap and HiPrep columns and in media packs.

Product	Binding capacity per column or per ml medium	Recommended working flow*	Maximum flow*	Working pH range**	Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar*
Strong anion exchangers					
HiTrap Q FF, 1 ml	3 mg (thyroglobulin, M_r 669 000) 120 mg (HSA, M_r 68 000) 110 mg (α–lactalbumin, M_r 14 300)	up to 1 ml/min	4 ml/min	2–12	0.3/43
HiTrap Q FF, 5 ml	15 mg (thyroglobulin, M_r 669 000) 600 mg (HSA, M_r 68 000) 550 mg (α–lactalbumin, M_r 14 300)	up to 5 ml/min	20 ml/min	2–12	0.3/43
HiPrep 16/10 Q FF, 20 ml	60 mg (thyroglobulin, M_r 669 000) 2400 mg (HSA, M_r 68 000) 2200 mg (α–lactalbumin, M_r 14 300)	2–10 ml/min	10 ml/min	2–12	0.15/22
Q Sepharose Fast Flow	3 mg/ml (thyroglobulin, M_r 669 000) 120 mg/ml (HSA, M_r 68 000) 110 mg/ml, (α–lactalbumin, M_r 14 300)	50–400 cm/h	750 cm/h	2–12	0.3/43
Strong cation exchangers					
HiTrap SP FF, 1 ml	50 mg (bovine COHb, M_r 69 000) 50 mg (human IgG, M_r 160 000) 70 mg (ribonuclease A, M_r 13 700)	up to 1 ml/min	4 ml/min	4–13	0.3/43
HiTrap SP FF, 5 ml	250 mg (bovine COHb, M_r 69 000) 250 mg (human IgG, M_r 160 000) 350 mg (ribonuclease A, M_r 13 700)	up to 5 ml/min	20 ml/min	4–13	0.3/43
HiPrep 16/10 SP FF, 20 ml	1000 mg (bovine COHb, M_r 69 000) 1000 mg (human IgG, M_r 160 000) 1400 mg (ribonuclease A, M_r 13 700)	2–10 ml/min	10 ml/min	4–13	0.15/22
SP Sepharose Fast Flow	50 mg/ml (bovine COHb, M_r 69 000) 50 mg/ml (human IgG, M_r 160 000) 70 mg/ml (ribonuclease A, M_r 13 700)	50–400 cm/h	750 cm/h	4–13	0.3/43
Weak anion exchangers					
HiTrap DEAE FF, 1 ml	100 mg (α–lactalbumin, M_r14 300) 110 mg (HSA, M_r 68 000)	up to 1 ml/min	4 ml/min	2–9	0.3/43

continues on following page

Product	Binding capacity per column or per ml medium	Recommended working flow*	Maximum flow*	Working pH range**	Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar*
HiTrap DEAE FF, 5 ml	500 mg (α–lactalbumin, M_r 14 300) 550 mg (HSA, M_r 68 000)	up to 5 ml/min	20 ml/min	2–9	0.3/43
HiPrep 16/10 DEAE FF, 20 ml	2000 mg (α–lactalbumin, M_r 14 300) 2200 mg (HSA, M_r 68 000)	2–10 ml/min	10 ml/min	2–9	0.15/22
DEAE Sepharose Fast Flow	100 mg/ml (α–lactalbumin, M_r 14 300) 110 mg/ml (HSA, M_r 68 000)	50–400 cm/h	750 cm/h	2–9	0.3/43
HiTrap ANX FF (high sub), 1 ml	43 mg (BSA, M_r 67 000) 5 mg (thyroglobulin, M_r 669 000)	up to 1 ml/min	4 ml/min	2–9	0.3/43
HiTrap ANX FF (high sub), 5 ml	215 mg (BSA, M_r 67 000) 25 mg (thyroglobulin, M_r 669 000)	up to 5 ml/min	20 ml/min	2–9	0.3/43
HiPrep 16/10 ANX FF (high sub), 20 ml	860 mg (BSA, M_r 67 000) 100 mg (thyroglobulin, M_r 669 000)	2–10 ml/min	10 ml/min	2–9	0.15/22
ANX Sepharose 4 Fast Flow (high sub)	43 mg/ml (BSA, M_r 67 000) 5 mg/ml (thyroglobulin, M_r 669 000)	50–300 cm/h	400 cm/h	2–9	0.1/14
Weak cation exchangers					
HiTrap CM FF, 1 ml	50 mg (ribonuclease A, M_r 13 700)	up to 1 ml/min	4 ml/min	6–10	0.3/43
HiTrap CM FF, 5 ml	250 mg (ribonuclease A, M_r 13 700)	up to 5 ml/min	20 ml/min	6–10	0.3/43
HiPrep 16/10 CM FF, 20 ml	1000 mg (ribonuclease A, M_r 13 700)	2–10 ml/min	10 ml/min	6–10	0.15/22
CM Sepharose Fast Flow	50 mg/ml medium (ribonuclease A, M_r 13 700)	50–400 cm/h	750 cm/h	6–10	0.3/43

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

***Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

****Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

Use prepacked HiTrap columns (1 ml or 5 ml) for media selection, method scouting, group separations, small scale purification, sample concentration or clean-up. Connect up to 3 HiTrap columns in series to scale-up.

Use prepacked HiPrep columns (20 ml) for method development, group separations, larger scale purification, sample concentration or clean-up. Connect several HiPrep columns in series to increase binding capacity.

For column packing:

Column	Volume	Bed height
Tricorn 10/100	up to 8 ml	up to 10 cm
Tricorn 10/150	up to 12 ml	up to 15 cm
Tricorn 10/200	up to 16 ml	up to 20 cm
XK 16/20	up to 30 ml	up to 15 cm
XK 26/20	up to 80 ml	up to 15 cm
XK 26/40	up to 196 ml	> 15 cm
XK 50/20	up to 274 ml	up to 14 cm
XK 50/30	up to 559 ml	up to 28.5 cm

Select a production column such as BPG or Chromaflow for larger volumes.

Purification examples

Media scouting

Using 1 ml HiTrap columns the most suitable matrix and charged group for a separation can be quickly and easily selected before optimization and scale-up. In Figure 57 a comparison of elution profiles for the same sample separated under identical conditions on three different media illustrates the differences in selectivity and resolution that can result from changing the charge group and the particle size. The most suitable medium can be selected and conditions optimized according to the requirements for the separation, for example to isolate a single, well-resolved peak or to maximize resolution between several peaks of interest.

Begin by scouting on the strong ion exchangers (Q, S or SP) in order to find the greatest differences in charge between the molecules of interest.

Columns: a) HiTrap SP XL 1 ml
b) HiTrap SP Sepharose Fast Flow 1 ml
c) HiTrap CM Sepharose Fast Flow 1 ml
Sample: 3 mg ribonuclease A (pI=9.3),
0.8 mg cytochrome C (pI=10.3),
0.8 mg lysozyme (pI>11)
Sample volume: 2 ml in start buffer
Start buffer: 20 mM sodium phosphate pH 6.8
Elution buffer: 20 mM sodium phosphate,
0.5 M NaCl pH 6.8
Flow: 1 ml/min (150 cm/h)
Gradient: 0% elution buffer (25 CV),
0–100% elution buffer (40 CV)

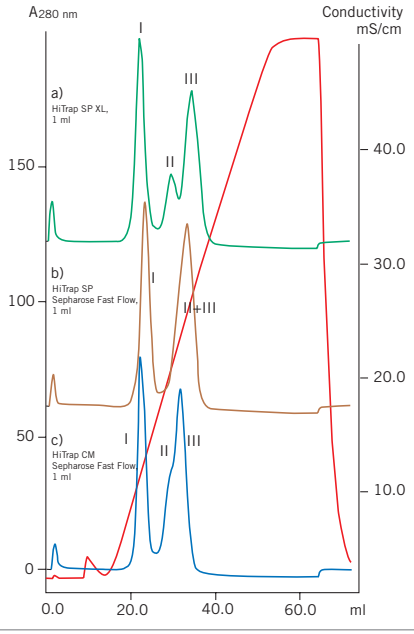


Fig. 57. Media scouting: separation of ribonuclease A (I), cytochrome C (II) and lysozyme (III) on HiTrap CM Sepharose Fast Flow 1 ml, HiTrap SP Sepharose Fast Flow 1 ml and HiTrap SP XL 1 ml.

Capture

Column: HiPrep 16/10 DEAE Sepharose FF (20 ml)
Sample: 200 ml clarified *E. coli* supernatant, diluted 1:2 with water pH 6.6, conductivity 2.6 mS/cm
Start buffer: 25 mM Tris-HCl, 10% glycerol, 1 mM EDTA, 2 mM DTT, pH 7.4
Elution buffer: 1 M NaCl, 25 mM Tris-HCl, 10% glycerol, 1 mM EDTA, 2 mM DTT, pH 7.4
Flow: 5 ml/min (150 cm/h)
Gradient: 0% elution buffer (6 CV)
0–50% elution buffer (20 CV)
50% elution buffer (1 CV)
100% elution buffer (2 CV)

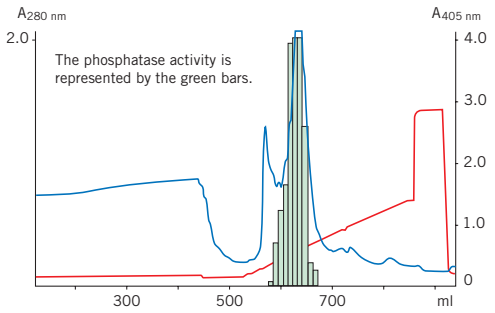


Fig. 58. A HiPrep 16/10 DEAE Sepharose Fast Flow column is used as the capture step to concentrate rPhosphatase and remove most of the contaminants.

Scaling-up

Figure 59 shows the ease with which separations can be scaled up on columns prepacked with Sepharose Fast Flow media. Beginning with a 1 ml HiTrap column the reproducibility of the separation has been maintained through a 20-fold scale-up.

Sample: 1. Conalbumin, 2 mg/ml
2. α-lactalbumin, 4 mg/ml
3. Soy trypsin inhibitor, 6 mg/ml
Sample volume: 1 CV (column volume)
a) 1 ml, b) 5 ml, c) 20 ml
Start buffer: 50 mM Tris-HCl, pH 7.3
Elution buffer: 50 mM Tris-HCl, 0.5 M NaCl, pH 7.3
Gradient: 0–100% elution buffer in 20 CV
a) 20 ml, b) 100 ml, c) 400 ml
Flow: 150 cm/h (1 ml/min using HiTrap 1 ml, 5 ml/min using HiTrap 5 ml and HiPrep 16/10 columns)

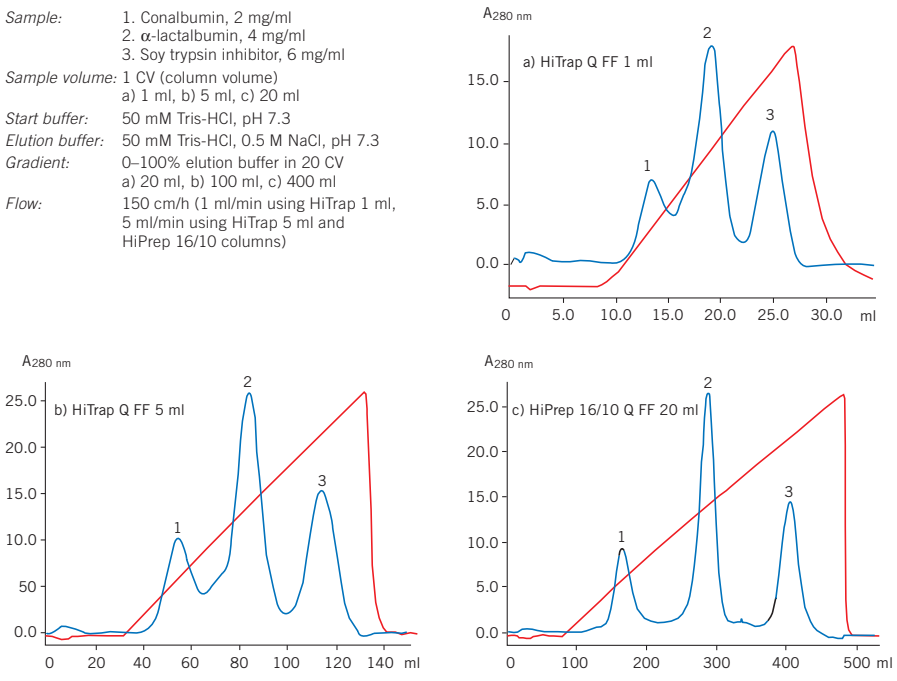
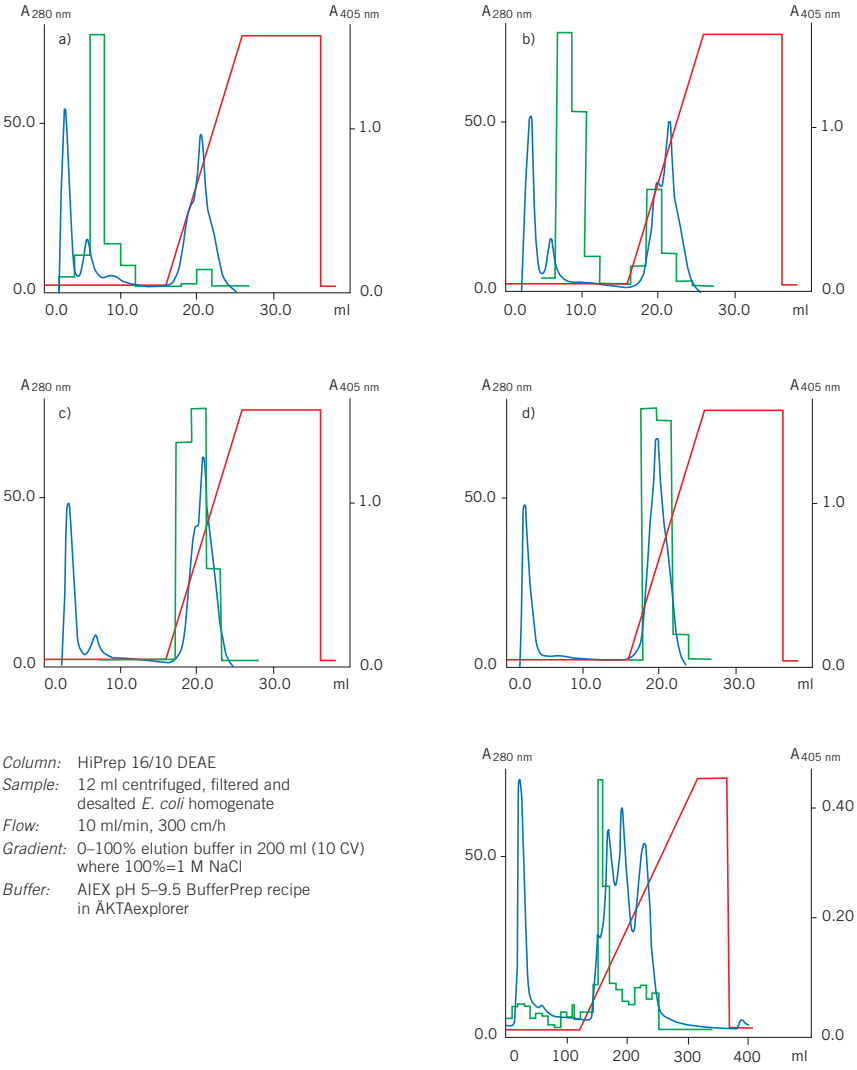


Fig. 59. 5-fold and 20-fold scale-up using prepacked Q Sepharose Fast Flow columns.

Column: HiTrap DEAE, 1 ml
Sample: 1 ml centrifuged, filtered and de salted *E. coli* homogenate
Flow: 2 ml/min, 310 cm/h
Gradient: 0–100% elution buffer in 10 ml (10 CV) where 100 %=1 M NaCl
Buffer: AIEX pH 5–9.5 BufferPrep recipe in ÄKTAexplorer

Alkaline phosphatase assay
1) Pipette 100 µl sample (fractions) + 100 µl substrate (SIGMA FAST pNPP substrate tablet set, N2270, prepared according to the manufacturer's protocol) into micro-titrer plate. Blank = 100 µl substrate + 100 µl buffer.
2) Incubate for 1 hr in the dark at room temperature.
3) Read the absorbance at 405 nm.



Column: HiPrep 16/10 DEAE
Sample: 12 ml centrifuged, filtered and desalted *E. coli* homogenate
Flow: 10 ml/min, 300 cm/h
Gradient: 0–100% elution buffer in 200 ml (10 CV) where 100%=1 M NaCl
Buffer: AIEX pH 5–9.5 BufferPrep recipe in ÄKTAexplorer

Fig. 60. Optimization and scale up on DEAE Sepharose Fast Flow.

Optimizing pH

When the most suitable medium has been selected for a separation, conditions can be optimized further by adjusting parameters such as pH. Figure 61 shows how increasing the pH on a column prepacked with CM Sepharose Fast Flow (HiPrep 16/10 CM FF) significantly improved resolution of a mixture of model proteins.

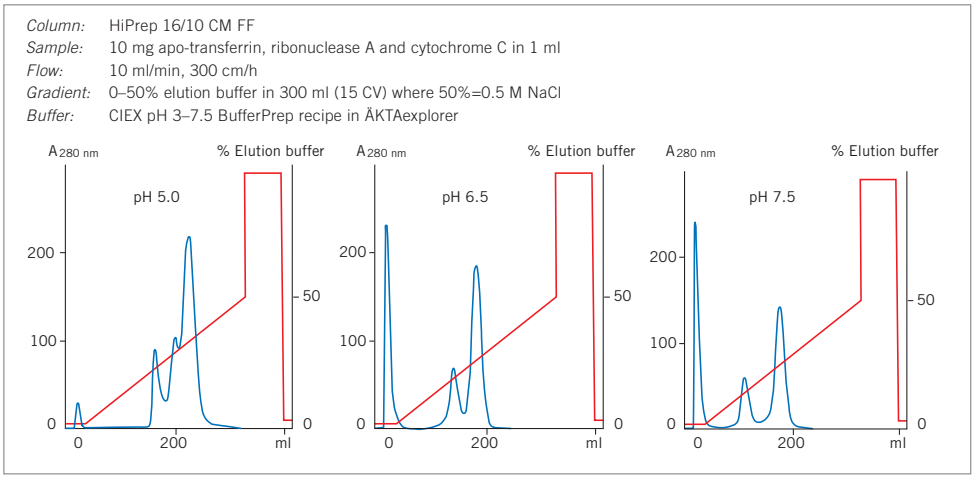


Fig. 61. Selecting optimal pH for separation of standard proteins on HiPrep 16/10 CM FF.

Sample concentration

It can be an advantage to concentrate a sample prior to gel filtration in order to minimize sample volume and facilitate a rapid, high resolution size separation. HiTrap columns offer a convenient, ready to use solution for sample concentration. Table 7 on page 89 gives examples of the high concentration factors achieved when concentrating proteins from very dilute starting material using HiTrap columns prepacked with Sepharose HP medium. Similar results can be achieved with HiTrap columns prepacked with Sepharose Fast Flow or Sepharose XL media.

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions using filters of 1 µm or less. To avoid formation of air bubbles in a packed column, maintain buffers and columns at a constant temperature before and during a run.

The pH of the start buffer should be at least 0.5–1 pH unit above the pI of the target substance when using an anion exchanger (Q, DEAE or ANX) and 0.5–1 pH unit below the pI of the target substance when using a cation exchanger (SP, CM). See Appendix 2 for recommendations on volatile and non-volatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

– **anion exchange (Q)**
start buffer: pH 8.0
elution buffer: start buffer including 1 M NaCl, pH 8.0

– **cation exchange (SP)**
start buffer: pH 6.0
elution buffer: start buffer including 1 M NaCl, pH 6.0

If selectivity is not satisfactory when using a strong ion exchanger (Q or SP), try a weak ion exchanger (DEAE, ANX or CM) instead.

Users of ÄKTAdesign systems with BufferPrep functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8 or cation exchange chromatography at pH 6.

First time use or after long term storage

1. To remove ethanol, wash with 1 column volume of distilled water at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 2 ml/min (HiPrep 20 ml), or at 50 cm/h for Sepharose Fast Flow packed in larger columns. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 column volumes of start buffer at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml) or 5 ml/min (HiPrep 20 ml).
3. Wash with 5 column volumes of elution buffer, same flow as step 2.
4. Wash with 5 column volumes of start buffer, same flow as step 2.
5. Run a blank elution before applying sample.

Separation by gradient elution

Flow: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 150 cm/h for Sepharose Fast Flow packed in larger columns. Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically-bound material.
6. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Flow: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 150 cm/h for Sepharose Fast Flow packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Elute with 5 column volumes of start buffer + NaCl at chosen ionic strength.
5. Repeat step 4 at higher ionic strengths until the target protein(s) has been eluted.
6. Wash with 5 column volumes of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically-bound material.
7. Re-equilibrate 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.

If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Refer to Chapter 2 for advice on optimizing the separation.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3. Note that this does not apply to HiTrap or HiPrep columns.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 40 cm/h with a contact time of 1–2 hours for Sepharose Fast Flow packed in larger columns.
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins, refer to Appendix 1.

Media characteristics

Composition:

- sulfopropyl (SP), carboxymethyl (CM), quaternary amino (Q) or diethylaminoethyl (DEAE) groups coupled to highly cross-linked 6% agarose via chemically stable ether bonds.
- diethylaminopropyl (ANX) group coupled to highly cross-linked 4% agarose via chemically stable ether bonds.

Product	Functional group	pH stability*	Mean particle size
Q Sepharose Fast Flow	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 1–14	90 µm
SP Sepharose Fast Flow	$-CH_2CH_2CH_2SO_3^-$	Long term: 4–13 Short term: 3–14	90 µm
DEAE Sepharose Fast Flow	$-O-CH_2CHOHCH_2N^+H(CH_2CH_3)_2$	Long term: 2–13 Short term: 1–14	90 µm
ANX Sepharose 4 Fast Flow	$-OCH_2CHOHCH_2OCH_2CHOHCH_2N^+H(CH_2CH_3)_2$	Long term: 3–13 Short term: 2–14	90 µm
CM Sepharose Fast Flow	$-O-CH_2COO^-$	Long term: 4–13 Short term: 2–14	90 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 the 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

For daily use, Sepharose Fast Flow media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), with additives such as non-ionic detergents, 70% ethanol, 1 M acetic acid and 30% isopropanol.

Sepharose Fast Flow can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.

Avoid cationic detergents with SP or CM Sepharose Fast Flow. Avoid anionic detergents with Q, DEAE or ANX Sepharose Fast Flow. Avoid oxidizing agents.

Storage

For column storage, wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the 20% ethanol solution for SP Sepharose Fast Flow. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at +4° C to +30° C in 20% ethanol. Do not freeze.

To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

Sepharose XL – for selected proteins that require very high binding capacity to increase productivity, easy scale-up

Use Sepharose XL media for purification of proteins when improved binding capacity compared to other Sepharose media has been confirmed for the selected protein.

Use Sepharose XL at the beginning of a purification scheme for initial capture when a high binding capacity and rapid separation is required for a selected protein from clarified samples.

Run columns packed with Sepharose XL on systems such as ÄKTAdesign, FPLC System and HPLC or systems using peristaltic pumps. Appendix 4 gives guidance on how to select the most suitable ÄKTAdesign system.

Purify viruses or viral vectors using Q Sepharose XL virus licensed.

Sepharose XL media are based on a matrix of 90 µm particles, made from 6% agarose and highly cross-linked for chemical and physical stability, substituted with quaternary ammonium (Q) or sulfopropyl (SP) groups. The ionic groups are bound to long, flexible dextran chains which have been coupled to the agarose. This increases the exposure of the Q or SP groups thereby raising the binding capacity to a very high level without restricting the passage of charged molecules. The strong ion exchange groups maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application. Particle size and bed volumes remain stable, despite changes in ionic strength or pH, to ensure fast separations at high flow rates with good resolution.

Purification options

Fig. 62. Q and S Sepharose XL are available in prepacked HiTrap and HiPrep columns, in media packs and in the Ion Exchange Selection Kit.

Product	Binding capacity per column or per ml medium	Recommended working flow*	Maximum flow*	Working pH range**	Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar*
Strong anion exchangers					
HiTrap Q XL, 1 ml	>130 mg (BSA, M_r 67 000)	up to 1 ml/min	4 ml/min	2–12	0.3/43
HiTrap Q XL, 5 ml	>650 mg (BSA, M_r 67 000)	up to 5 ml/min	20 ml/min	2–12	0.3/43
HiPrep 16/10 Q XL, 20 ml	>2600 mg (BSA, M_r 67 000)	2–10 ml/min	10 ml/min	2–12	0.15/22
Q Sepharose XL and Q Sepharose XL virus licensed****	>130 mg/ml (BSA, M_r 67 000)	300–500 cm/h	700 cm/h	2–12	0.3/3
Strong cation exchangers					
HiTrap SP XL, 1 ml	>160 mg (lysozyme, M_r 14 500)	up to 1 ml/min	4 ml/min	4–13	0.3/43
HiTrap SP XL, 5 ml	>800 mg (lysozyme, M_r 14 500)	up to 5 ml/min	20 ml/min	4–13	0.3/43
HiPrep 16/10 SP XL, 20 ml	>3200 mg (lysozyme, M_r 14 500)	2–10 ml/min	10 ml/min	4–13	0.15/22
SP Sepharose XL	>160 mg/ml (lysozyme, M_r 14 500)	300–500 cm/h	700 cm/h	4–13	0.3/43

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

***Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

****Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

***** Important information: Separating viral particles with Q Sepharose XL may require a license under United States patent 6,537,793 B2 and foreign equivalents owned by Gencell SAS. Such a license is not included with the purchase of Q Sepharose XL, but is included with the purchase of Q Sepharose XL virus licensed products. Purchasers of Q Sepharose XL virus licensed products are granted a free limited license under US patent 6,537,793 B2 and foreign equivalents owned by Gencell SAS to separate viral particles solely through use of the product purchased.*

Use prepacked HiTrap columns (1 ml or 5 ml) for media selection, method scouting, group separations, small scale purification, sample concentration or clean-up. Connect up to 3 HiTrap columns in series to scale-up.

Use prepacked HiPrep columns (20 ml) for method development, group separations, larger scale purification, sample concentration or clean-up. Connect several HiPrep columns in series to increase binding capacity.

For column packing:

Column	Volume	Bed height
Tricorn 10/100	up to 8 ml	up to 10 cm
Tricorn 10/150	up to 12 ml	up to 15 cm
Tricorn 10/200	up to 16 ml	up to 20 cm
XK 16/20	up to 30 ml	up to 15 cm
XK 26/20	up to 80 ml	up to 15 cm
XK 26/40	up to 196 ml	> 15 cm
XK 50/20	up to 274 ml	up to 14 cm
XK 50/30	up to 559 ml	up to 28.5 cm

Select a production column such as BPG or Chromaflow for larger volumes.

Purification examples

Media selection

Using 1 ml HiTrap columns the most suitable matrix and charged group for a separation to be selected quickly and easily. In Figure 63 a comparison of elution profiles for the same sample separated under identical conditions on three different media illustrates the differences in selectivity and resolution that can result from changing the charge group and matrix. The most suitable medium can be selected and conditions optimized according to the requirements for the purification. In this example Sepharose XL resolves the three components and optimization of elution conditions could further improve the resolution. However, any of these media would be suitable if the aim was to isolate the first major peak (ribonuclease A).

Columns:	a) HiTrap SP XL 1 ml b) HiTrap SP Sepharose Fast Flow 1 ml c) HiTrap CM Sepharose Fast Flow 1 ml
Sample:	3 mg ribonuclease A (pI=9.3), 0.8 mg cytochrome C (pI=10.3), 0.8 mg lysozyme (pI>11)
Sample volume:	2 ml in start buffer
Start buffer:	20 mM sodium phosphate pH 6.8
Elution buffer:	20 mM sodium phosphate, 0.5 M NaCl pH 6.8
Flow:	1 ml/min (150 cm/h)
Gradient:	0% elution buffer (25 CV), 0–100% elution buffer (40 CV)

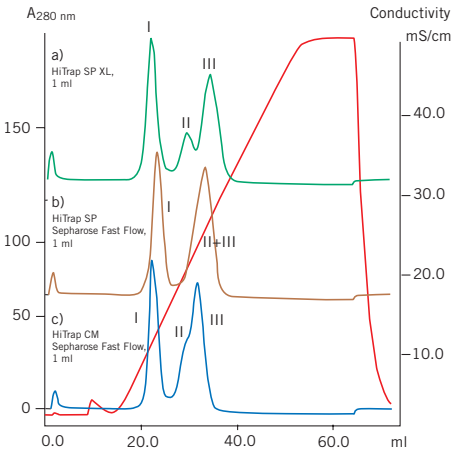


Fig. 63. Media scouting: separation of ribonuclease A (I), cytochrome c (II) and lysozyme (III) on a range of anion exchange HiTrap columns.

Capture

Capture of alkaline phosphatase from a clarified lysate of *E. coli* using a HiTrap Q XL 1 ml column is shown in Figure 64. Separation was monitored at A_{280} nm and phosphatase activity assayed by a spectrophotometric method at A_{405} nm

Columns:	HiTrap Q XL 1 ml
Sample:	2 ml *E.coli* lysate clarified by centrifugation
Sample volume:	2 ml
Start buffer:	20 mM Tris-HCl pH 7.4
Elution buffer:	20 mM Tris-HCl, 0.5 M NaCl pH 7.4
Flow:	1 ml/min (150 cm/h)
Gradient:	0% elution buffer (30 CV), 0–100% elution buffer (40 CV)

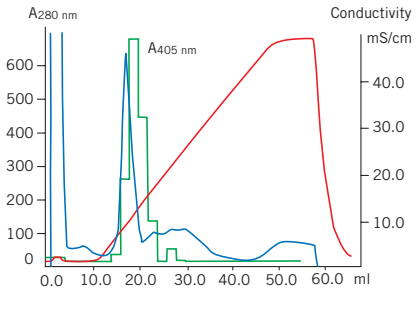


Fig. 64. Clarified *E. coli* lysate on HiTrap Q XL, absorbance values at 450 nm relate to phosphatase activity in eluted fractions.

Capture and scale-up

Figure 65 shows a pilot scale purification performed on a Sepharose XL ion exchanger. The separation was developed on Q Sepharose XL packed in an XK 16/20 column in order to select optimal pH and to determine maximum binding capacity available. Adding $CaCl_2$ to the sample precipitated DNA and so increased the binding capacity for the target protein. Final loading was reduced to 75% of the maximum capacity and the result verified before scaling-up to the larger column.

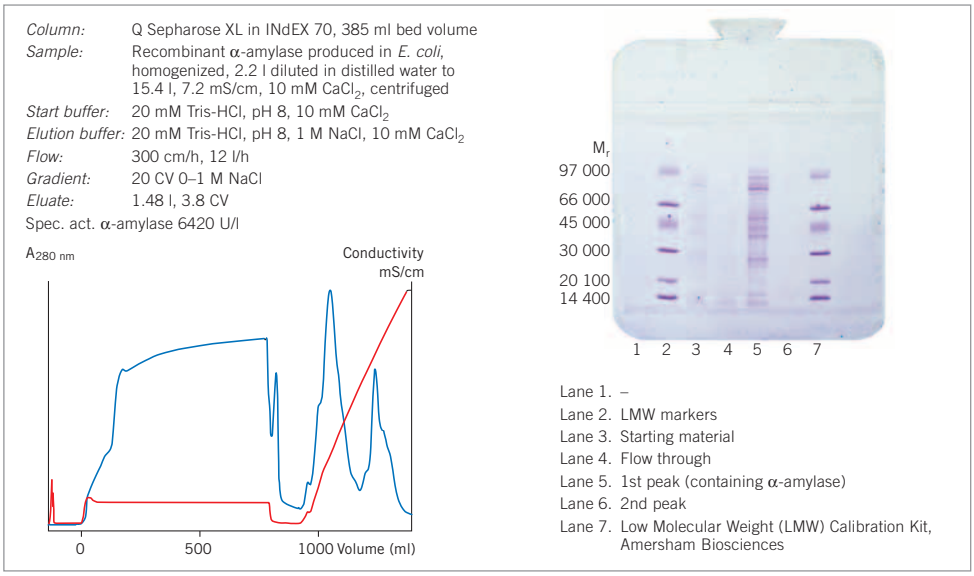


Fig. 65. Capture of recombinant α-amylase from *E. coli* on Q Sepharose XL pilot scale column together with SDS-PAGE (Phast System, Coomassie Brilliant Blue staining) of starting material and eluted fractions (reduced).

Viral purification

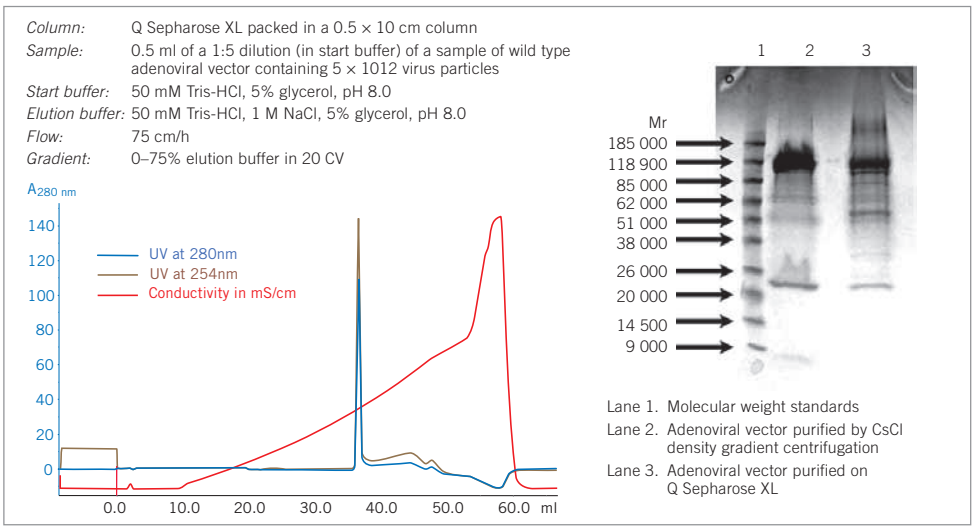


Fig. 66. Q Sepharose XL virus licensed provides an alternate to traditional cesium chloride density gradient centrifugation used for purification of viruses and viral vectors.

Sample concentration

It can be an advantage to concentrate a sample prior to gel filtration in order to minimize sample volume and facilitate a rapid, high resolution size separation. HiTrap columns offer a convenient, ready to use solution for sample concentration. Table 7 on page 89 gives examples of the high concentration factors achieved when concentrating proteins from very dilute starting material using HiTrap columns prepacked with Sepharose HP medium. Similar results can be achieved with HiTrap columns prepacked with Sepharose Fast Flow or Sepharose XL media.

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. Use the instructions given here as a basis from which to optimize a separation.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Samples must be fully dissolved and free from particles or other material likely to interfere with the separation. Refer to Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions using filters of 1 µm or smaller. To avoid formation of air bubbles in a packed column, maintain buffers and columns at a constant temperature before and during a run.

The pH of the start buffer should be at least 0.5–1 pH unit above the pI of the target substance when using an anion exchanger (Q) and 0.5–1 pH unit below the pI of the target substance when using a cation exchanger (S). See Appendix 2 for recommendations on volatile and non-volatile buffer systems for anion and cation exchangers.

For samples with unknown charge properties, try the following:

– **anion exchange (Q)**
  start buffer: pH 8.0
  elution buffer: start buffer including 1 M NaCl, pH 8.0

– **cation exchange (SP)**
  start buffer: pH 6.0
  elution buffer: start buffer including 1 M NaCl, pH 6.0

Users of ÄKTAdesign systems with BufferPrep functionality can select one of the buffer recipes recommended for anion exchange chromatography at pH 8 or cation exchange chromatography at pH 6.

First time use or after long term storage

1. To remove ethanol, wash with 1 column volume of distilled water at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml), or at 50 cm/h for Sepharose XL packed in larger columns. This step ensures removal of ethanol and avoids the risk of precipitation if buffer salts were to come into contact with the ethanol. The step can be omitted if precipitation is not likely to be a problem.
2. Wash with 5 column volumes of start buffer at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 150 cm/h for Sepharose XL packed in larger columns.
3. Wash with 5 column volumes of elution buffer, same flow as step 2.
4. Wash with 5 column volumes of start buffer, same flow as step 2.
5. Run a blank elution before applying sample.

Separation by gradient elution

Flow: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 150 cm/h for Sepharose XL packed in larger columns. Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes and an increasing ionic strength up to 0.5 M NaCl (50%B).
5. Wash with 5 column volumes of 1 M NaCl (100%B) to elute any remaining ionically-bound material.
6. Re-equilibrate 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Separation by step elution

Flow: 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 150 cm/h for Sepharose XL packed in larger columns.

Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable.
2. Adjust the sample to the chosen starting pH and ionic strength and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the baseline, eluent pH and conductivity are stable i.e. when all unbound material has washed through the column.
4. Elute with 5 column volumes of start buffer + NaCl at chosen ionic strength.
5. Repeat step 4 at higher ionic strengths until the target protein(s) has been eluted.
6. Wash with 5 column volumes of a high salt solution (1 M NaCl in start buffer) to elute any remaining ionically-bound material.
7. Re-equilibrate with 5–10 column volumes of start buffer or until eluent pH and conductivity reach the required values.

Save time by using higher flow rates during the high salt wash and re-equilibration steps. Do not exceed the maximum recommended flow for the medium.

If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable.

Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl at 1 ml/min (HiTrap 1 ml), 5 ml/min (HiTrap 5 ml), 5 ml/min (HiPrep 20 ml) or at 40 cm/h with a contact time of 1–2 hours for Sepharose XL packed in larger columns.
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins, refer to Appendix 1.

Media characteristics

Composition: sulfopropyl (SP) or quaternary amino (Q) groups attached via chemically stable ether bonds to long, flexible dextran chains that are covalently coupled to highly cross-linked 6% agarose.

Product	Functional group	pH stability*	Mean particle size
Q Sepharose XL	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 2–14	90 µm
SP Sepharose XL	$-CH_2CH_2CH_2SO_3^-$	Long term: 4–13 Short term: 3–14	90 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 the 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

For daily use, Sepharose XL media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), with additives such as non-ionic detergents, 70% ethanol, 1 M acetic acid and 30% isopropanol.

Sepharose XL can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.

Avoid cationic detergents with SP Sepharose XL. Avoid anionic detergents with Q Sepharose XL. Avoid oxidizing agents.

Storage

For column storage, wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the storage solution for SP Sepharose XL. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Whenever possible, use the storage and shipping device if supplied by the manufacturer. Store unused media at +4° C to +30° C in 20% ethanol. Do not freeze.

To avoid formation of air bubbles in a packed column, ensure that column and buffers are at the same temperature when preparing for a run.

Sepharose Big Beads: purification from crude, viscous samples at large scale

Use Sepharose Big Beads for purification of proteins from crude, viscous samples.

Use Sepharose Big Beads when handling large volumes of crude or viscous samples that must be bound rapidly and when resolution is less important.

Use Sepharose Big Beads for capture steps, when viscosity and back-pressure may limit the throughput attainable with ion exchangers of smaller particle size.

Run columns packed with Sepharose Big Beads on systems such as ÄKTAdesign, FPLC System and HPLC or systems using peristaltic pumps. Appendix 4 gives guidance on how to select the most suitable ÄKTAdesign system.

Sepharose Big Beads are ion exchangers designed for large-scale industrial applications. The media are based on 100–300 µm, cross-linked 6% agarose particles, substituted with quaternary ammonium (Q) or sulfopropyl (SP) groups. The large particle size, together with a high degree of cross-linking for extreme physical and chemical stability, ensures that high flow rates can be maintained when processing very viscous samples. For example, a flow of 500 cm/h can be maintained in an industrial process at viscosities up to 2.5 times the viscosity of water. More dilute samples can be run at 1000 cm/h. Particle size and bed volumes remain stable, despite changes in ionic strength or pH. The strong ion exchange groups (Q and SP) maintain their charge over a broad pH range, allowing selection of the most suitable pH for each application.

Figures 67 and 68 show the excellent flow characteristics and typical binding capacities for Big Bead media.

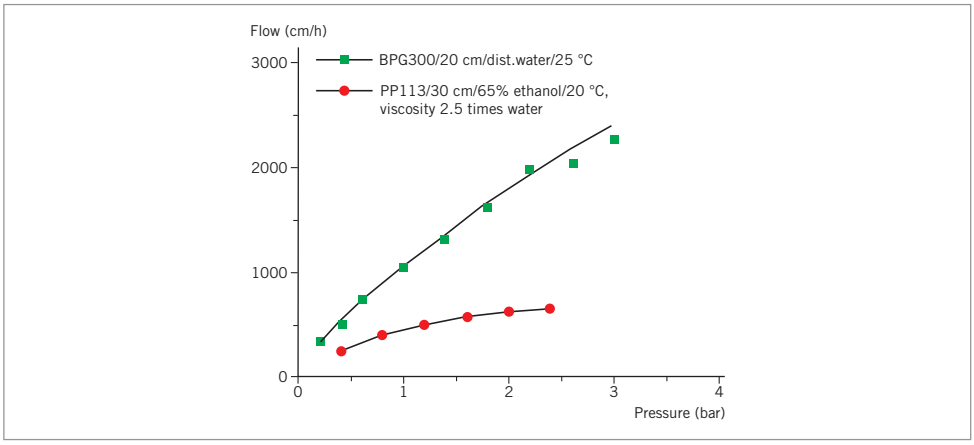


Fig. 67. Sepharose Big Beads allow high flow rates with high viscosity samples.

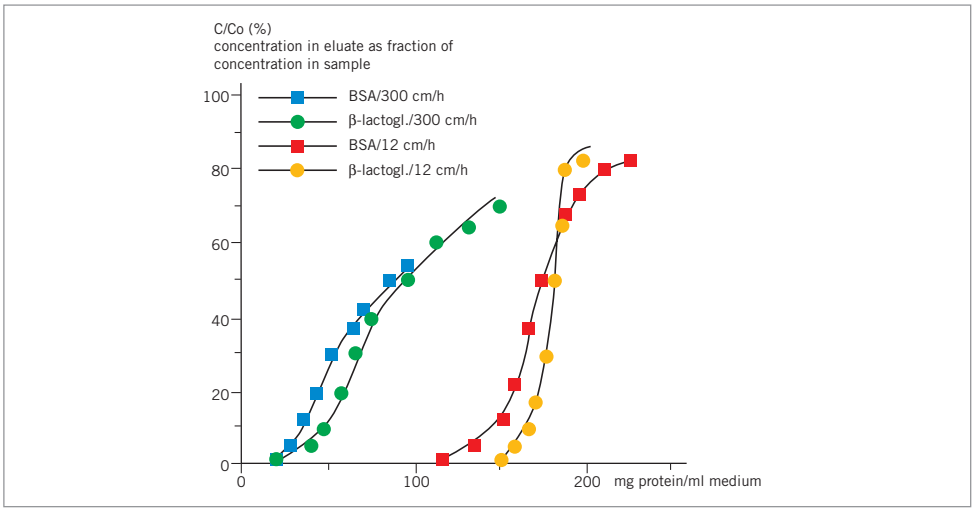


Fig. 68. Typical binding capacities of SP Sepharose Big Beads. Binding capacity measured in acetate pH 5 for bovine serum albumin and formate pH 4.1 for β-lactoglobulin at linear flows of 12 and 300 cm/h.

Purification options

Product	Binding capacity/ml medium	Recommended working flow*	Maximum flow*	Working pH range**	Maximum operating back pressure *** (MPa/psi) *1 MPa=10 bar*
Strong anion exchangers					
Q Sepharose Big Beads	tested for each specific application	up to 300 cm/h	1800 cm/h	2–12	0.3/43
Strong cation exchanger					
SP Sepharose Big Beads	tested for each specific application	up to 300 cm/h	1800 cm/h	4–13	0.3/43

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

***Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

****Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

For column packing during method development, particularly when handling crude, viscous samples:

XK 16/20	up to 30 ml	up to 15 cm
XK 26/20	up to 80 ml	up to 15 cm
XK 26/40	up to 196 ml	> 15 cm
XK 50/20	up to 274 ml	up to 14 cm
XK 50/30	up to 559 ml	up to 28.5 cm

Select a production scale column such as BPG or Chromaflow for larger volumes. Sepharose Big Beads can be packed in large scale columns by applying constant pressure between 1–3 bar, by slurry sedimentation followed by adaptor compression, or by suction packing. Follow the instructions supplied with the medium.

Performing a separation

Guidelines for selection of media, buffer, pH and ionic strength conditions and method optimization are given in Chapter 2. See Appendix 2 for recommendations on volatile and non-volatile buffer systems.

Correct sample and buffer preparation is essential in order to achieve optimal separation and avoid any deterioration in column performance. Refer to Chapter 2 and Appendix 1 for recommendations and advice.

Filter buffers *after* all salts and additives have been included. Use high quality water and chemicals. Filter solutions through 1 µm filters. To avoid formation of air bubbles in a packed column, maintain buffers and columns at a constant temperature before and during a run.

First time use or after long term storage

1. Wash with 5 column volumes of distilled water at 300 cm/h.
2. Wash with 5 column volumes of start buffer, same flow as step 1.
3. Wash with 5 column volumes of elution buffer, same flow as step 1.
4. Wash with 5 column volumes of start buffer, same flow as step 1.
5. Run a blank elution before applying sample.

Gradient or step elution

Conditions for a large scale purification using Sepharose Big Beads will be determined during method development and relate to the specific application. Refer to Chapter 2 for advice on optimizing a separation. Typical separation flow rates should be 200–500 cm/h.

If ionic detergents have been used, wash the column with 5 column volumes of distilled water, followed by 2 column volumes 2 M NaCl. Re-equilibrate with at least 10 column volumes of start buffer until the UV baseline, eluent pH and/or conductivity are stable. Organic solvents such as ethanol can be used to remove non-ionic detergents. When selecting an organic solvent, check the chemical stability of the medium to determine a suitable concentration.

Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 3.

Cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl at 40 cm/h for a contact time of 1–2 hours.
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until eluent pH and conductivity have reached the required values.

To remove precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins, refer to Appendix 1.

Media characteristics

Composition: sulfopropyl (SP) or quaternary amino (Q) groups coupled to highly cross-linked 6% agarose via chemically stable ether bonds.

Product	Functional group	pH stability*	Mean particle size
Q Sepharose Big Beads	$-CH_2N^+(CH_3)_3$	Long term: 2–12 Short term: 2–14	200 µm
SP Sepharose Big Beads	$-CH_2CH_2CH_2SO_3^-$	Long term: 4–13 Short term: 3–14	200 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 the 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

For daily use, Sepharose Big Beads media are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), with additives such as non-ionic detergents, 70% ethanol, 1 M acetic acid, 30% acetonitrile and 30% isopropanol.

Sepharose Big Beads can be used with organic solvents such as dimethylsulfoxide, dimethylformamide, tetrahydrofuran, acetone, chloroform, dichloromethane, dichloroethane and dichloroethane/pyridine (50:50) as well as polar solvents and aqueous/organic isolutions. The water in the medium can be exchanged by the alternative solvent with very little effect on the pore size of the matrix.

Avoid cationic detergents with SP Sepharose Big Beads. Avoid anionic detergents with Q Sepharose Big Beads. Avoid oxidizing agents.

Storage

For column storage, wash with 2 column volumes of distilled water followed by 2 column volumes of 20% ethanol. Include 0.2 M sodium acetate in the storage solution for SP Sepharose Big Beads. For small scale columns, degas the ethanol/water mixture thoroughly, for large scale columns ensure that an air trap is included before the column. Add storage solution at a low flow rate, checking the back pressure as the column equilibrates. Alternatively, store at neutral pH in buffer containing 20% ethanol or in 0.01 M NaOH.

Store at room temperature or, for long periods, store at +4° C to +8° C. Ensure that the column is sealed well to avoid drying out. Store unused media at +4° C to +30° C in 20% ethanol. Do not freeze.

Chapter 4
Ion exchange in a Purification Strategy (CIPP)

To ensure efficient, reproducible purification giving the required degree of purity, it is beneficial to develop a multi-step process using the purification strategy of Capture, Intermediate Purification and Polishing (CIPP), shown in Figure 69.

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. An important first step for any purification is correct sample preparation and this is covered in more detail in Appendix 1 and Chapter 2.

Ion exchange chromatography (IEX) plays a significant and highly flexible role in most multi-step purification schemes. If a specific affinity medium is not available or if little is known about the target molecule, IEX is recommended as the first step to consider for any purification. The technique can be used for capture, intermediate purification or polishing, according to the demands of the specific application. Since IEX offers different selectivities (using anion or cation exchangers) and since the pH of the purification can be modified to alter the charge characteristics of the sample components, it is possible to use the technique more than once in the same purification scheme. In addition IEX can be used with step-wise elution for a rapid capture step or with gradient elution to achieve the highest resolution in a polishing step.

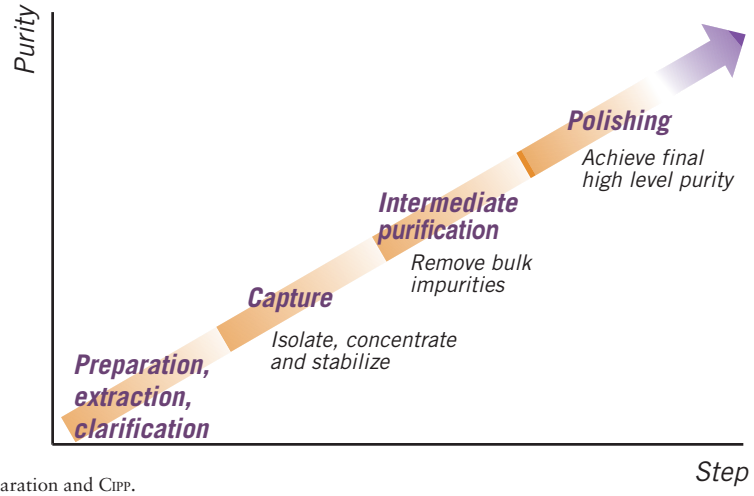


Fig. 69. Preparation and CIPP.

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. The objective is to *achieve final purity* by removing any remaining trace impurities or closely related substances.

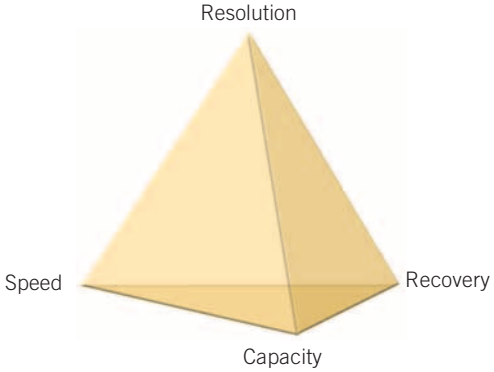
The optimal selection and combination of purification techniques for *Capture, Intermediate Purification and Polishing* is crucial for an efficient purification.

Selection and combination of purification techniques

Proteins are purified using purification techniques that separate according to differences in specific properties, as shown in Table 8.

Table 8. 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 and selectivity 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 9.

Table 9. 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 9).

Ammonium sulfate, often used for sample clarification and concentration (see Appendix 1), 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 70.

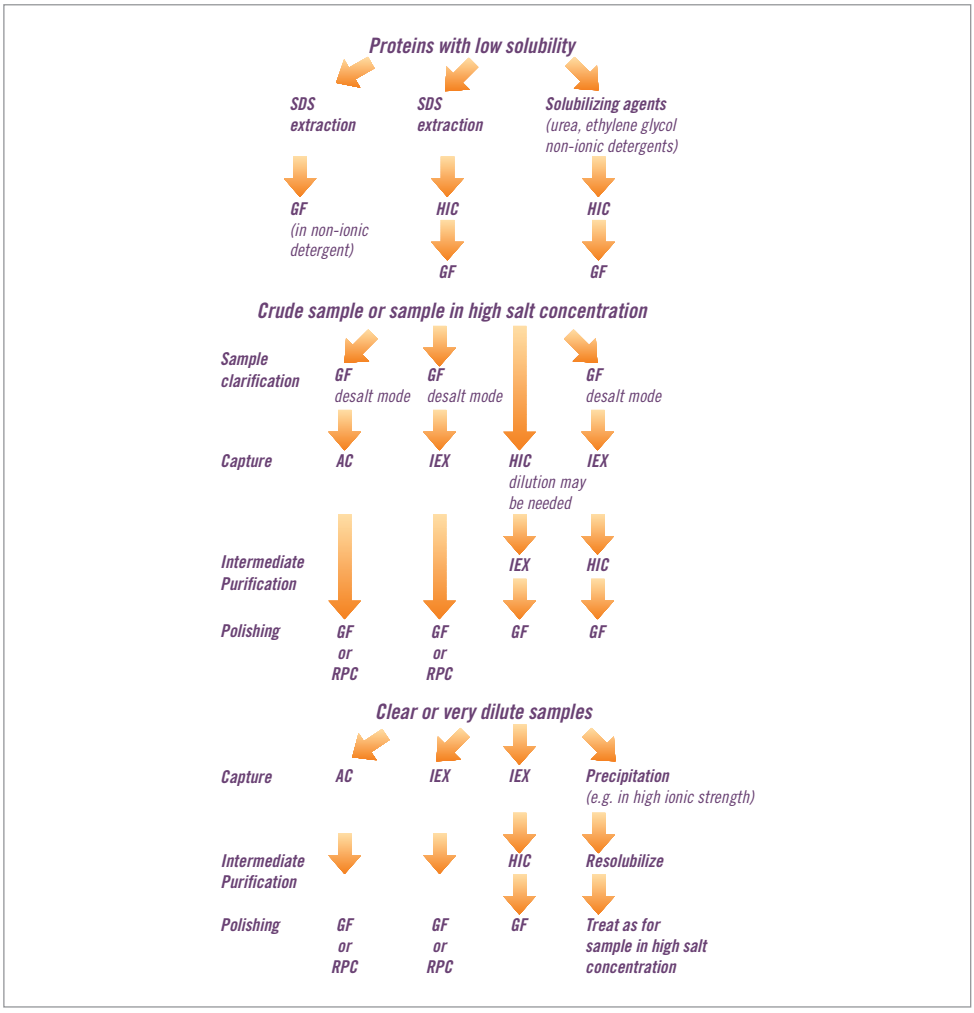


Fig. 70. Logical combinations of chromatography techniques.

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.

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

Ion exchange as a capture step

When IEX chromatography is used as a capture step, the objective is to quickly adsorb the protein(s) of interest from the crude sample and isolate them from critical contaminants such as proteases and glycosidases. The target protein(s) are concentrated and transferred to an environment which will conserve potency/activity. Removal of other critical contaminants may also be achieved by careful optimization of pH and elution conditions.

The focus is on capacity and speed in a capture step. It may be advisable to compromise on the potential resolution that can be achieved by an IEX separation in order to maximize the capacity and/or speed of the separation in this first step.

IEX media for capture steps should offer high speed and high capacity.

1. Sepharose Fast Flow (90 µm particle size) – good resolution for crude mixtures at any scale using flows up to 300 cm/h and offering a wide range of selectivities.
2. Sepharose XL (90 µm particle size) – high capacity, good resolution for capture of selected proteins at laboratory and process scale using flows up to 300 cm/h.
3. Sepharose Big Beads (200 µm particle size) – for viscous samples that preclude the use of IEX media with smaller particle size, using flows up to 300 cm/h, or for fast separations of very large sample volumes when resolution is of less importance, using flows up to 1000 cm/h.

If only milligram quantities of product are needed and the capture step will not be scaled up, use high performance media such as MonoBeads or MiniBeads according to the capacity required. Also, if the starting material is reasonably clean, a single step purification on MonoBeads may be sufficient to achieve required purity at laboratory scale.

Select start conditions that avoid adsorption of contaminants and so help to maximize the binding capacity for the target protein(s). This will facilitate a fast, simple step elution of the concentrated target protein(s).

Figure 71 shows optimization of a capture step used for purification of a recombinant enzyme, deacetooxycephalosporin C synthase (DAOCS). Since this enzyme is oxygen-sensitive, it was important to rapidly remove the most harmful contaminants from the relatively unstable target protein. The isoelectric point of DAOCS (pI = 4.8) made an anion exchanger the most suitable choice. Columns from the HiTrap IEX Selection Kit were screened to select the most suitable medium (results not shown) before optimizing the separation on a larger HiPrep 16/10 Q XL column. A detailed description of the entire purification process for DAOCS can be found in Application Note 18-1128-91, available from Amersham Biosciences.

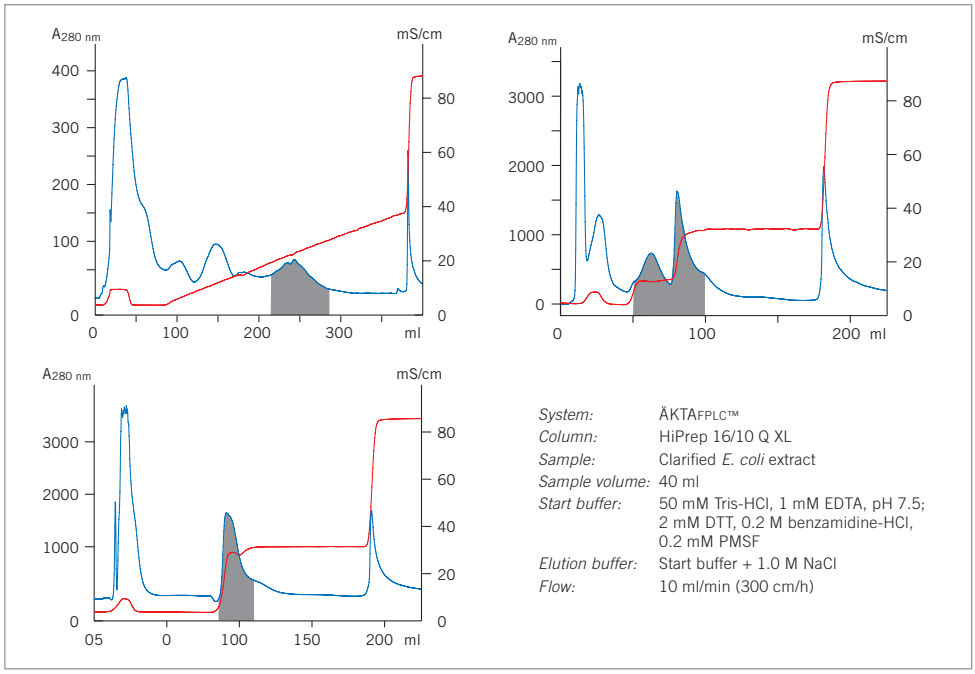


Fig. 71. Capture step using IEX and optimization of conditions. The elution position of DAOCS is shaded.

Ion exchange for intermediate purification

When IEX is used for intermediate purification, the objective is to remove most of the significant impurities such as proteins, nucleic acids, endotoxins and viruses.

In a typical intermediate purification step, speed is less critical since sample volume has been reduced and damaging contaminants have been removed during capture. Focus is on capacity and resolution in order to maintain productivity (amount of target protein processed per column in unit time) and to achieve as high selectivity (purity) as possible. Consequently, a gradient elution will usually be required.

Use a technique with a selectivity that is complementary to that used in the capture step.

IEX media for intermediate purification should offer high capacity and high resolution with a range of complementary selectivities:

1. Sepharose High Performance (34 µm particle size) – high resolution using flows up to 150 cm/h.
2. SOURCE 15 (15 µm particle size) – high throughput, high resolution for laboratory or large-scale applications using flows up to 1800 cm/h.
3. SOURCE 30 (30 µm particle size) – an alternative to SOURCE 15 for large-scale applications when flows up to 2000 cm/h can be used.
4. Sepharose Fast Flow (90 µm particle size) – fast separations, good resolution using flows up to 300 cm/h, broad range of selectivities.

If only milligram quantities are required and the intermediate purification step will not be scaled-up, use MonoBeads or MiniBeads according to the capacity required.

Optimize the selectivity of the medium to ensure high binding capacity and to maximize resolution. Use continuous gradient or multi-step elution conditions.

Figure 72 shows a process scheme developed for purification of epidermal growth factor (EGF), expressed as an extra-cellular product from *Saccharomyces cerevisiae*. As shown the scheme was developed and optimized at laboratory scale before being transferred to large scale production. Purity and yield were maintained when the process was scaled up (Figure 73). Starting material was a clarified supernatant supplied by Chiron-Cetus Corp., Emeryville, CA, USA. Q Sepharose High Performance provides a high resolution intermediate purification step after capturing the sample by HIC and prior to polishing by GF on Superdex 75 prep grade. Note the decrease in particle size to further improve resolution as the purification moves from a capture step using a Sepharose Fast Flow matrix to the Sepharose High Performance matrix.

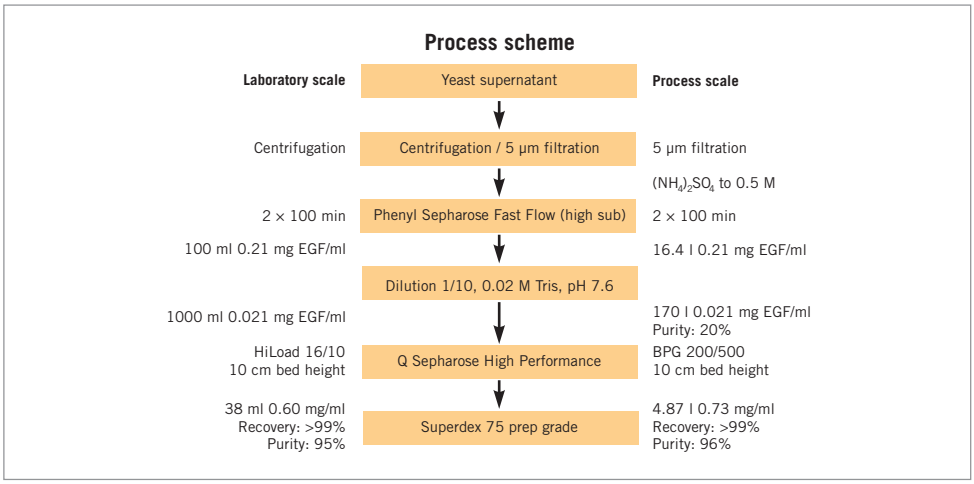


Fig. 72. Process scheme for purification of EGF.

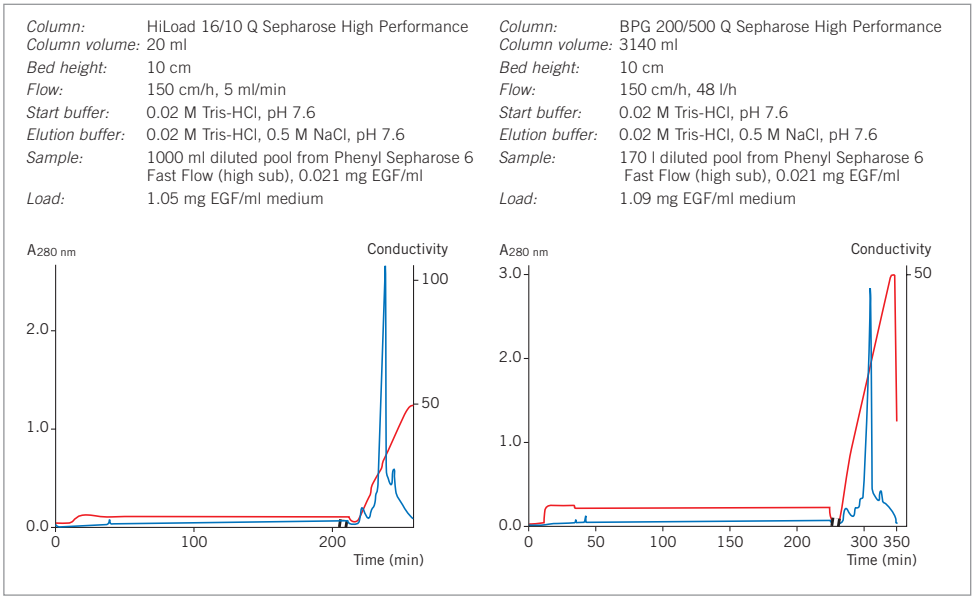


Fig. 73. Elution pattern, purity and yield are maintained when scaling-up from a HiLoad column to a BPG column.

Ion exchange as a polishing step

When IEX is used for polishing, most impurities have been removed except for trace amounts or closely related substances such as structural variants of the target protein, nucleic acids, viruses or endotoxins. The purpose of the separation is to reduce these variants and trace contaminants to acceptable levels for the application. In contrast to capture steps where a fast, high capacity, step elution is most commonly used, a polishing step will therefore focus on achieving the highest possible resolution. An example of this approach is shown in Figure 74 in which Mono S 5/50 GL was used to separate a recombinant DNA binding protein, transposase TniA, from minor contaminants remaining after partial purification by anion exchange and heparin affinity chromatography. The final product was >90% pure.

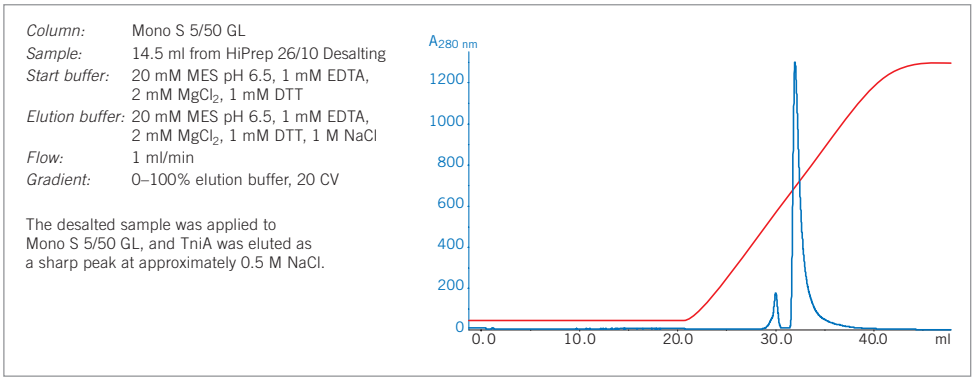


Fig. 74. High resolution cation exchange chromatography on Mono S 5/50 GL.

IEX media for **polishing** steps should offer highest resolution:

1. MiniBeads (3 µm particle size) – polishing at microscale when highest resolution is essential.
2. MonoBeads (10 µm particle size) – polishing at laboratory scale when highest resolution is essential and a higher capacity than MiniBeads is required.
3. SOURCE 15 (15 µm particle size) – rapid, high resolution polishing for laboratory or large scale applications using flows up to 1800 cm/h.
4. SOURCE 30 (30 µm particle size) – an alternative to SOURCE 15 for large scale applications when flows up to 2000 cm/h can be used.

Optimize the gradient elution to maximize selectivity. Use high efficiency media with small bead sizes to improve resolution.

Alternative techniques for polishing steps

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 75.

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

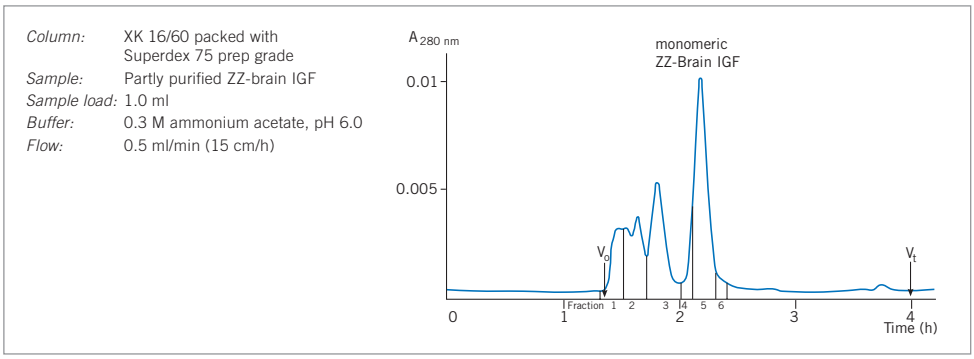


Fig. 75. Final polishing step: separation of dimers and multimers on Superdex 75 prep grade.

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.

Chapter 5
Chromatofocusing: principles and methods

Introduction

This chapter introduces the principles and methods of chromatofocusing, a chromatography technique that separates proteins according to differences in their isoelectric point (pI).

Use chromatofocusing as a polishing step for partially purified samples. The fewer components in the sample, the better the chance for a well-resolved separation of individual proteins. For specific applications, chromatofocusing can resolve molecules where pI values differ by as little as 0.02 pH units. This high resolving ability can be particularly useful for the separation of very similar substances.

Use chromatofocusing for high resolution, analytical separations.

Use chromatofocusing as a complementary technique when proteins have not been resolved by other chromatography techniques such as charge (using ion exchange), size or hydrophobicity.

As explained in Chapter 1, each protein has its own unique *pH versus net charge relationship* which can be visualized as a *titration curve*. This curve reflects how the overall net charge of the protein changes according to the pH of the surroundings. *The pI of each protein is the pH at which the protein has zero surface charge*. Proteins with different pIs can be separated by being passed through a chromatofocusing column (packed with *a specifically-designed medium*) while a pH gradient is generated on the column by *specifically-designed and matched amphoteric buffers*. Proteins elute in order of their isoelectric points, as shown in Figure 76.

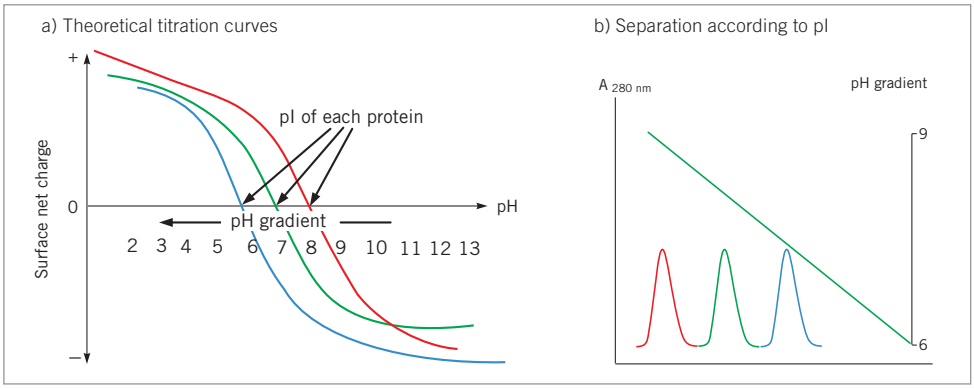


Fig. 76. Separating proteins according to their isoelectric points by chromatofocusing.

In order to achieve consistently high resolution, generation of the linear pH gradient requires an even buffering capacity over the entire pH range used for a separation. Hence the need for specifically designed buffers (Polybuffer 74, Polybuffer 96 or Pharmalyte™ 8–10.5) and media substituted with charged, buffering amines (Mono P™, PBE 118 or PBE 94).

Although chromatofocusing can be a high resolution technique, it is less suitable for the isolation of proteins that precipitate irreversibly at or near their isoelectric point since these proteins are likely to precipitate on the column if they reach a high enough concentration.

Separation mechanism: on-column generation of pH gradients and protein behavior

In order to separate proteins according to their different pIs, the chromatofocusing medium is equilibrated with start buffer at a pH slightly above the highest pH required. An elution buffer (adjusted to the lowest pH required) is passed through the column and begins to titrate the amines on the medium and the proteins. As the buffer flows through the column, the pH is lowered and a moving, *descending* pH gradient is generated (Figure 77).

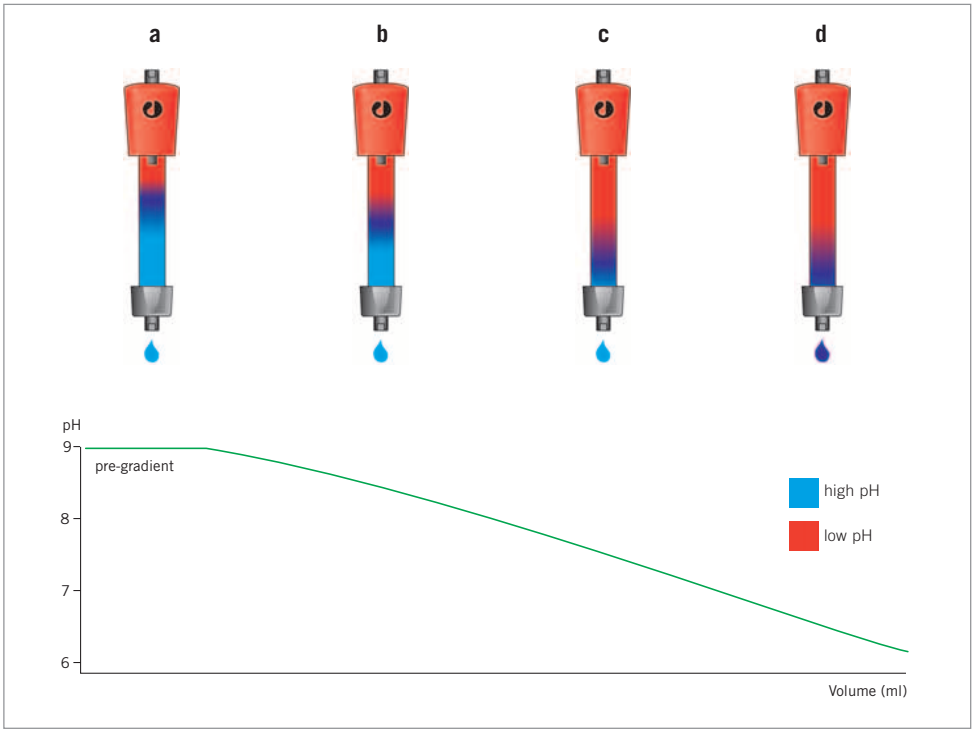


Fig. 77. Development of a pH gradient in a chromatofocusing column. The column is pre-equilibrated with start buffer at high pH (a) and elution with Polybuffer at low pH (b,c,d,) generates a descending linear pH gradient.

After a pre-gradient volume of elution buffer has passed, sample (in start buffer) is applied to the column. The proteins in the sample are titrated (pH adjusted) as soon as they are introduced into the column. Proteins in the sample that are at a pH *above* their pI are negatively-charged and retained near the top of the column (binding to the positively-charged amine groups). Any proteins that are at a pH *below* their pI begin to migrate down the column with the buffer flow and will not bind until they reach a zone where the pH is above their pI. This is the beginning of the separation process that is illustrated in Figure 78.

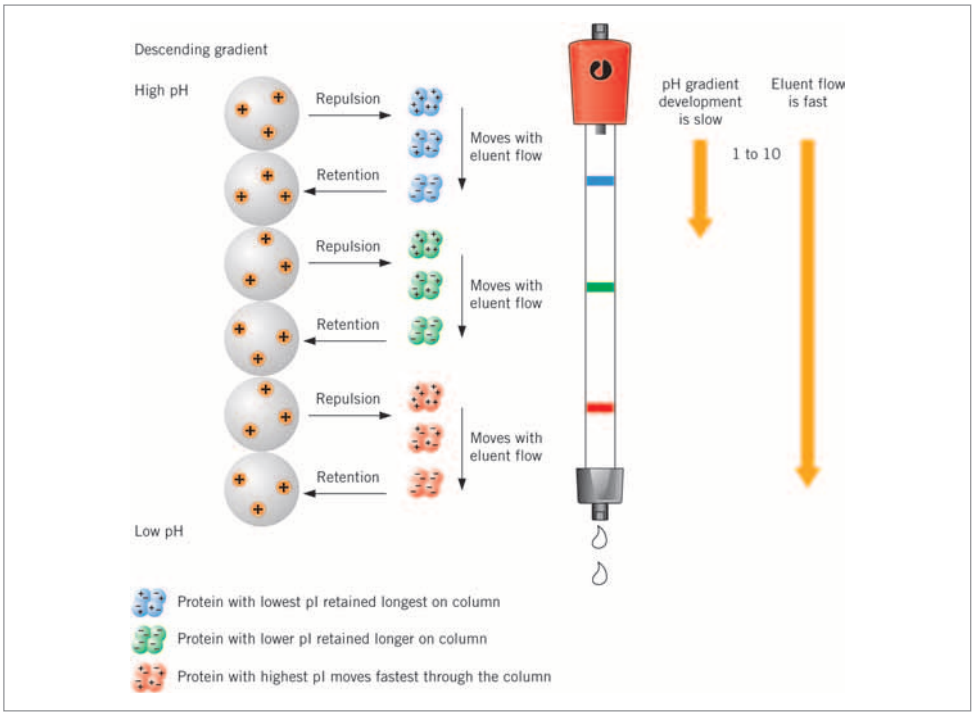


Fig. 78. Proteins with different pIs separate as they pass through the column. Molecules with the same isoelectric point are focused in narrow bands during the separation.

As the pH continues to decrease near the top of the column, any protein that drops *below* its pI becomes positively charged, is repelled by the positively-charged amine groups, and begins to migrate down the column with the elution buffer, travelling faster than the speed at which the pH gradient moves down the column. However, as the protein migrates down the column, the pH of the surroundings increases. When the protein reaches a zone in which the pH is *above* its pI, it becomes negatively charged and binds to the column again. The protein remains bound until the developing pH gradient reduces the local pH when, again, the protein drops below its pI, becomes positively charged, and begins to move down the column, catching up with the gradient. This process continues until the protein is eluted from the column at a pH near to its pI (when it has almost no net charge).

Figure 78 illustrates the *focusing effect* which takes place during a separation and contributes significantly to the high resolution achievable with chromatofocusing. In a descending pH gradient, a protein can exist in three charged states: positive, negative or neutral and is constantly changing its charged state as the pH gradient develops and the protein travels through different pH zones on the column. Molecules at the rear of a zone will migrate more rapidly than those at the front, gradually forming narrower bands of proteins, each band comprised of one or more proteins of the same isoelectric point.

Thus, during chromatofocusing, proteins with different pIs migrate at different rates down the column as the pH gradient develops, continually binding and dissociating while being focused into narrow bands and finally eluted. The protein with the highest pI elutes first and the protein with the lowest pI will elute last.

Media selection

Use prepacked, Mono P for fast separations (up to ten times faster than PBE).

Use Mono P 5/200 GL for highest resolution separations.

Use Mono P 5/50 GL rapid scouting of elution conditions (pH gradients) or for separations not requiring highest resolution.

Use PBE 94 for scaling up from Mono P. Note that, under the same separation conditions, resolution may be lower due to the longer diffusion times within larger particles. Re-optimization of the gradient and flow rate should give similar resolution.

Use PBE 118 when pH gradients above pH 9 are required.

Mono P is based on the same matrix as Mono Q and Mono S (see Chapter 3). The 10 µm MonoBeads particles are substituted with tertiary and quaternary amines and the small bead size contributes significantly to the high resolution that can be achieved.

Polybuffer exchangers, PBE 118 and PBE 94, are based on Sepharose CL-6B, a cross-linked agarose matrix. The charged secondary, tertiary and quaternary amine groups are coupled to monosaccharide units in the 90 µm Sepharose CL-6B particles by ether linkages. For all chromatofocusing media the charged groups have been selected to give an even buffering capacity across a broad pH range. The buffering capacity and titration curves for PBE 118, PBE 94 and Mono P are shown in Figures 79 and 80.

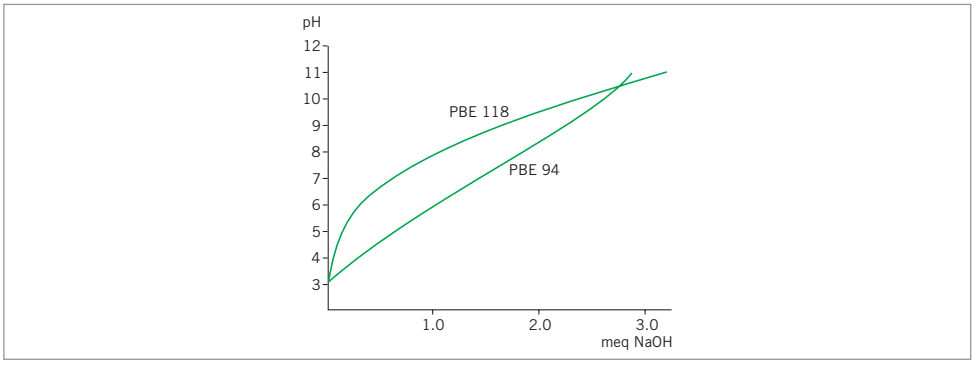


Fig. 79. Titration of 10 ml aliquots of PBE 118 and PBE 94 in 1 M KCl against NaOH shows the smooth buffering capacity of these media over a broad pH range.

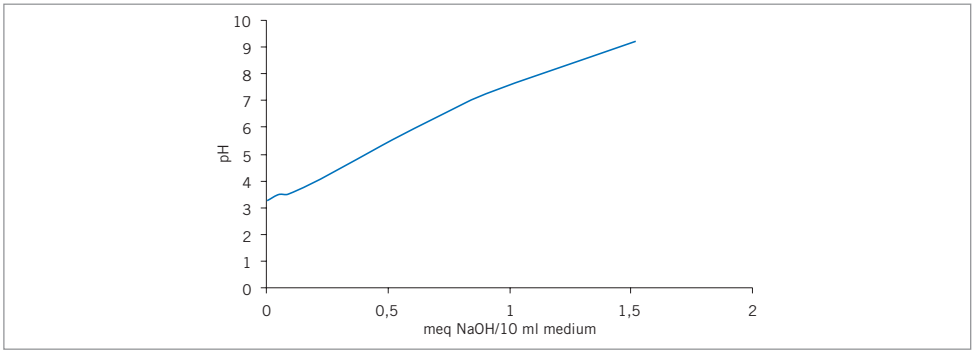


Fig. 80. Titration curve of Mono P column shows smooth buffering capacity.

Buffer selection

Use Polybuffer 74 for any pH gradient between 7 and 4. Use a pH gradient 7–4 when starting to work with proteins of unknown pI.

Use Polybuffer 96 for pH gradients that should begin above pH 7 e.g. when the proteins of interest have a pI above or close to 7.

Use Pharmalyte pH 8–10.5 when higher pH values are required.

Refer to section 'Selection of pH gradient and buffers' for further details.

Polybuffers are specifically designed to form pH gradients by titration of the charged groups on PBE 118, PBE 94 and Mono P media. Polybuffers are mixtures of selected amphoteric buffering substances of different pI and pKa values. Each mixture is designed to give an even buffering capacity across a wide pH range, in order to generate the required linear pH gradient. The titration curves for Polybuffer 96 and Polybuffer 74 are shown in Figure 81.

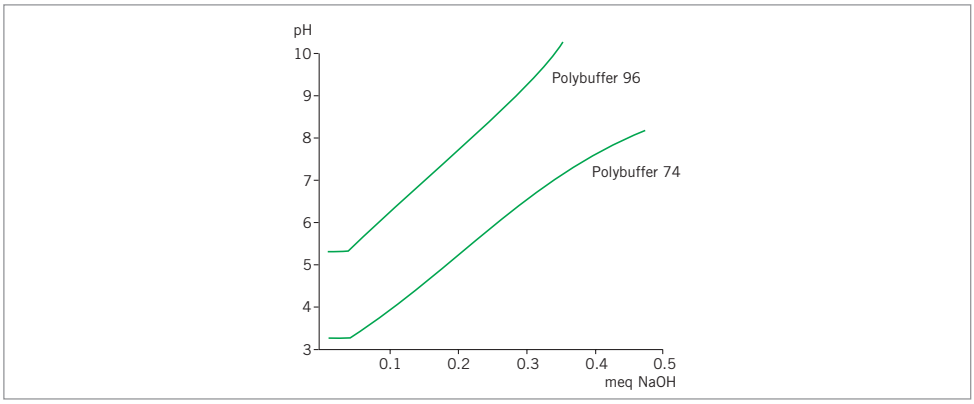


Fig. 81. Titration of 2 ml Polybuffer with 0.1 M NaOH.

Purification options

Product	Maximum loading capacity	Recommended operating flow*	Maximum flow**	Working pH range**	Maximum operating back pressure *** (MPa/psi) 1 MPa=10 bar
Mono P 5/50 GL	10 mg/column	0.5–1.5 ml/min (305–450 cm/h)	3.0 ml/min	4–9	4/580
Mono P 5/200 GL	40 mg/column	0.5–1.5 ml/min (305–450 cm/h)	2.0 ml/min	4–9	4/580
PBE 118	1–20 mg/ml medium	30–40 cm/h	115 cm/h	8–11	0.015/2
PBE 94	1–20 mg/ml medium	30–40 cm/h	115 cm/h	4–9	0.015/2

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

***Working pH range refers to the pH interval where the medium binds protein as intended or as needed for elution without adverse long term effects.*

****Maximum operating back pressure refers to the pressure above which the medium begins to compress.*

Buffer options

Buffer	pH range	Compatible with medium
Polybuffer 74	7–4	PBE 94 Mono P
Polybuffer 96	9–6	PBE 94 Mono P
Pharmalyte pH 8–10.5	11–8	PBE 118

Purification examples

High resolution

The potential for high resolution separation of proteins with otherwise similar properties makes chromatofocusing an excellent technique for the study of different isoforms. For example, haemoglobins can be separated into 4 distinguishable sub-groups, each with a different isoelectric point (Figure 82). The isoelectric points of sub-groups A and F differ by only 0.05 pH units, yet the peaks are well resolved. The focusing effect of the linear pH gradient and excellent peak symmetry (achieved by using small, uniform particles in a well-packed column) combine to give a high resolution separation.

Sample: Partially purified haemoglobins
Column: Mono P 0.5 cm × 20 cm
Start buffer: 0.025 M triethanolamine-methanesulfonic acid, pH 8.1
Flow: 1 ml/min (305 cm/h)
Elution buffer: Polybuffer 96-methanesulfonic acid, diluted in distilled water 1:16, pH 6.65

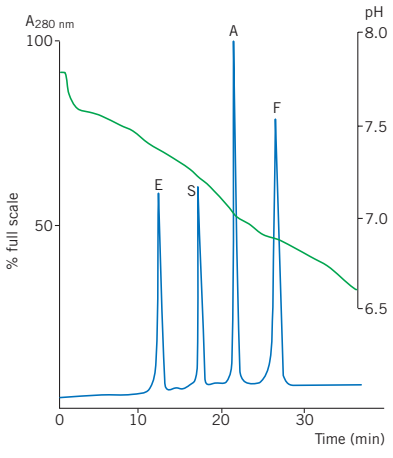


Fig. 82. Chromatofocusing of haemoglobin variants.

Under ideal conditions with a very well packed column, Polybuffer exchangers can give almost as good resolution as Mono P. Figure 83 shows an example in which model proteins that differ by as little as 0.02 pH units in their pI values have been resolved on a Polybuffer exchanger.

Sample: Whale myoglobin (2 mg), horse myoglobin (2 mg), carboxyhaemoglobin (2 mg)
Column: PBE 94, 1 cm × 18 cm bed height
Start buffer: 0.025 M ethanolamine, pH 9.5
Flow: 15 cm/h (0.2 ml/min)
Elution buffer: Polybuffer 96, pH 7, dilution 1:10 from stock buffer

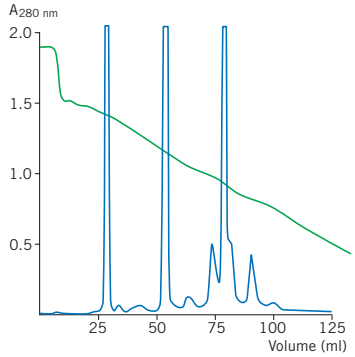


Fig. 83. Chromatofocusing of standard protein mixture.

High selectivity

Figure 84 demonstrates the high selectivity of chromatofocusing that enables detection of subtle changes in individual proteins. In this application researchers were able to follow the desialylation of transferrin as sialic acid groups were removed by neurominidase.

Sample: Iron-saturated diferric transferrin
Column: Mono P 0.5 cm × 20 cm
Start buffer: 0.025 M bis-Tris HCl, pH 6.45, 10% betaine hydrate, 3 mM $FeCl_3$
Flow: 1 ml/min (305 cm/h)
Elution buffer: Polybuffer 74 diluted 1:10 in distilled water, pH 5, 10% betaine hydrate, 3 mM $FeCl_3$

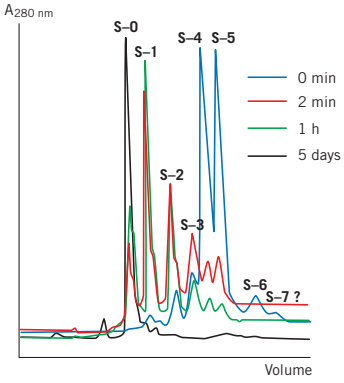


Fig. 84. Following the desialylation of transferrin.

Polishing step

Figure 85 shows an example of chromatofocusing being used for polishing in a purification strategy (in a polishing step most impurities have been removed and the objective is to achieve final purity by removing any trace impurities or closely related substances). In this example highly pure leukotriene A4 hydrolase was required for functional and structural studies. The enzyme was expressed as $(His)_6$-tagged LTA4 hydrolase in *E. coli*. After harvesting the cells and removing nucleic acids, nickel ion affinity chromatography was used as a specific protein capture step and the eluted fraction subjected to a polishing step on Mono P.

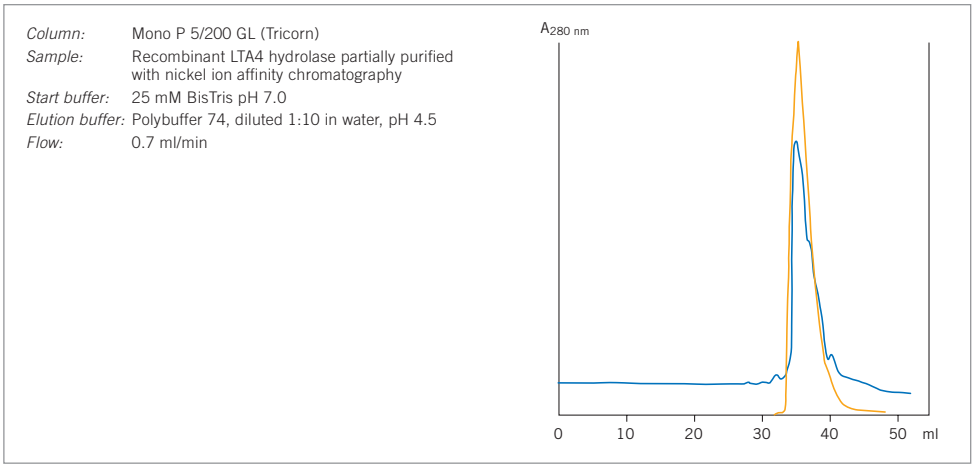


Fig. 85. Purification of leukotriene A4 hydrolase. Results courtesy of Eva Ohlson, Karolinska Institutet, Stockholm, Sweden.

Packing a column

Prepacked columns are likely to give the highest resolution and the most reproducible results, particularly with the MonoBead matrix used in Mono P 5/50 GL and Mono P 5/200 GL.

Efficient packing of a chromatofocusing column is critical to achieve the best results. Follow carefully the instructions for column packing given in Appendix 3 when packing PBE 94 or PBE 118.

Pack columns in the start buffer to be used for the separation (see Table 10, 11, 12 or 13) using long, narrow columns such as Tricorn 10/300. The amount of medium required will depend on the amount of sample, the nature of the sample and contaminants and the degree of resolution required. For most separations 20–30 ml Polybuffer exchanger will be sufficient to separate from 1–200 mg protein/pH unit in the gradient.

Degas the start buffer and the slurry in order to avoid air bubbles which can interfere with the separation.

Check column efficiency as described in Appendix 3. It may be worth checking the packing using a colored marker. Use bovine cytochrome c which is strongly repelled from the medium due to its high isoelectric point (pI=10.5). The progress of the protein band through the column can be visualized in order to check for any distortion caused by poor column packing or air bubbles.

Selection of pH gradient and buffers

No gradient forming equipment is required for chromatofocusing since the gradient is generated on the column as buffer and medium interact. The upper limit of the gradient is defined by the pH of the start buffer and the lower limit of the gradient is defined by the pH of the elution buffer. Polybuffers perform best over pH intervals of 3 pH units or less and the narrowest pH intervals are likely to give the highest resolution.

The *gradient volume* is defined by the strength of the elution buffer (low buffer concentrations give gradual pH changes and good separations between peaks). The optimal gradient volume may need to be determined by experimentation. Recommended gradient volumes and buffers are indicated in Tables 10, 11, 12 and 13. The *pre-gradient volume* is the volume of elution buffer that passes through the column before the pH begins to decrease, hence the total volume of buffer required is always greater than the gradient volume.

Start buffers: avoid the use of polyvalent buffers. Use monovalent or amphoteric buffers with a net charge > 0 and ≤ +1and ionic strength of 25 mM. Set the pH of the start buffer to 0.4 pH units above the desired pH in order to compensate for fluctuations in pH at the start of the run. These fluctuations are caused by slight differences in the conductivity of start and elution buffers.

Avoid buffers at concentrations below 25 mM as they will require larger gradient volumes with longer elution times and give broader, less concentrated protein peaks. More concentrated buffers would give steeper gradients, but could result in loss of resolution as peaks would be eluted closer together.

Gradients ending at pH 9 are not recommended when using Mono P since this medium has low buffering capacity above pH 9.0.

Gradients for proteins of known pI

Choose a pH interval so that the protein of interest is eluted after running 30–50% of the pH gradient. Narrow pH intervals will give optimal resolution.

Gradients for proteins of unknown pI

Since most proteins have isoelectric points in the range of 7–4, start with pH interval 7–4 on PBE 94 or Mono P. Proteins with a pI above 7 will pass through the column and can be collected and re-run at a higher pH if desired. Once a suitable pH range is established, select the most suitable pH gradient and compatible medium to achieve the optimal resolution.

Save time by using the shorter Mono P 5/50 GL column to test for a suitable pH gradient before transferring to Mono P 5/200 GL to achieve the highest resolution. Routine separations on Mono P 5/50 GL may also be satisfactory if highest resolution is not essential.

Table 10. Recommended buffer systems for broad pH intervals using Mono P 5/50 GL or Mono P 5/200 GL.

			Approx. volumes (ml)			
			Mono P 5/200 GL		Mono P 5/50 GL	
pH	Start buffer	Elution buffer (100 ml)	Total volume	Pre-gradient volume	Total volume	Pre-gradient volume
9–7	0.025 M diethanolamine, pH 9.5, HCl	1.0 ml Pharmalyte 8–10.5, 5.2 ml Polybuffer 96, pH 7.0, HCl	34	7	11	2
9–6	0.025 M diethanolamine, pH 9.5, HCl	10 ml Polybuffer 96, pH 6.0, HCl	34	9	19	2
9–6	0.075 M Tris, pH 9.3, CH_3COOH	10 ml Polybuffer 96, pH 6.0, CH_3COOH	30	3	17	2
8–6	0.025 M triethanolamine, pH 8.3, CH_3COOH	0.21 ml Pharmalyte 8–10.5, 9.0 ml Polybuffer 96, pH 6.0, CH_3COOH	37	7	15	4
8–5	0.025 M triethanolamine, pH 8.3, iminodiacetic acid*	3.0 ml Polybuffer 96, 7.0 ml Polybuffer 74, pH 5.0, iminodiacetic acid*	47	6	15	3
7–5	0.025 M bis-Tris, pH 7.1, HCl	10 ml Polybuffer 74, pH 5.0, HCl	26	3	13	3
7–4	0.025 M bis-Tris, pH 7.1, iminodiacetic acid*	10 ml Polybuffer 74, pH 4.0, iminodiacetic acid*	46	3	19	3
6–4	0.025 M bis-Tris, pH 6.3, HCl	10 ml Polybuffer 74, pH 4.0, HCl	39	3	16	3

**Use a saturated solution of iminodiacetic acid to adjust the pH of the buffer.*

Table 11. Recommended buffer systems for narrow pH intervals using Mono P 5/50 GL and Mono P 5/200 GL.

			Approx. volumes (ml)			
			Mono P 5/200 GL		Mono P 5/50 GL	
pH	Start buffer	Elution buffer (100 ml)	Total volume	Pre-gradient volume	Total volume	Pre-gradient volume
9–8	0.025 M diethanolamine, pH 9.4, HCl	1.0 ml Pharmalyte 8–10.5, 5.2 ml Polybuffer 96, pH 8.0, HCl	28	3	10	3
8.5–7.5	0.025 M Tris, pH 8.8, CH_3COOH	0.11 ml Pharmalyte 8–10.5, 9.5 ml Polybuffer 96, pH 7.5, CH_3COOH	29	4	10	4
8–7	0.025 M triethanolamine, pH 8.3, HCl	10 ml Polybuffer 96, pH 7.0, HCl	29	5	10	4
7.5–6.5	0.025 M methylimidazole, pH 7.6, CH_3COOH	10 ml Polybuffer 96, pH 6.5, CH_3COOH	27	9	11	6
7–6	0.025 M bis-Tris, pH 7.0, CH_3COOH	9.5 ml Polybuffer 96, 0.5 ml Polybuffer 74, pH 6.0, CH_3COOH	28	10	12	5
6.5–5.5	0.025 M bis-Tris, pH 6.7, CH_3COOH	4.0 ml Polybuffer 96, 6.0 ml Polybuffer 74, pH 5.5, CH_3COOH	23	5	9	3
6–5	0.025 M bis-Tris, pH 6.4, HCl	10 ml Polybuffer 74, pH 5.0, HCl	25	3	10	3
5.5–4.5	0.025 M piperazine, pH 6.3, HCl or iminodiacetic acid*	10 ml Polybuffer 74, pH 4.5, HCl or iminodiacetic acid*	24	3	10	3
5–4	0.025 M N-methylpiperazine, pH 5.7, HCl or iminodiacetic acid*	10 ml Polybuffer 74, pH 4.0, HCl or iminodiacetic acid*	27	7	11	3

**Use a saturated solution of iminodiacetic acid to adjust the pH of the buffer.*

Table 12. Recommended buffer systems for gradients with PBE 118.

pH*	Start buffer	Elution buffer	Dilution factor	Approx. volume (in column volumes)	
				Total volume	Pre-gradient volume
10.5–9	0.025 M triethylamine, pH 11, HCl	Pharmalyte 8–10.5, pH 9	1:45	-	-
10.5–8	0.025 M triethylamine, pH 11, HCl	Pharmalyte 8–10.5, pH 8, HCl	1:45	13	1.5
10.5–7	0.025 M triethylamine, pH 11, HCl	Pharmalyte 8–10.5, pH 7, HCl	1:45	13.5	2

Table 13. Recommended buffer systems for gradients with PBE 94.

pH	Start buffer	Elution buffer	Dilution factor	Approx. volume (in column volumes)	
				Total volume	Pre-gradient volume
9–8	0.025 M ethanolamine, pH 9.4, HCl	Pharmalyte 8–10.5, pH 8.0, HCl	1:45	12	1.5
9–7	0.025 M ethanolamine, pH 9.4, HCl	Polybuffer 96, pH 7.0, HCl	1:10	14	2
9–6	0.025 M ethanolamine, pH 9.4, CH_3COOH	Polybuffer 96, pH 6.0, CH_3COOH	1:10	12	1.5
8–7	0.025 M Tris, pH 8.3, HCl	Polybuffer 96, pH 7.0, HCl	1:13	10.5	1.5
8–6	0.025 M Tris, pH 8.3, CH_3COOH	Polybuffer 96, pH 6.0, CH_3COOH	1:13	12	3
8–5	0.025 M Tris, pH 8.3, CH_3COOH	Polybuffer 74 (70%) + Polybuffer 96 (30%) pH 5.0, CH_3COOH	1:10	10.5	2
7–6	0.025 M imidazole, pH 7.4, CH_3COOH	Polybuffer 96, pH 6.0, CH_3COOH	1:13	10	3
7–5	0.025 M imidazole, pH 7.4, HCl	Polybuffer 74 pH 5.0, HCl	1:8	14	2.5
7–4	0.025 M imidazole, pH 7.4, HCl	Polybuffer 74, pH 4.0, HCl	1:8	14	2.5
6–5	0.025 M histidine, pH 6.2, HCl	Polybuffer 74, pH 5.0, HCl	1:10	10	2
6–4	0.025 M histidine, pH 6.2, HCl	Polybuffer 74, pH 4.0, HCl	1:8	9	2
5–4	0.025 M piperazine, pH 5.5, HCl	Polybuffer 74, pH 4.0, HCl	1:10	12	3

Selection of counter-ions

As can be seen in the buffer tables, the most commonly used counter-ion is chloride. Other monovalent counter-ions can be used, but it is essential that these anions have a pKa at least two pH units below the lowest point of the chosen gradient.

Acetate is not recommended as a counter-ion for Polybuffer 74 because it has a high pKa. Multivalent counter-ions with a net charge below -1 are not recommended. Note that iminodiacetic acid is a multivalent ion.

Bicarbonate ions can result from the presence of atmospheric CO_2 or badly stored buffers. All high pH, amine-containing buffers adsorb atmospheric CO_2 and so generate bicarbonate ions which can disrupt a pH gradient by causing a fluctuation or plateau in the region of pH 5.5–6.5, depending on conditions.

These effects are most marked with Polybuffer 96 in pH gradients ending at pH 6, and can be minimized by using acetate as the counter-ion or setting the lower limit to pH 6.5. Adsorption of CO_2 can be minimized by storing solutions under nitrogen in tightly sealed bottles and at 3–8° C in the dark.

Buffer preparation

Use high quality water and chemicals. Filter buffers through 0.45 µm or 0.22 µm filters under vacuum to ensure that the solutions are thoroughly degassed. The presence of air bubbles in the column can significantly interfere with resolution.

Ensure that all buffer components are stored correctly to avoid adsorption of CO_2 (especially those containing amines). Use fresh Polybuffer from a previously unopened bottle whenever possible. Store previously opened solutions under nitrogen in tightly sealed bottles at +4 °C and in the dark to minimize adsorption of CO_2.

Prepare and use all buffers carefully at the same temperature to ensure correct pH and ionic strength. Use fresh buffers whenever possible. Buffers stored under refrigeration must reach the temperature at which they were prepared before running a separation.

The concentration of the start buffer is especially important when working at low ionic strength where microenvironments can vary by as much as 1 pH unit. Alterations in ionic strength may occur if readjustments to pH are made when buffers have been overtitrated with acid. Ensure that start and elution buffers are at the same low ionic strength in order to avoid large pH changes at the beginning or end of a pH gradient.

1. Select start and elution buffers from Tables 10, 11, 12 or 13 according to the pH gradient required and the medium to be used.
2. Calculate the required buffer volumes according to the column volume used. For the elution buffer, dilute Polybuffer and/or Pharmalyte in distilled water to approximately 95% of the final volume required.
3. Titrate buffers to the correct pH with the listed acid (e.g. 1–2 M or saturated iminodiacetic acid). Always perform the titration on a maximum volume before adding a few milliliters of water to reach the final volume.
4. When the final pH is reached, add distilled water to bring the total volume to 100 ml.

For shallower gradients within the same pH interval, prepare the elution buffer as normal, but dilute to a larger volume. Note that proteins elute with increased volumes when using diluted eluents so pre-gradient and total volumes also need to be increased.

Sample preparation

Correct sample preparation is extremely important. 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 medium. Appendix 1 contains an overview of sample preparation techniques.

Samples must be clear and free from particulate matter, particularly when working with Mono P. For small sample volumes a syringe-tip filter of cellulose acetate or PVDF can be sufficient for sample filtration.

If the pH of the sample is too low or different buffers are used, both gradient and resolution may be affected. Depending on the sample volume, use a HiTrap Desalting or HiPrep 26/10 Desalting column to remove high salt concentrations and/or transfer the sample into start buffer, see page 156. The pH of the sample is not critical if the buffer concentration of the sample is very low. It may be possible to simply dilute the sample if pH and final volume are not critical.

Since chromatofocusing is a binding technique, the volume of the sample is not significant as long as all of the sample can be applied and focused before the proteins of interest begin to elute. As a general rule, do not load a sample volume that is greater than half of the total column volume.

Performing a separation

Recommended flow: 0.5–1.5 ml/min, 305–450 cm/h (Mono P 5/50 GL, Mono P 5/200 GL), 30–40 cm/h (PBE 118, PBE 94).

Start and elution buffers: see Tables 10, 11, 12 or 13.

Monitor eluent at 280 nm since Polybuffers absorb at wavelengths below 280 nm. Monitor pH throughout the run as fluctuations can occur that may affect the separation, for example, due to the presence of CO_2 in the buffer.

Using a column for the first time or after long term storage:

1. Inject 0.5 column volumes 5 M NaOH
2. Equilibrate with start buffer until the buffer leaving the column is at the same pH as the start buffer.
3. Apply 0.5 column volumes 2 M salt solution containing the same anion as used in the acid for pH titration of the start buffer.
4. Re-equilibrate the column with start buffer until eluent leaving the column is at the start pH.

To check for pH fluctuations or other disturbances caused by incorrect buffer conditions or contaminants, consider performing a blank run using elution buffer (total volume as indicated in Tables 10, 11, 12 or 13).

Using a column for repeated runs:

1. Equilibrate with start buffer until buffer leaving the column is at the same pH as the start buffer. See Table 10, 11, 12 or 13 for recommended pre-gradient volumes.
2. Adjust sample to pH of start buffer or exchange sample into start buffer using, for example, a HiTrap Desalting column, see page 156.
3. Apply elution buffer. Note that there is a pre-gradient volume that will be eluted before the pH gradient begins (see Tables 10, 11, 12 or 13 for typical pre-gradient volumes).
4. Apply sample.
5. Apply elution buffer using gradient volumes as indicated in Tables 10, 11, 12 or 13. Check for pH fluctuations or other disturbances during elution.
6. Wash with 2 column volumes 2 M NaCl to elute any material still bound to the column.
7. Re-equilibrate with 5 column volumes start buffer until UV absorbance and pH/conductivity values are stable.

To maintain a clean column, inject 0.5 column volumes of 1 M NaOH every tenth run (or more frequently if required). The injection of NaOH can be followed by 0.5 column volumes of 75% acetic acid.

If the fractions are to be analyzed by reversed phase chromatography, Polybuffer may interact with the pairing ions used during the run. If the pairing ions are non-hydrophobic Polybuffer will elute in the void volume and the retained samples can be eluted with a gradient of organic solvent. However, if the pairing ions are very hydrophobic, Polybuffer will be retained on the column and give an absorption peak at wavelengths below 280 nm during an elution.

If organic solvents must be used, note the following:

- Before running a column, check the solubility of sample and all buffers.
- The pH interval required may be altered since the pKa values of the charged groups in the buffers, Polybuffer and chromatofocusing medium will be increased.
- Perform a blank gradient to ensure maintenance of a linear pH gradient. Linearity is likely to vary more at the high and low end of a pH interval 9–4. Adjust to the highest pH in the useful buffering range of the start buffer substance and adjust the elution buffer to approximately 0.5 pH units higher than recommended in the buffer Tables 10, 11, 12 or 13.

Optimization

- If results suggest that there may be a problem of sample solubility during the separation (see Troubleshooting) include additives such as betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) in the start and elution buffers to improve solubility. These additives should not affect the separation, but may need to be removed at a later stage thus adding an extra step to the purification strategy.
- To improve resolution:
  - –dilute Polybuffer (up to 1:20), but note that this will increase the pre-gradient and total gradient volumes so the time at which components elute will change and the volume of an eluted peak may increase
  - –decrease sample load
  - –decrease flow (flow rates as low as 0.25 ml/min have been used successfully on Mono P columns)
- To improve selectivity, use a shallower gradient (increase gradient volume), but note that this will result in broader peaks because each pH interval will occupy a larger space on the column. However, the resolution within a pH interval will increase.
- To change selectivity, use alternative buffers, salts or counter-ions.

If resolution is satisfactory, it may be possible to increase flow rate in order to speed up the separation. Flow rates up to 1.5 ml/min have been used successfully with Mono P columns without loss of resolution. Figure 86 shows the effect of increasing flow on the separation of a test mixture of proteins on PBE 118.

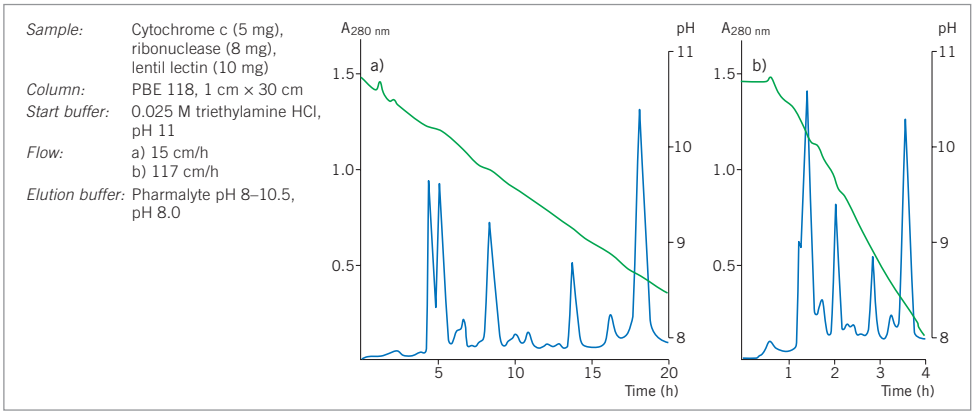


Fig. 86. Effect of flow on resolution during chromatofocusing.

Troubleshooting

A common difficulty encountered during chromatofocusing is that some proteins precipitate when they reach high concentrations at or near their isoelectric point. On-column precipitation causes symptoms such as increased back pressure, apparent loss of sample and even blockage of the column.

To avoid precipitation it may be possible to reduce the sample load so that the proteins do not reach sufficiently high concentrations, include additives such as betaine to improve sample solubility or to alter the pH range of the separation to avoid precipitation. Alternatively, remove the proteins causing the problem by using another chromatography technique before chromatofocusing.

If the protein of interest precipitates reversibly, then it will elute later than expected. If the protein of interest precipitates irreversibly at its isoelectric point, then chromatofocusing is not a suitable technique for purification.

A second factor that can easily affect results is the linearity of the pH gradient. The presence of CO_2, especially in the start buffer, can distort the pH gradient and disrupt the separation. It is essential to monitor the pH gradient throughout the run to ensure a linear pH gradient and a satisfactory separation. Figure 87 demonstrates the significant effect that excess CO_2 can have on the formation of a pH gradient. Store buffers under nitrogen or argon (to minimize the adsorption of CO_2 which occurs with high pH solutions) and always degas before use.

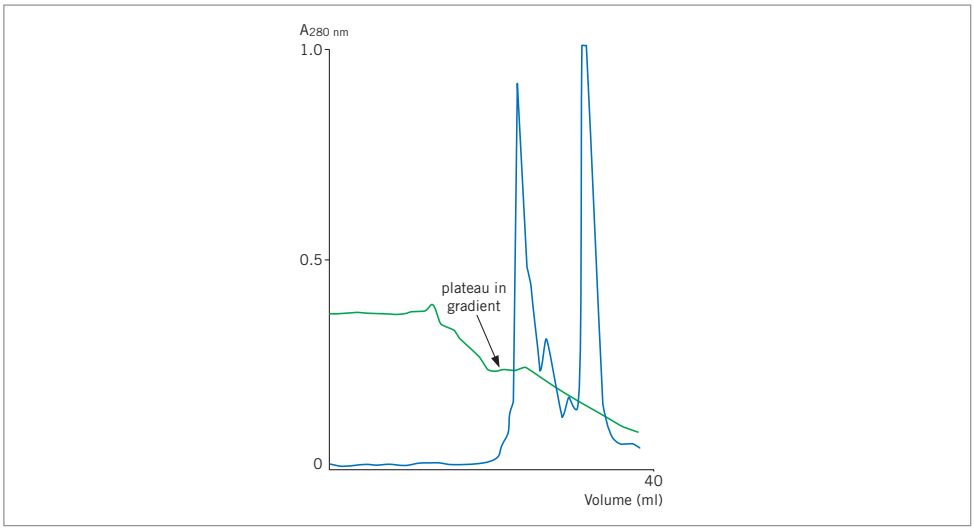


Fig. 87. A plateau in a pH gradient during chromatofocusing caused by excess CO_2 in the start buffer.

These difficulties and others that may occasionally be encountered during chromatofocusing are listed in the troubleshooting table that follows.

Table 14. Troubleshooting.

Situation	Cause	Remedy
Reduced or no flow through the column.	Outlet closed or pumps not working.	Open outlet. Check pumps for signs of leakage (if using a peristaltic pump, check tubing also).
	Blocked filter, end-piece, adaptor or tubing.	Remove and clean or replace if possible.
	Lipoproteins or protein aggregates have precipitated.	Remove lipoproteins and aggregates during sample preparation (see Appendix 1). Follow cleaning procedures, Appendix 10.
	Proteins are precipitating at their isoelectric point.	Follow cleaning procedures, Appendix 10. Reduce sample load. Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers. Change pH range to avoid precipitation. If protein contaminants are precipitating, remove with an alternative technique before chromatofocusing.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10.
Column does not reach start pH during equilibration.	-	Inject 4 column volumes 2 M NaOH or a higher concentration of start buffer, re-equilibrate with start buffer.
Blank gradient does not reach desired pH.	Incorrect eluent composition.	Check eluent pH. Prepare fresh eluent.
	Eluent contains CO_2.	Degas all buffers, use fresh Polybuffer and restart.
Peaks poorly resolved.	Gradient fluctuates, eluent contains CO_2.	Degas all buffers, use fresh Polybuffer and restart.
	Gradient fluctuates, incorrect counterion.	Use recommended counterion.
	Protein precipitation	Follow cleaning procedures, Appendix 10. during separation. Reduce sample load. Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers. Change pH range to avoid precipitation. If protein contaminants are precipitating, remove with an alternative technique before chromatofocusing.
	Sub-optimal elution conditions.	See optimization, page 143.
	Large mixing spaces in or after column.	Reduce all post-column volumes.
	Column poorly packed.	Check column efficiency (see Appendix 1). Repack if needed. Use prepacked columns.
	Lipoproteins or protein aggregates have precipitated.	Remove lipoproteins and aggregates during sample preparation (see Appendix 3). Follow cleaning procedures, Appendix 10.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10.
Protein does not bind.	Column not equilibrated sufficiently in start buffer.	Check that pH of eluent matches pH of start buffer. Prolong equilibration step if necessary.
	Start pH too low.	Increase pH of start buffer.
	Ionic strength of eluent too high.	Check conditions against recommended buffer composition.
	Ionic strength of sample too high.	Use a desalting column e.g. HiTrap Desalting to remove excess salt (see page 156). Sample dilution may be an alternative within required limits for pH and volume.

continues on following page

Situation	Cause	Remedy
	Column is contaminated with lipoproteins or proteins.	Change or clean filter. Follow cleaning procedures, Appendix 10.
Proteins do not bind or elute as expected.	Sample has changed during storage.	Prepare fresh sample.
	Sample or buffer conditions are different from previous runs.	Check sample and buffer conditions.
	Microbial growth has occurred in the column.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10.
Protein elutes at a lower than expected pH.	Sample precipitating during separation.	Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers. Change pH range to avoid precipitation.
Protein of interest binds, but does not elute.	Incorrect eluent composition.	Check pH of eluent after the run.
	Precipitation during separation.	Follow cleaning procedures, Appendix 10. pH of elution buffer is too high, use lower pH range. Reduce sample load. Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers.
Low recovery of activity, but normal recovery of protein.	Protein may be unstable or inactive in the buffer.	Determine 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.
Protein yield lower 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 precipitation	Follow cleaning procedures, Appendix 10. during separation. Reduce sample load. Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers. Change pH range to avoid precipitation.
	Polybuffer interfering with Lowry protein assay.	Remove Polybuffer, page 148 or use a Bradford assay.
More sample is recovered than expected.	Different assay conditions used before and after the chromatographic step.	Use same assay conditions for all assays.
	Polybuffer interfering with Lowry protein assay.	Remove Polybuffer, page 148 or use a Bradford assay.
	Protein co-eluting with other substances.	Optimize conditions to improve resolution. Check buffer conditions used for assay before and after the run.
More activity is recovered than was applied to the column.	Different assay conditions used before and after the chromatography step.	Use same assay conditions for all assays.
	Removal of inhibitors during separation.	

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Situation	Cause	Remedy
Back pressure increases during a run or during successive runs.	Precipitation of protein in the column filter and/or at top of the bed.	Follow cleaning procedures, Appendix 10. Reduce sample load. Add betaine (10% w/v), taurine (4% w/v) or glycerol (1–2%) to buffers. Change pH range to avoid precipitation. If protein contaminants are precipitating, remove with an alternative technique before chromatofocusing.
	Bed compressed.	If possible, repack column or use a new column.
	Microbial growth.	Store in the presence of 20% ethanol to prevent microbial growth when not in use. Always filter buffers. Follow cleaning procedures, Appendix 10.
	Turbid sample.	Improve sample preparation (see Appendix 1). Improve sample solubility: add betaine (max. 10% w/v at 25 °C), taurine (max. 4% w/v at 25 °C, below pH 8.5) or glycerol (1–2%). Or for hydrophobic samples, add ethylene glycol, urea, detergents or organic solvents*.
Air in the column.	Buffers not properly degassed. Column packed or stored at cool temperature and then warmed up.	Take special care if buffers are used after storage in a fridge or cold-room. Do not allow column to warm up due to sunlight or heating system. Degas buffers thoroughly. Flush column with distilled water and wash with 20% ethanol.
Cracks in the bed.	Large air leak in column.	Check all connections for leaks. Repack column if possible (see Appendix 3).
Unexpected peaks or spikes in chromatogram.	Buffer impurities.	Clean the buffer by running it through a precolumn. Use high quality reagents.
	Air bubble trapped in UV monitor flow cell.	Use degassed buffers.
Peaks appear on blank gradients.	Incomplete elution of previous sample.	Wash the column according to recommended methods.
Distorted peaks.	Uneven temperature distribution on the column.	Remove from any heat source e.g. sunlight through a window.

**Polar organic solvents such as methanol, ethanol and acetonitrile can be used at concentrations from 0–20%, but this may cause some proteins to lose their biological activity. The presence of organic solvents may also affect the low end of a linear pH gradient.*

Removing Polybuffer

For most practical applications it is not necessary to remove Polybuffer since the amount that elutes with any sample is extremely low. Polybuffers do not interfere with enzyme assays or amino acid analysis, but they may interfere with certain protein assays such as Lowry.

Polybuffers can be removed from protein samples using a gel filtration medium with a suitable fractionation range. Figure 88 shows separation of Polybuffer and protein using either Sephacryl™ S-100 HR or Superdex 75. In this example Sephacryl S-100 gives the best separation between the protein myoglobin and Polybuffer. It is recommended to follow any separation by monitoring the absorbance of Polybuffer (A_{215} nm) as well as the absorbance of the eluting protein (A_{280} nm, A_{254} nm or A_{215} nm) in order to optimize the running conditions and ensure effective separation. For practical and theoretical information on gel filtration chromatography, refer to the handbook *Gel Filtration–Principles and Methods* available from Amersham Biosciences.

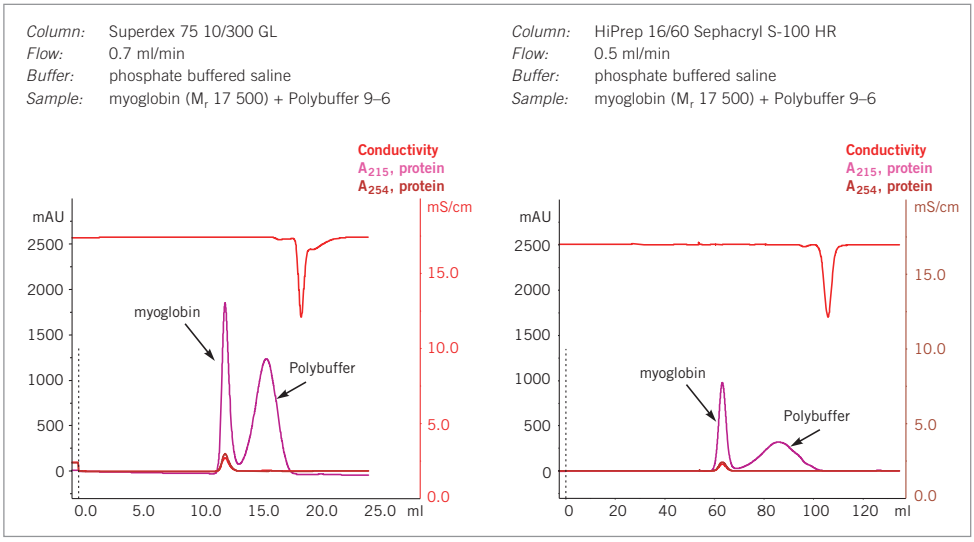


Fig. 88. Separation of Polybuffer from protein using gel filtration.

Cleaning

Mono P 5/50 GL, Mono P 5/200 GL, PBE 94, PBE 118

Since certain proteins may precipitate at or near their isoelectric point, blockage of the top filter on a chromatofocusing column is often the most common reason for an increase in back pressure. Reverse the flow direction and run through 2 column volumes of elution buffer at 0.5 ml/min (Mono P) or 30 cm/h (PBE). Return to normal flow direction, run through 5 column volumes of elution buffer.

To remove severe contamination (often indicated by an increase in column back pressure) proceed as follows:

Reverse flow direction and run the following sequence of solutions at a flow rate of 0.25–0.50 ml/min.

1. Wash with 4 column volumes 1 M NaCl.
2. Rinse with 2 column volumes distilled water.
3. Wash with 4 column volumes 1 M NaOH.
4. Rinse with 2 column volumes distilled water.
5. Wash alternately with 0.5 column volumes 0.1 M HCl (PBE) or 1 M HCl (Mono P) and 2 column volumes distilled water until the elution profile is constant.
6. Wash with 4 column volumes 1 M NaCl.
7. Reverse flow direction and re-equilibrate column in start buffer.

If back pressure remains high, change top filter.

Save time by monitoring any cleaning procedure to check for elution of contaminants. Depending on the nature of the contaminants, the following cleaning solutions can also be used: 100% isopropanol, 20% acetonitrile, 2 M NaOH, 75% acetic acid, 20% ethanol, 100% methanol or up to 6 M guanidine hydrochloride, cationic or non-ionic detergents. Always rinse with at least 2 column volumes of distilled water after using any of these cleaning solutions. When using organic solvents, wash the column using sawtooth gradients e.g. run from 0–100% solvent in 5 column volumes then from 100–0% in 5 column volumes, including 1% trifluoroacetic acid in the water and organic solvent.

If column performance is still not restored, inject a solution of 1 mg/ml pepsin in 0.1 M acetic acid containing 0.5 M NaCl and leave overnight at room temperature or one hour at 37 °C. Depending on the contaminant, other enzymes may also be used, *e.g.* DNase. After any enzymatic treatment, repeat the steps to remove severe contamination described previously.

Media characteristics

Product	Functional group	pH stability*	Mean particle size
Mono P 5/50 GL	Tertiary and quarternary amines	Long term: 2–12 Short term: 2–14	10 µm (monosized)
Mono P 5/200 GL	Tertiary and quarternary amines	Long term: 2–12 Short term: 2–14	10 µm (monosized)
PBE 118	Tertiary and quarternary amines	Long term: 3–13 Short term: 2–14	90 µm
PBE 94	Tertiary and quarternary amines	Long term: 2–12 Short term: 1–14	90 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 the 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

Mono P 5/50 GL, Mono P 5/200 GL

Mono P is stable in all commonly used, aqueous buffers in the range of pH 2–12, and in the presence of additives such as denaturing agents (8 M urea or 6 M guanidine hydrochloride) and non-ionic or cationic detergents.

Avoid oxidizing agents and anionic detergents.

PBE 94, PBE 118

Polybuffer exchangers are stable in all commonly used, aqueous buffers in the range of pH 3–12 and compatible with urea and other strong dissociating agents.

Avoid oxidizing agents and anionic detergents.

Storage

Mono P 5/50 GL, Mono P 5/200 GL, PBE 94, PBE pH 8–10.5

If the column is to be stored for more than two days after use, wash with 5 column volumes of distilled water and 5 column volumes of 20% ethanol.

Polybuffer 96, Polybuffer 74, Pharmalyte 118

Store at 3–8 °C in the dark, preferably under nitrogen. Avoid microbial contamination. To minimize adsorption of CO_2, store solutions under nitrogen in tightly sealed bottles after use.

Never store chromatofocusing media in 1 M HCl or 1 M NaOH. Avoid solutions containing charged groups.

Chromatofocusing and CIPP

Chromatofocusing can give high resolution of quite complex mixtures, but best results will be obtained when working with samples containing few components.

Chromatofocusing is therefore best suited as a polishing step in a purification strategy (see Figure 85) when high resolution of similar components is required and the majority of contaminants have been removed. Refer to Chapter 5 for details on CIPP, a strategic approach to purification.

Appendix 1

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 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.
- 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.

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	30 or 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 on page 156.

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 volatilen 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 89.

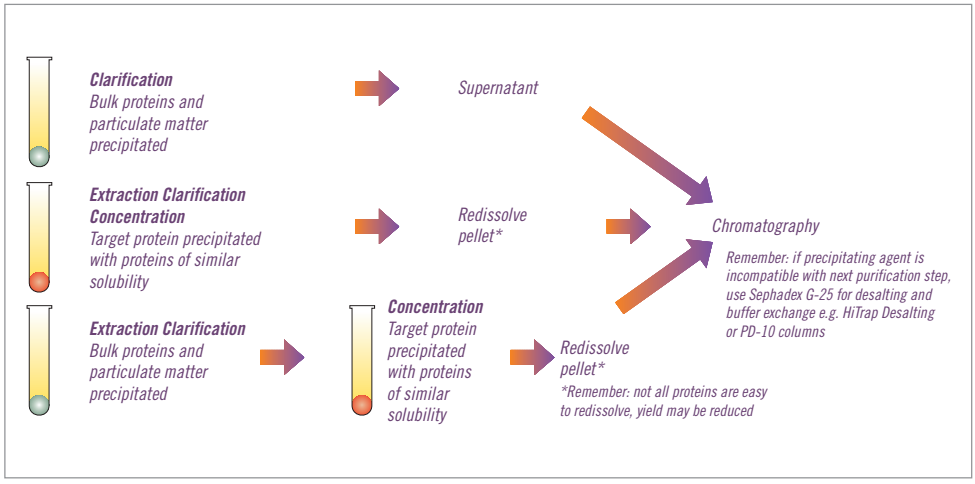


Fig. 89. Three ways to use precipitation.

Examples of precipitation agents are reviewed in Table 15. The most common precipitation method using ammonium sulphate is described in more detail.

Table 15. Examples of precipitation techniques.

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulphate	As described below.	> 1 mg/ml proteins especially immuno-globulins.	Stabilizes proteins, no denaturation, supernatant can go directly to HIC. Helps to reduce lipid content.
Dextran sulphate	Add 0.04 ml 10% dextran sulphate 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 sulphate.
Polyethylene glycol (PEG, M_r > 4000)	Up to 20% w/vol	Plasma proteins.	No denaturation, supernatant goes directly to IEX or AC, complete removal may be difficult. Stabilizes proteins.
Acetone (cold)	Up to 80% vol/vol at ±0 °C. Collect pellet after centrifugation at full speed in an Eppendorf™ centrifuge.		May denature protein irreversibly. Useful for peptide precipitation or concentration of sample for electrophoresis.
Polyethyleneimine	0.1% w/v		Precipitates aggregated nucleoproteins.
Protamine sulphate	1% w/v		Precipitates aggregated nucleoproteins.
Streptomycin sulphate	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 sulphate precipitation

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

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

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

Solutions needed for precipitation:

Saturated ammonium sulphate solution (add 100 g ammonium sulphate 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 sulphate 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 sulphate 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 sulphate is removed during clarification/buffer exchange steps with Sephadex G-25, using desalting columns (see page 156).

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

The quantity of ammonium sulphate required to reach a given degree of saturation varies according to temperature. Table 16 shows the quantities required at +20 °C.

Table 16. Quantities of ammonium sulphate 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 sulphate to add (grams) per liter of solution at +20 °C**																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

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 17 gives examples of common denaturing agents.

Table 17.

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 sulphate	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.

Buffer exchange and desalting

Dialysis is frequently mentioned in the literature as a technique to remove salt or other small molecules and to exchange 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, to perform a 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.

Desalting columns are used not only to remove low molecular weight contaminants, such as salt, but also for buffer exchange before or after different chromatographic steps and for the rapid removal of reagents to terminate a reaction.

Sample volumes up to 30% of the total volume of the desalting column can be processed. Sample concentration does not influence the separation as long as the concentration of proteins does not exceed 70 mg/ml when using normal aqueous buffers. The sample should be fully dissolved. Centrifuge or filter to remove particulate material.

For small sample volumes it may be possible to dilute the sample with the start buffer that is to be used for chromatographic purification, but cell debris and particulate matter must still be removed.

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.

Figure 90 shows a typical buffer exchange and desalting separation. The process can be monitored by following changes in UV absorption and conductivity.

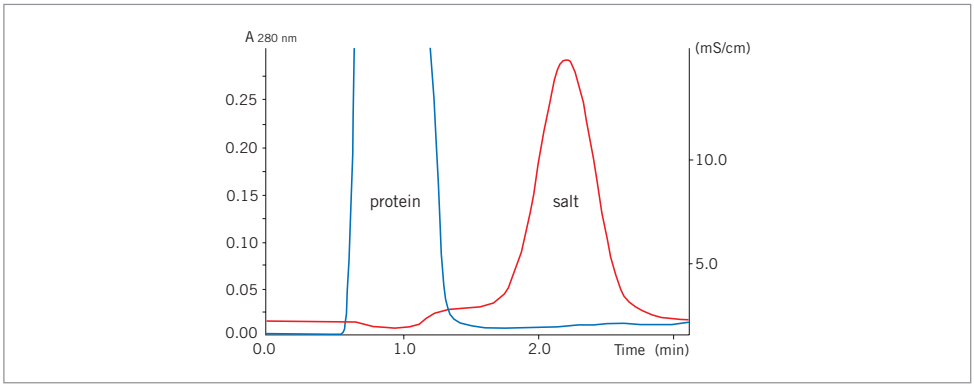


Fig. 90. Buffer exchange of mouse plasma (10 ml) on HiPrep 26/10 Desalting.

For laboratory scale operations, Table 18 shows a selection guide for prepacked, ready to use desalting and buffer exchange columns.

Table 18. Selection guide for desalting and buffer exchange.

Column	Sample volume	Sample elution volume
MicroSpin™ G-25	0.1–0.15 ml	0.1–0.15 ml
PD-10 (gravity feed column)	1.5–2.5 ml	2.5–3.5 ml
HiTrap Desalting 5 ml	0.25–1.5 ml	1.0–2.0 ml
HiPrep 26/10 Desalting	2.5–15 ml	7.5–20 ml

To desalt larger sample volumes:

- connect up to 5 HiTrap Desalting 5 ml columns in series to increase the sample volume capacity, e.g. 2 columns: sample volume 3 ml, 5 columns: sample volume 7.5 ml.
- connect up to 4 HiPrep 26/10 Desalting columns in series to increase the sample volume capacity, e.g. 2 columns: sample volume 30 ml, 4 columns: sample volume 60 ml. Even with 4 columns in series, the sample can be processed in 20 to 30 minutes, at room temperature, in aqueous buffers.

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

Alternative 1: Manual desalting with HiTrap Desalting 5 ml using a syringe

1. Fill the syringe with buffer. Remove the stop plug. 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 remove completely 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 19.

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.

The maximum recommended sample volume is 1.5 ml. See Table 19 for the effect of reducing the sample volume applied to the column.

Table 19. Recommended sample and elution volumes using a syringe or Multipipette™.

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

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.

Figure 91 shows a typical result obtained from ÄKTAprime. The UV (protein) and conductivity (salt) traces enable pooling of the desalted fractions.

Sample: $(His)_6$ protein eluted from HiTrap Chelating HP with sodium phosphate 20 mM, sodium chloride 0.5 M, imidazole 0.5 M, pH 7.4
Column: HiTrap Desalting 5 ml
Buffer: Sodium phosphate 20 mM, sodium chloride 0.15 M, pH 7.0

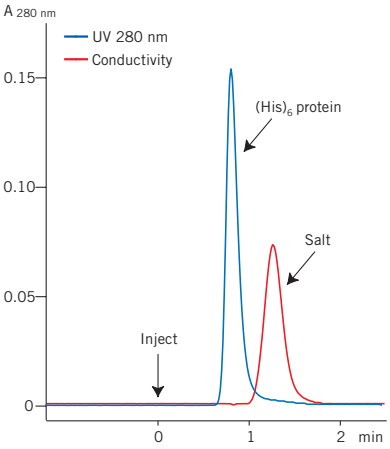


Fig. 91. Desalting of a $(His)_6$ fusion protein on ÄKTAprime.

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 sulphate 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 under Buffer exchange and desalting.

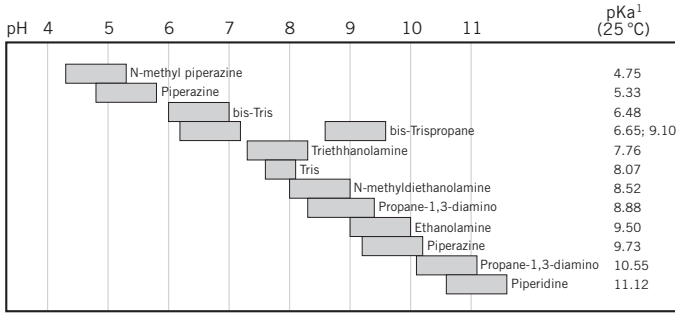
Removal of low molecular weight contaminants

If samples contain a high level of low molecular weight contaminants, use a desalting column before the first chromatographic purification step, as described under Buffer exchange and desalting.

Appendix 2

Non-volatile and volatile buffer systems

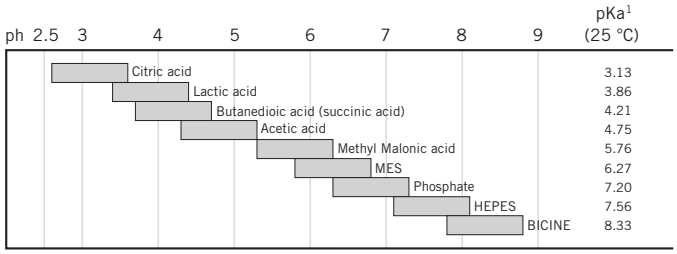
Non-volatile buffers for anion exchange chromatography



pH interval	Substance	Conc. (mM)	Counter-ion	pKa (25 °C)[1]	d(pKa)/dT (°C)
4.3–5.3	N-Methylpiperazine	20	Cl^-	4.75	-0.015
4.8–5.8	Piperazine	20	Cl^- or $HCOO^-$	5.33	-0.015
5.5–6.5	L-Histidine	20	Cl^-	6.04	
6.0–7.0	bis-Tris	20	Cl^-	6.48	-0.017
6.2–7.2; 8.6–9.6	bis-Tris propane	20	Cl^-	6.65; 9.10	
7.3–8.3	Triethanolamine	20	Cl^- or CH_3COO^-	7.76	-0.020
7.6–8.6	Tris	20	Cl^-	8.07	-0.028
8.0–9.0	N-Methyldiethanolamine	20	SO_4^{2-}	8.52	-0.028
8.0–9.0	N-Methyldiethanolamine	50	Cl^- or CH_3COO^-	8.52	-0.028
8.4–9.4	Diethanolamine	20 at pH 8.4	Cl^-	8.88	-0.025
		50 at pH 8.8			
8.4–9.4	Propane 1,3-Diamino	20	Cl^-	8.88	-0.031
9.0–10.0	Ethanolamine	20	Cl^-	9.50	-0.029
9.2–10.2	Piperazine	20	Cl^-	9.73	-0.026
10.0–11.0	Propane 1,3-Diamino	20	Cl^-	10.55	-0.026
10.6–11.6	Piperidine	20	Cl^-	11.12	-0.031

[1] *Ref: Handbook of chemistry and physics, 83rd edition, CRC, 2002–2003.*

Non-volatile buffers for cation exchange chromatography



pH interval	Substance	Conc. (mM)	Counter-ion	pKa (25 °C)[1]	d(pKa)/dT (°C)
1.4–2.4	Maleic acid	20	Na^+	1.92	
2.6–3.6	Methyl malonic acid	20	Na^+ or Li^+	3.07	
2.6–3.6	Citric acid	20	Na^+	3.13	-0.0024
3.3–4.3	Lactic acid	50	Na^+	3.86	
3.3–4.3	Formic acid	50	Na^+ or Li^+	3.75	+0.0002
3.7–4.7; 5.1–6.1	Succinic acid	50	Na^+	4.21; 5.64	-0.0018
4.3–5.3	Acetic acid	50	Na^+ or Li^+	4.75	+0.0002
5.2–6.2	Methyl malonic acid	50	Na^+ or Li^+	5.76	
5.6–6.6	MES	50	Na^+ or Li^+	6.27	-0.0110
6.7–7.7	Phosphate	50	Na^+	7.20	-0.0028
7.0–8.0	HEPES	50	Na^+ or Li^+	7.56	-0.0140
7.8–8.8	BICINE	50	Na^+	8.33	-0.0180

[1] *Ref: Handbook of chemistry and physics, 83rd edition, CRC, 2002–2003.*

Volatile buffer systems

pH range	Buffer system	Counter-ion	pKa-values for buffering ions[1]
3.3–4.3	Formic acid	H^+	3.75
3.3–4.3; 4.8–5.8	Pyridine/formic acid	$HCOO^-$	3.75; 5.25
3.3–4.3; 9.3–10.3	Trimethylamine/formic acid	$HCOO^-$	4.75; 9.81
4.3–5.8	Pyridine/acetic acid	CH_3COO^-	4.75; 5.25
4.3–5.3; 9.3–10.3	Trimethylamine/acetic acid	CH_3COO^-	4.75; 9.81
3.3–4.3; 8.8–9.8	Ammonia/formic acid	$HCOO^-$	3.75; 9.25
4.3–5.3; 8.8–9.8	Ammonia/acetic acid	CH_3COO^-	4.75; 9.25
5.9–6.9; 9.3–10.3	Trimethylamine/carbonate	CO_3^{2-}	6.35; 9.81
5.9–6.9; 8.8–9.8	Ammonium bicarbonate	HCO_3^-	6.35; 9.25
5.9–6.9; 8.8–9.8	Ammonium carbonate/ammonia	CO_3^{2-}	6.35; 9.25
5.9–6.9; 8.8–9.8	Ammonium carbonate	CO_3^{2-}	6.35; 9.25
4.3–5.3: 7.2–8.2	N-ethylmorpholine/acetate	$HCOO^-$	4.75; 7.72

[1] *Ref: Handbook of chemistry and physics, 83rd edition, CRC, 2002–2003.*

Appendix 3

Column packing and preparation

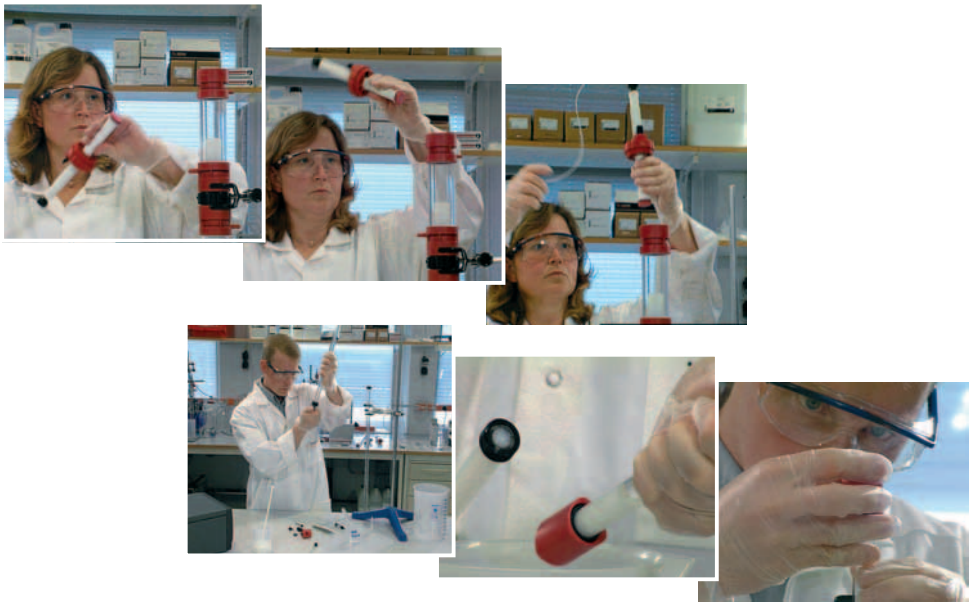
Prepacked columns from Amersham Biosciences will ensure reproducible results and the highest performance.

Use small prepacked columns for media scouting and method optimization, to increase efficiency in method development e.g. HiTrap IEX Selection Kit.

Efficient column packing is essential for IEX separation, especially when using gradient elution. A poorly packed column gives rise to poor and uneven flow, band broadening, and loss of resolution. If column packing is required, the following guidelines will apply at all scales of operation:

- With a high binding capacity medium, use short, wide columns (typically 5–15 cm bed height) for rapid purification, even with low linear flow.
- The amount of IEX medium required will depend on the binding capacity of the medium and the amount of sample. Binding capacities for each medium are given in this handbook and supplied with the product instructions. Estimate the amount of medium required to bind the sample of interest and use five times this amount to pack the column. The amount of medium required can be reduced if resolution is satisfactory.
- Once separation parameters have been determined, scale up a purification by increasing the diameter of the column to increase column volume. Avoid increasing the length of the column as this will alter separation conditions.

IEX media can be packed in either Tricorn or XK columns available from Amersham Biosciences. A step-by-step demonstration of column packing can be seen in "The Movie", available in CD format (see Ordering Information).



1. Equilibrate all materials to the temperature at which the separation will be performed.
2. Eliminate air by flushing column end pieces with the recommended buffer. Ensure no air is trapped under the column net. Close column outlet leaving 1–2 cm of buffer in the column.
3. Gently resuspend the medium.

Note that IEX media from Amersham Biosciences are supplied ready to use. Decanting of fines that could clog the column is unnecessary.

Avoid using magnetic stirrers since they may damage the matrix.

4. Estimate the amount of slurry (resuspended medium) required on the basis of the recommendations supplied.
5. Pour the required volume of slurry into the column. Pouring down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
6. Immediately fill the column with buffer.
7. Mount the column top piece and connect to a pump.
8. Open the column outlet and set the pump to the desired flow rate.

When slurry volume is greater than the total volume of the column, connect a second glass column to act as a reservoir (see Ordering information for details). This ensures that the slurry has a constant diameter during packing, minimizing turbulence and improving column packing conditions.

If the recommended flow rate cannot be obtained, use the maximum flow rate the pump can deliver.

Do not exceed the maximum operating pressure of the medium or column.

9. Maintain the packing flow rate for at least 3 column volumes after a constant bed height is obtained. Mark the bed height on the column.

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

10. Stop the pump and close the column outlet. Remove the top piece and carefully fill the rest of the column with buffer to form an upward meniscus at the top.
11. Insert the adaptor into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adaptor slowly down the column (the outlet of the adaptor should be open) until the mark is reached. Lock the adaptor in position.
13. Connect the column to the pump and begin equilibration. Re-position the adaptor if necessary.

The medium must be thoroughly washed to remove the storage solution, usually 20% ethanol. Residual ethanol may interfere with subsequent procedures.

Many media equilibrated with sterile phosphate-buffered saline containing an antimicrobial agent may be stored at +4 °C for up to 1 month, but always follow the specific storage instructions supplied with the product.

Column selection

Tricorn and XK columns are fully compatible with the high flow rates achievable with modern media and a broad range of column dimensions are available. Columns most suitable for packing IEX media are listed under the column packing section for each IEX medium (Chapter 3). In most cases the capacity of the IEX medium and the amount of sample to be purified will determine the column size required. For a complete listing refer to the Amersham Biosciences BioDirectory™ or web catalog (*www.chromatography.amershambiosciences.com*) or visit *www.tricorncolumns.com* for more details on Tricorn columns.

Column packing and efficiency

Column efficiency is expressed as the number of theoretical plates per meter chromatography bed (N) or as H (height equivalent to a theoretical plate, HETP), which is the bed length (L) divided by the plate number. Since column efficiency is related to the band broadening which can occur on a column, it can be calculated from the expression:

$$N = 5.54 \times \left(\frac{V_R}{w_h}\right)^2$$

V_R = volume eluted from the start of sample application to the peak maximum

w_h = peak width measured as the width of the recorded peak at half of the peak height

H is calculated from the expression:

$$H = \frac{L}{N}$$

L = height of packed bed.

Measurements of V_R and w_h can be made in distance (mm) or volume (ml) but both parameters must be expressed in the same unit.

Column performance should be checked at regular intervals by injecting acetone to determine column efficiency (N) and peak symmetry (assymetry factor, A_s). Since the observed value for N depends on experimental factors such as flow rate and sample loading, comparisons must be made under identical conditions. In IEX, efficiency is measured under isocratic conditions by injecting acetone (which does not interact with the medium) and measuring the eluted peak as shown in Figure 92.

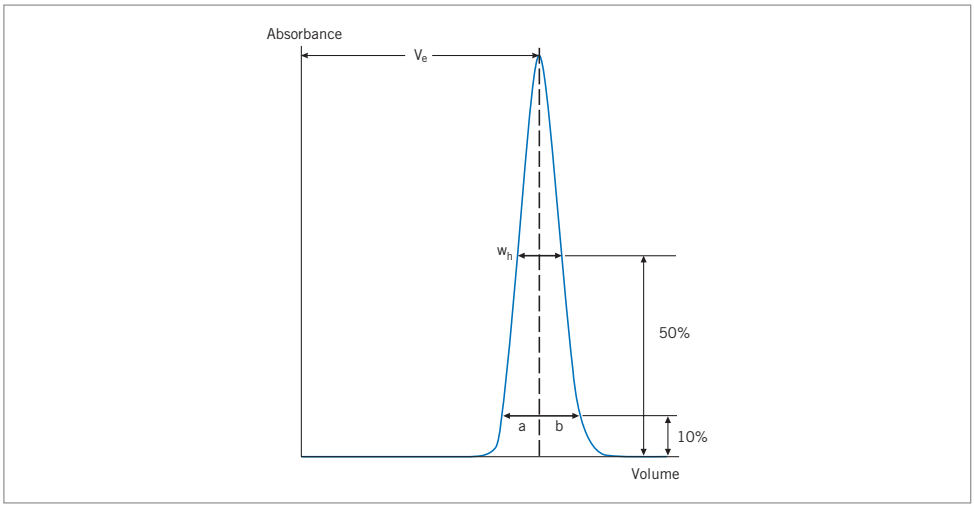


Fig. 92.

As a general rule, a good H value is about two to three times the average particle diameter of the medium being packed. For a 90 µm particle, this means an H value of 0.018–0.027 cm.

The symmetry factor (As) is expressed as:

$$A_s = \frac{b}{a}$$

where

a = 1st half peak width at 10% of peak height

b = 2nd half peak width at 10% of peak height

A_s should be as close as possible to 1. A reasonable A_s value for a short column as used in IEX is 0.80–1.80.

An extensive leading edge is usually a sign that the medium is packed too tightly and extensive tailing is usually a sign that the medium is packed too loosely.

Run at least two column volumes of buffer through a newly packed column to ensure that the medium is equilibrated with start buffer. Use pH monitoring to check the pH of the eluent.

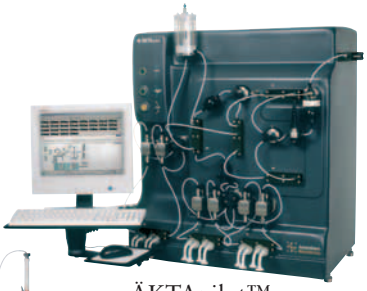
Appendix 4

Selection of purification equipment

Simple IEX chromatography, such as elution by a step-gradient, can be performed using a syringe or peristaltic pump with prepacked HiTrap columns. A chromatography system is required in order to achieve a high resolution separation using accurately controlled linear gradient elution, to take advantage of the high flow rates of modern media, or when the same column is to be used for many runs.

Way of working	Standard ÄKTAdesign configurations					Syringe or peristaltic pump + HiTrap column
	Pilot	Explorer 100	Purifier 10	FPLC	Prime	
Simple, step-gradient elution	✓	✓	✓	✓	✓	✓
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	✓	✓				
Proven sanitary design for cGMP with easily exchanged wetted parts	✓					

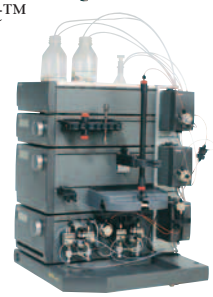
ÄKTAprime



ÄKTApilot™

ÄKTAexplorer

ÄKTAFPLC



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.

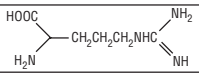
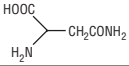
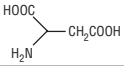
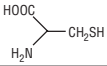
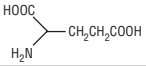
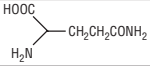
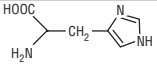
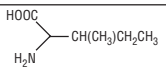
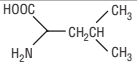
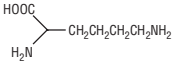
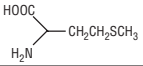
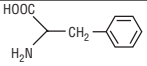
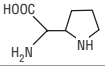
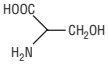
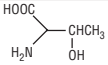
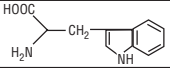
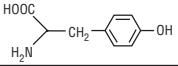
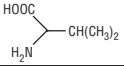
Column pressures

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

Formula	M_r	Middle unit residue ($-H_2O$) 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 20).

Table 20.

% 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*
Gradient:	5–15%	14 000–200 000
	5–20%	10 000–200 000
	10–20%	10 000–150 000

* The larger proteins fail to move significantly into the gel.

For information and advice on electrophoresis techniques, please refer to the section Additional reading and reference material.

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 and available on *www.amershambiosciences.com*

- 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 the *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 page 156).

General recommendations for purified proteins

- Store as a precipitate in high concentration of ammonium sulphate, 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 page 156).

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.

Appendix 10

Column cleaning

Correct preparation of samples and buffers and application of a high salt wash (1 M NaCl) at the end of each separation should keep most columns in good condition. However, reduced performance, a slow flow rate, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove contaminants.

It is recommended to reverse the direction of flow during column cleaning so that contaminants do not need to pass through the entire length of the column. The number of column volumes and time required for each cleaning step may vary according to the degree of contamination. If the cleaning procedure to remove common contaminants does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Care should be taken when changing a filter as this may affect the column packing and interfere with performance.

Removal of common contaminants

The following procedure should be satisfactory to remove common contaminants:

1. Wash with at least 2 column volumes of 2 M NaCl (see Table 21 for recommended flow).
2. Wash with at least 4 column volumes of 1 M NaOH (same flow as step 1).
3. Wash with at least 2 column volumes of 2 M NaCl (same flow as step 1).
4. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and eluent pH are stable.
5. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 1) until pH and conductivity values have reached the required values.

Removal of precipitated proteins, lipids, hydrophobically bound proteins or lipoproteins

To remove precipitated proteins

1. Inject 1 column volume of pepsin (1 mg/ml in 0.5 M NaCl, 0.1 M acetic acid). Leave overnight at room temperature or for one hour at 37 °C.
2. Rinse with at least 2 column volumes of distilled water (see Table 21 for recommended flow) until the UV-baseline and the eluent pH are stable.
3. Wash with at least 4 column volumes of start buffer or storage buffer, same flow as step 2, until eluent pH and conductivity have reached the required values.

Alternatively,

1. Wash with 2 column volumes of 6 M guanidine hydrochloride (see Table 21 for recommended flow).
2. Wash immediately with at least 5 column volumes of buffer at pH 7–8 (see Table 21 for recommended flow).
3. Rinse with at least 2 column volumes of distilled water (same flow as step 2) until the UV-baseline and eluent pH are stable.
4. Wash with at least 4 column volumes of start buffer or storage buffer (same flow as step 2) until pH and conductivity values have reached the required values.

Table 21. Recommended flow according to medium, column dimensions and eluent.

Column (volume) or medium	Water, buffer, 2 M NaCl or 1 M NaOH*	6 M guanidine hydrochloride	70% ethanol or 30% isopropanol
MiniBeads (0.24 ml)	0.2 ml/min	0.1 ml/min	0.1 ml/min
MiniBeads (0.8 ml)	0.2 ml/min	0.1 ml/min	0.1 ml/min
MonoBeads (1.7 ml)	0.2 ml/min	0.1 ml/min	0.1 ml/min
MonoBeads (1 ml)	0.5 ml/min	0.25 ml/min	0.25 ml/min
MonoBeads (8 ml)	2 ml/min	1 ml/min	1 ml/min
MonoBeads (20 ml)	5 ml/min	2.5 ml/min	2.5 ml/min
SOURCE 15 4.6/100 PE	0.2 ml/min	0.1 ml/min	0.1 ml/min
RESOURCE 1 ml	1 ml/min	0.5 ml/min	0.5 ml/min
RESOURCE 6 ml	6 ml/min	3 ml/min	3 ml/min
SOURCE in larger columns**	40 cm/h	20 cm/h	20 cm/h
HiTrap (1 ml)	1 ml/min	0.5 ml/min	0.5 ml/min
HiTrap (5 ml)	5 ml/min	2.5 ml/min	2.5 ml/min
HiPrep (20 ml)	5 ml/min	2.5 ml/min	2.5 ml/min
HiLoad (20 ml)	3 ml/min	2.5 ml/min	2.5 ml/min
HiLoad (53 ml)	8 ml/min	5 ml/min	5 ml/min
Sepharose High Performance in larger columns**	40 cm/h	20 cm/h	20 cm/h
Sepharose Fast Flow in larger columns**	40 cm/h	20 cm/h	40 cm/h
Sepharose XL in larger columns**	40 cm/h	20 cm/h	40 cm/h
Sepharose Big Beads**	40 cm/h	40 cm/h	40 cm/h

** If contamination is thought to be significant, use a lower flow rate to increase the contact time when using 1 M NaOH.*

*** When cleaning larger columns, allow a contact time of 1–2 hours for any solution that is used as an initial cleaning step.*

To remove lipids, hydrophobically bound proteins or lipoproteins

Organic solvents or detergents may be required to completely remove contaminants of this type.

Before using organic solvents, wash the medium with at least 4 column volumes of distilled water to avoid any salts precipitating on the column.

When applying organic solvents or solutions it may be necessary to reduce the flow rate to avoid over-pressuring the column.

Use cleaning solutions such as up to 100% isopropanol, up to 100% methanol, up to 100% acetonitrile, up to 2 M NaOH, up to 75% acetic acid, up to 100% ethanol, ionic or non-ionic detergents.

Always check for solvent compatibility in the instructions supplied with the medium or column.

Avoid anionic detergents with Q, DEAE and ANX charged groups. Avoid cationic detergents with S, SP and CM charged groups.

Examples of cleaning procedures:

1. Wash with 4 column volumes of up to 70% ethanol or 30% isopropanol (see Table 21 for recommended flow).
2. Rinse with at least 2 column volumes of distilled water (see Table 21 for recommended flow) until the UV-baseline and eluent pH are stable.
3. Wash immediately with 3 column volumes of start buffer (same flow as step 2).

Alternatively,

1. Wash with 2 column volumes of detergent in a basic or acidic solution, e.g. 0.1–0.5% non-ionic detergent in 0.1 M acetic acid (see Table 21 for recommended flow).
2. Rinse with 5 column volumes 70% ethanol to remove residual detergent (seeTable 21 for recommended flow).
3. Rinse with at least 2 column volumes of distilled water (same flow as step 1) until the UV-baseline and the eluent pH are stable.
4. Wash with 3 column volumes of start buffer (same flow as step 1).

Additional reading

	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
Gel filtration Handbook: Principles and Methods	18-1022-18
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
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
Column Packing Video (PAL)	17-0893-01
Column Packing Video (NTSC)	17-0894-01
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

Many of these items can be downloaded from ***www.chromatography.amershambiosciences.com***.
Other useful web links include: ***www.hitrap.com*** and ***www.tricorncolumns.com***.

References

	Code No.
Reference lists are available from ***www.chromatography.amershambiosciences.com***	
Reference list HiTrap Desalting	18-1156-70
Reference list HiPrep 26/10 Desalting	18-1156-89
Reference list HiLoad Q Sepharose HP and HiLoad Q Sepharose FF	18-1156-98
Reference list HiLoad SP Sepharose HP and HiLoad SP Sepharose FF	18-1156-99
Reference list HiPrep 16/10 CM FF	18-1156-91
Reference list HiPrep 16/10 DEAE FF	18-1156-90
Reference list HiPrep 16/10 Q FF	18-1156-92
Reference list HiTrap Q HP and HiTrap Q FF	18-1156-82
Reference list HiTrap SP HP and HiTrap SP FF	18-1156-83
Reference list HiTrap CM FF	18-1156-84
Reference list HiTrap DEAE FF	18-1156-85
Reference list Mono Q (2000–2002)	18-1166-18
Reference list Mono S (1998–2002)	18-1166-16
Reference list MiniBeads (1998–2002)	18-1166-17

Ordering information

Ion exchange

SOURCE, Sepharose High Performance, Sepharose Fast Flow. Sepharose XL and Sepharose Big Beads are all available as BioProcess media for large scale production. Please contact your local Amersham Biosciences representative for details.

Product	Quantity	Code No.
MiniBeads		
Mini Q PC 3.2/3*	1 × 0.24 ml	17-0686-01
Mini S PC 3.2/3*	1 × 0.24 ml	17-0687-01
Mini Q 4.6/50 PE	1 × 0.8 ml	17-5177-01
Mini S 4.6/50 PE	1 × 0.8 ml	17-5178-01
MonoBeads		
Mono Q 5/50 GL	1 × 1 ml	17-5166-01
Mono Q 10/100 GL	1 × 8 ml	17-5167-01
Mono Q 4.6/100 PE	1 × 1.7 ml	17-5179-01
Mono Q HR 16/10	1 × 20 ml	17-0506-01
Mono S 5/50 GL	1 ×1 ml	17-5168-01
Mono S 10/100 GL	1 × 8 ml	17-5169-01
Mono S 4.6/100 PE	1 × 1.7 ml	17-5180-01
Mono S HR 16/10	1 × 20 ml	17-0507-01
SOURCE		
RESOURCE Q	1 x 1 ml	17-1177-01
RESOURCE Q	1 x 6 ml	17-1179-01
SOURCE 15Q 4.6/100 PE	1 x 1.7 ml	17-5181-01
SOURCE 15Q	10 ml	17-0947-20
SOURCE 15Q	50 ml	17-0947-01
SOURCE 15Q	200 ml	17-0947-05
SOURCE 30Q	10 ml	17-1275-10
SOURCE 30Q	50 ml	17-1275-01
SOURCE 30Q	200 ml	17-1275-05
RESOURCE S	1 × 1 ml	17-1178-01
RESOURCE S	1 × 6 ml	17-1180-01
SOURCE 15S 4.6/100 PE	1 × 1.7 ml	17-5182-01
SOURCE 15S	10 ml	17-0944-10
SOURCE 15S	50 ml	17-0944-01
SOURCE 15S	200 ml	17-0944-05
SOURCE 30S	10 ml	17-1273-20
SOURCE 30S	50 ml	17-1273-01
SOURCE 30S	200 ml	17-1273-02
Sepharose High Performance		
HiTrap Q HP	5 × 1 ml	17-1153-01
HiTrap Q HP	5 × 5 ml	17-1154-01
HiLoad 16/10 Q Sepharose High Performance	1 × 20 ml	17-1064-01
HiLoad 26/10 Q Sepharose High Performance	1 × 53 ml	17-1066-01
Q Sepharose High Performance	75 ml	17-1014-01

Product	Quantity	Code No.
Sepharose High Performance (continued)		
HiTrap SP HP	5 × 1 ml	17-1151-01
HiTrap SP HP	5 × 5 ml	17-1152-01
HiLoad 16/10 SP Sepharose High Performance	1 × 20 ml	17-1137-01
HiLoad 26/10 SP Sepharose High Performance	1 × 53 ml	17-1138-01
SP Sepharose High Performance	75 ml	17-1087-01
HiTrap IEX Selection Kit** ** Q Sepharose Fast Flow, DEAE Sepharose Fast Flow, SP Sepharose Fast Flow, CM Sepharose Fast Flow, ANX Sepharose 4 Fast Flow (high sub), Q Sepharose XL and SP Sepharose XL	7 × 1 ml	17-6002-33
Sepharose Fast Flow		
HiTrap Q FF	5 × 1 ml	17-5053-01
HiTrap Q FF	5 × 5 ml	17-5156-01
HiPrep 16/10 Q FF	1 × 20 ml	17-5190-01
Q Sepharose Fast Flow	25 ml	17-0510-10
Q Sepharose Fast Flow	300 ml	17-0510-01
HiTrap SP FF	5 × 1 ml	17-5054-01
HiTrap SP FF	5 × 5 ml	17-5157-01
HiPrep 16/10 SP FF	1 × 20 ml	17-5192-01
SP Sepharose Fast Flow	25 ml	17-0729-10
SP Sepharose Fast Flow	300 ml	17-0729-01
HiTrap DEAE FF	5 × 1 ml	17-5055-01
HiTrap DEAE FF	5 × 5 ml	17-5154-01
HiPrep 16/10 DEAE FF	1 × 20 ml	17-5090-01
DEAE Sepharose Fast Flow	25 ml	17-0709-10
DEAE Sepharose Fast Flow	500 ml	17-0709-01
HiTrap CM FF	5 × 1 ml	17-5056-01
HiTrap CM FF	5 × 5 ml	17-5155-01
HiPrep 16/10 CM FF	1 × 20 ml	17-5091-01
CM Sepharose Fast Flow	25 ml	17-0719-10
CM Sepharose Fast Flow	500 ml	17-0719-01
HiTrap ANX FF (high sub)	5 × 1 ml	17-5162-01
HiTrap ANX FF (high sub)	5 × 5 ml	17-5163-01
HiPrep 16/10 ANX FF (high sub)	1 × 20 ml	17-5191-01
ANX Sepharose 4 Fast Flow (high sub)	25 ml	17-1287-10
ANX Sepharose 4 Fast Flow (high sub)	500 ml	17-1287-01
Sepharose XL		
HiTrap Q XL	5 × 1 ml	17-5158-01
HiTrap Q XL	5 × 5 ml	17-5159-01
HiPrep 16/10 Q XL	1 × 20 ml	17-5092-01
Q Sepharose XL	300 ml	17-5072-01
Q Sepharose XL virus licensed	25 ml	17-5437-10
HiTrap SP XL	5 × 1 ml	17-5160-01
HiTrap SP XL	5 × 5 ml	17-5161-01
HiPrep 16/10 SP XL	1 × 20 ml	17-5093-01
SP Sepharose XL	300 ml	17-5073-01

Product	Quantity	Code No.
Sepharose Big Beads		
Q Sepharose Big Beads	1 l	17-0989-03
SP Sepharose Big Beads	1 l	17-0657-03
Desalting columns		
HiTrap Desalting	5 × 5 ml	17-1408-01
HiTrap Desalting	100 × 5 ml	11-0003-29
HiPrep 26/10 Desalting	1 × 20 ml	17-5087-01
HiPrep 26/10 Desalting	4 × 20 ml	17-5087-02
PD-10 Desalting columns	30	17-0857-01
PD-10 Desalting columns	50	17-0735-01
Column Packing CD		
The Movie	1	18-1165-33
Empty Columns		
Complete information on the range of Tricorn columns is available at ***www.tricorncolumns.com***		
Tricorn 10/100 column Tricorn columns are delivered with a column tube, adaptor unit, end cap, a filter kit containing adaptor and bottom filters and O-rings, two stop plugs, adaptor lock and filter holder, and two M6 connectors for connection to FPLC systems if required.	1	18-1163-15
XK 16/20 column	1	18-8773-01
XK 26/20 column	1	18-1000-72
XK 50/20 column 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.	1	18-1000-71
Empty Disposable PD-10 Desalting columns	50/pk	17-0435-01
LabMate PD-10 Buffer Reservoir	1	18-3216-03
Accessories and spare parts		
For a complete listing refer to Amersham Biosciences Biodirectory or ***www.chromatography.amershambiosciences.com***		
Packing Connector XK 16	1	18-1153-44
Packing Connector XK 26	1	18-1153-45
Tricorn packing equipment 10/100 includes Tricorn packing connector 10–10, Tricorn 10/100 glass tube, bottom unit and stop plug.	1	18-1153-25
Tricorn packing connector 10–10* Connects extra glass column to a Tricorn 10 column to act as a packing reservoir for efficient packing.	1	18-1153-23

Chromatofocusing

Product	Quantity	Code No.
Mono P 5/50 GL	1 × 1 ml	17-5170-01
Mono P 5/200 GL	1 × 4 ml	17-5171-01
PBE 118 Polybuffer exchanger	200 ml	17-0711-01
PBE 94 Polybuffer exchanger	200 ml	17-0712-01
Polybuffer 74	250 ml	17-0713-01
Polybuffer 96	250 ml	17-0714-01
Pharmalyte pH 8–10.5	25 ml	17-0455-01

Product index

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