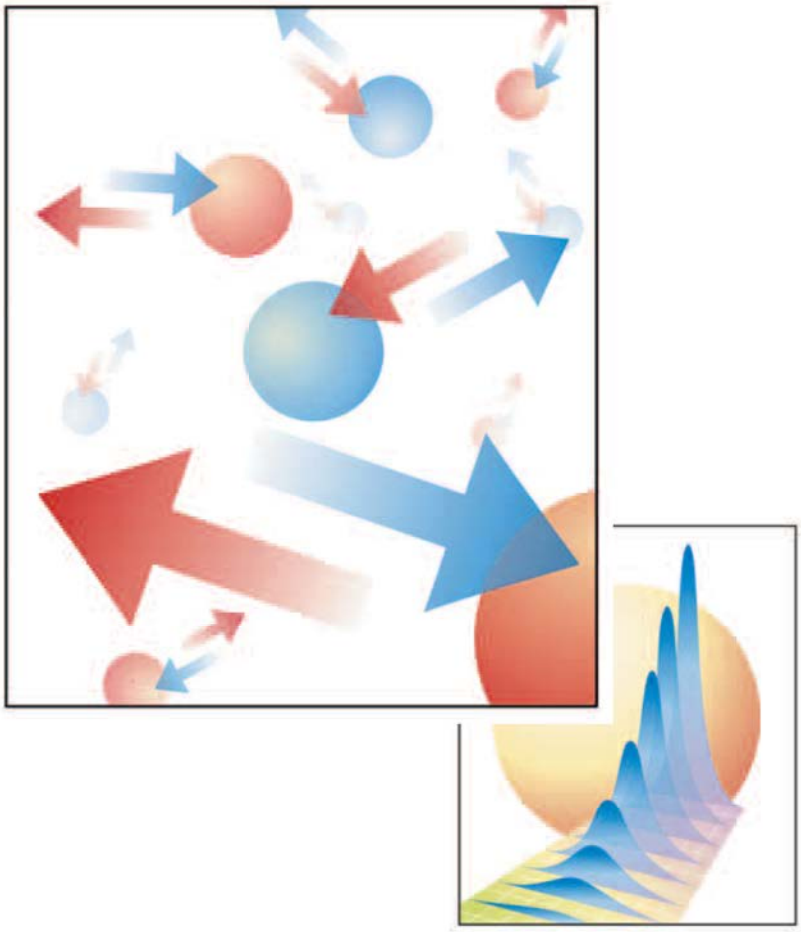
Ion Exchange Chromatography & Chromatofocusing

Principles and Methods



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Ion Exchange Chromatography & Chromatofocusing

Principles and Methods

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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)

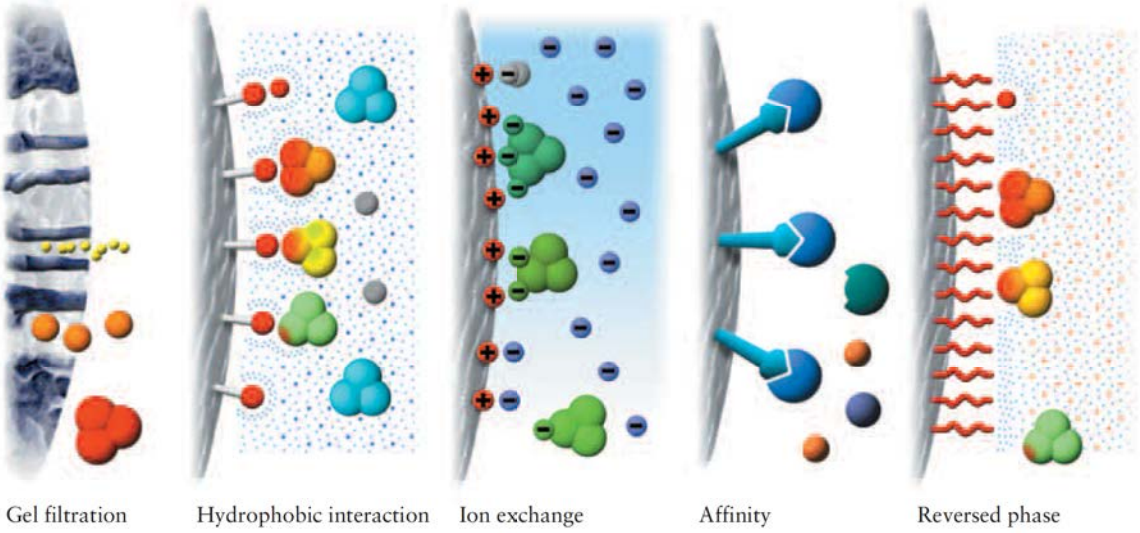


Fig. 1. Separation principles in chromatographic purification.

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.

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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.

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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

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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).

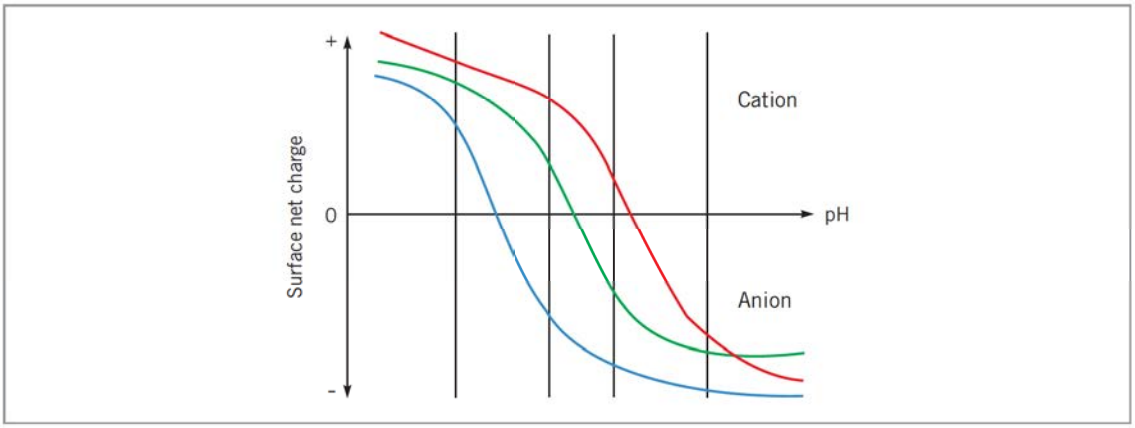


Fig. 2. Theoretical protein titration curves, showing how net surface charge varies with pH.

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.

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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*.

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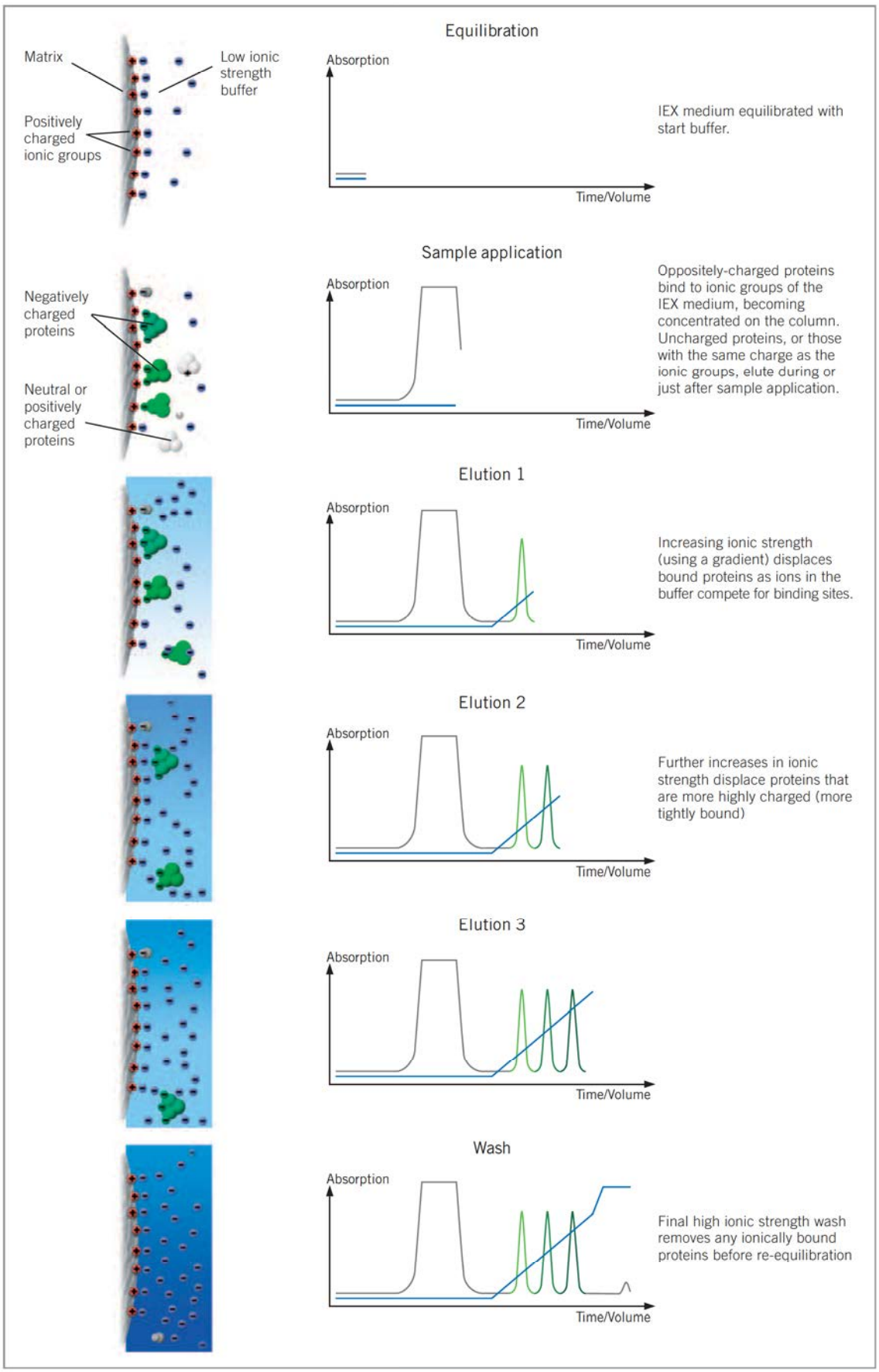


Fig. 3. Principles of an anion exchange separation.

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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.

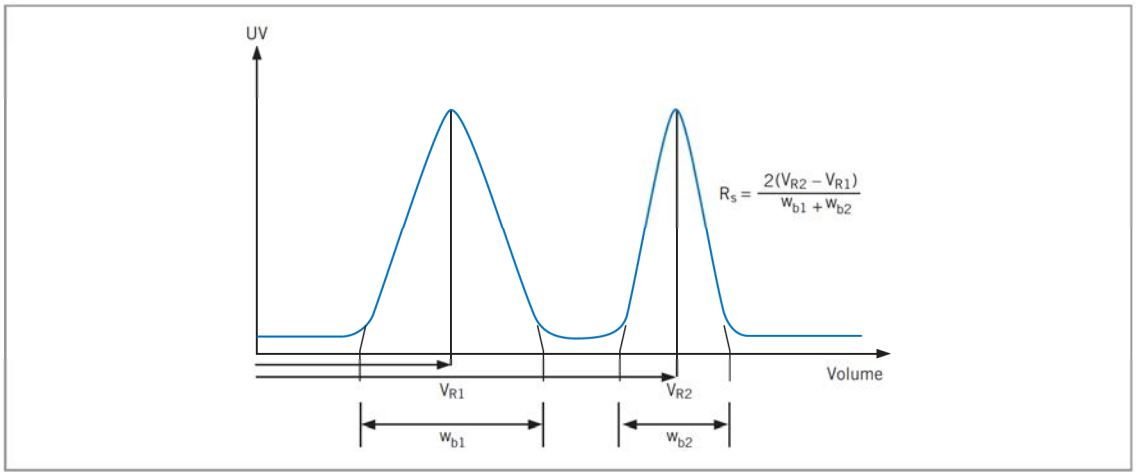


Fig. 4. Determination of the resolution (R_s) between two peaks.

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%.

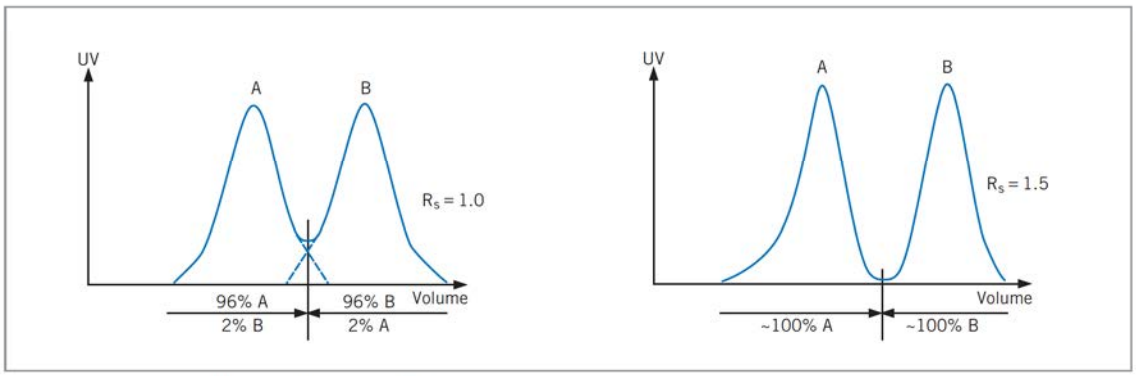


Fig. 5. Separation results with different resolutions.

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.

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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.

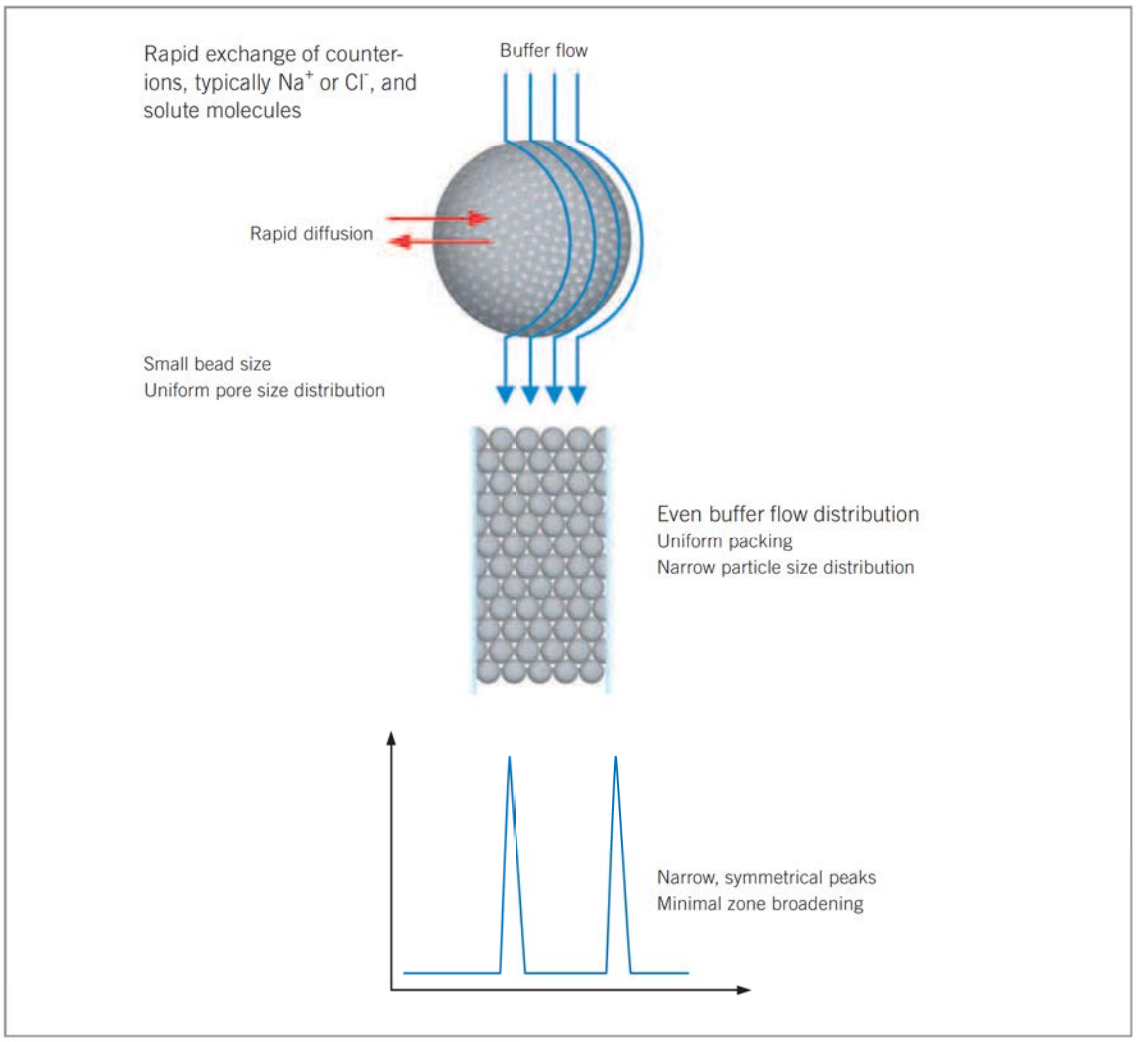


Fig. 6. Factors that effect column efficiency.

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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.

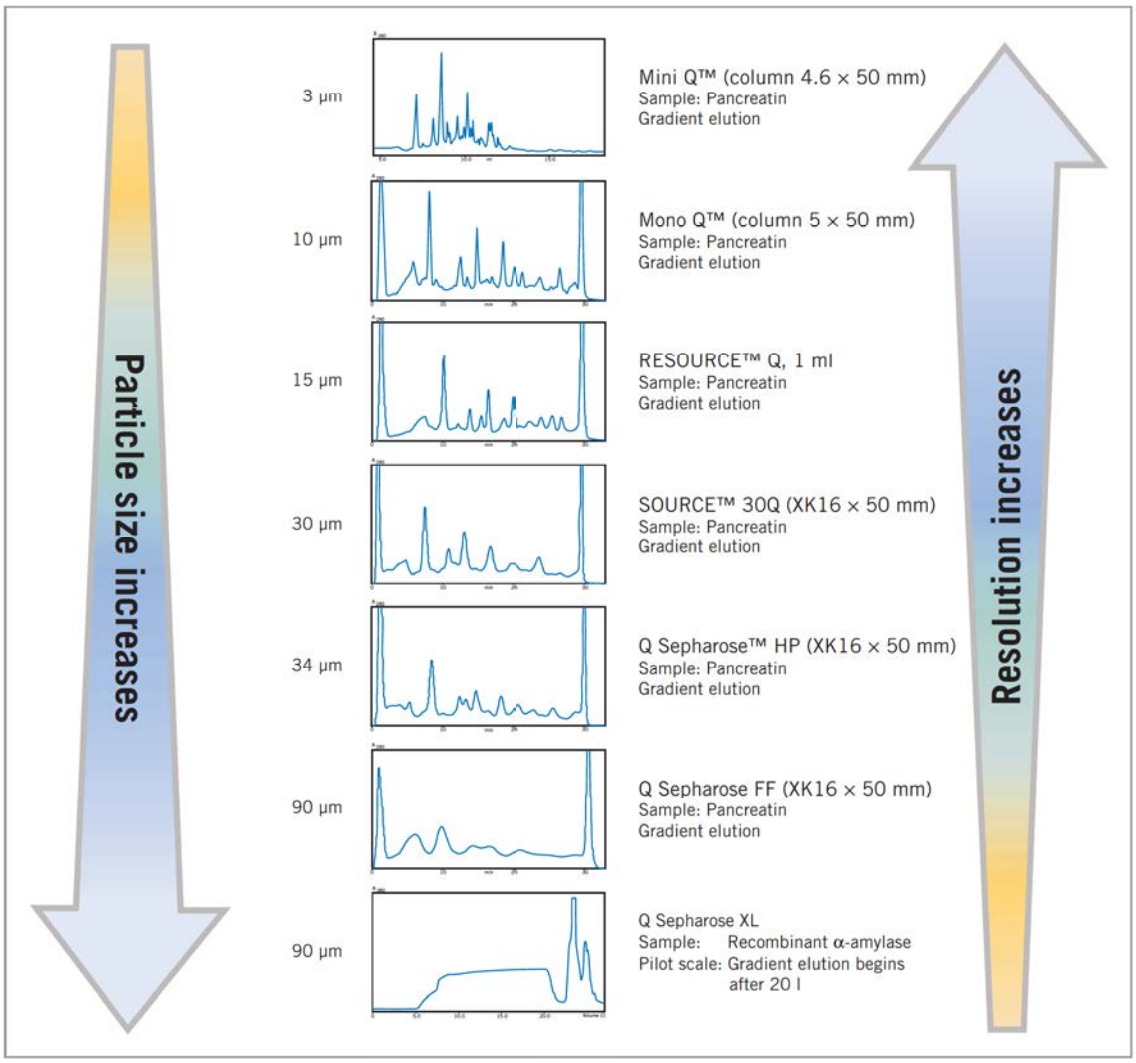


Fig. 7. The influence of particle size and selectivity on final resolution.

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.

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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.

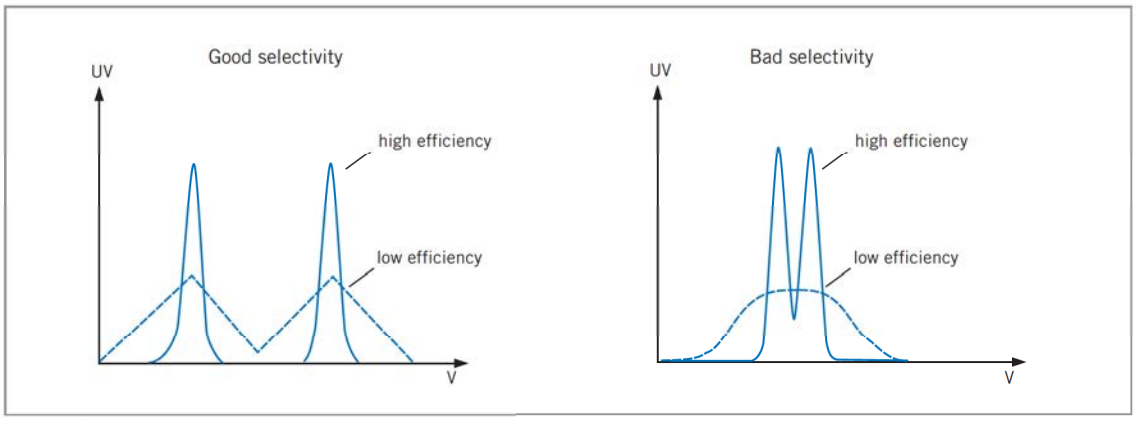


Fig. 8. Effect of selectivity and efficiency on resolution.

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.

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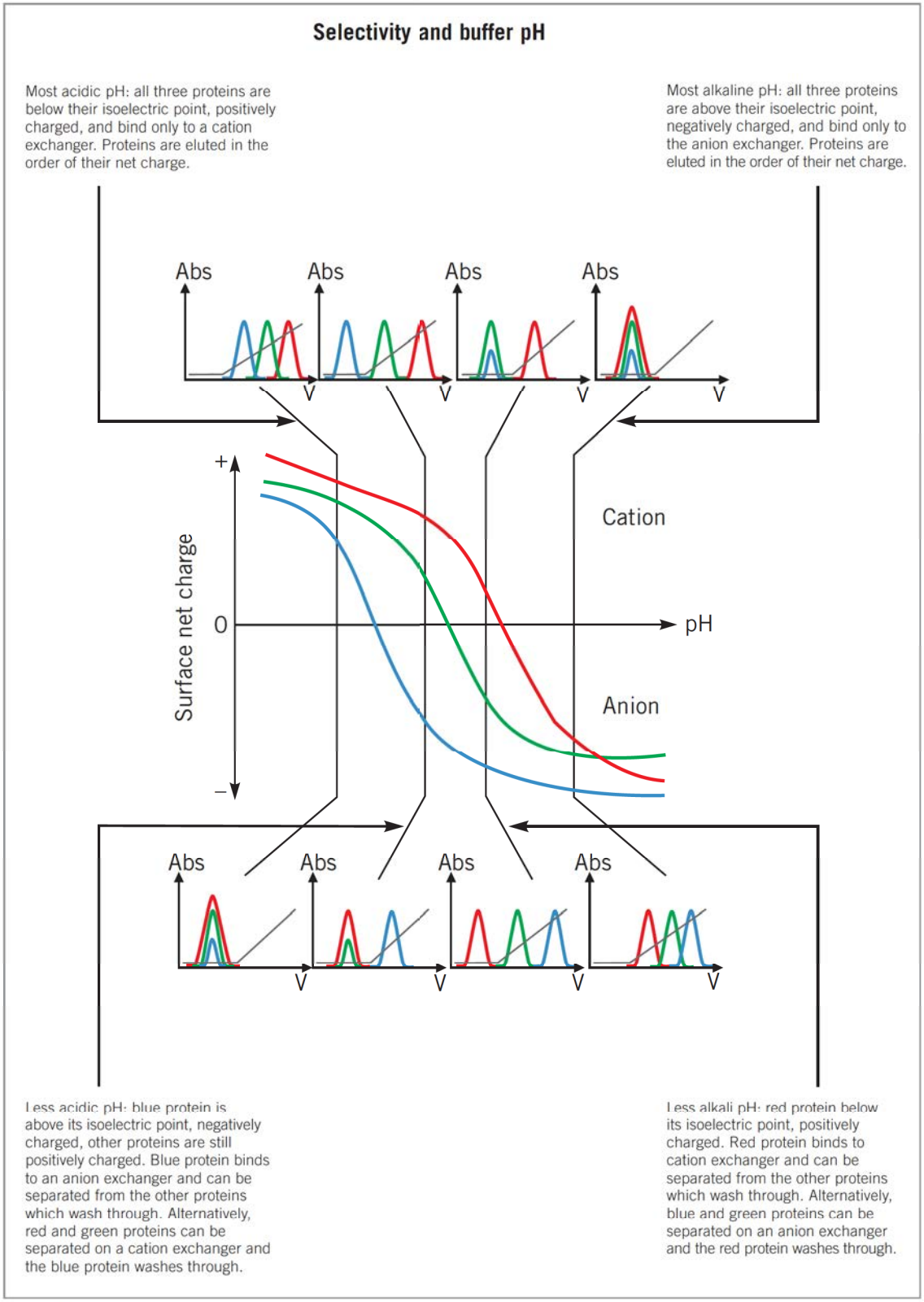


Fig. 9. Effect of pH on protein binding and elution patterns.

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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.

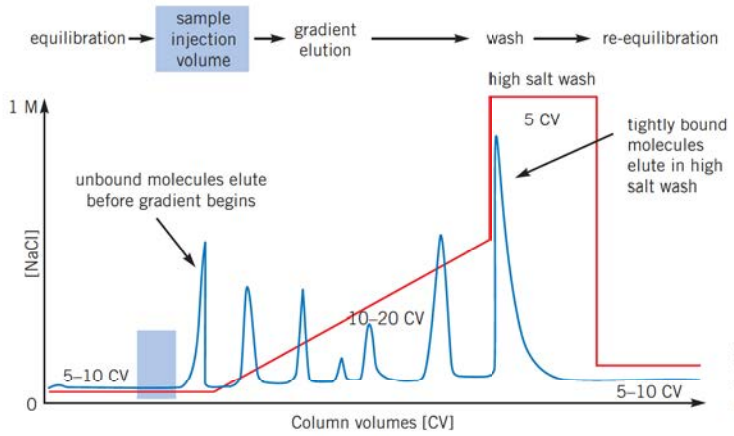


Fig. 10a. Typical high resolution IEX separation using linear gradient elution (25–45 column volumes).

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).

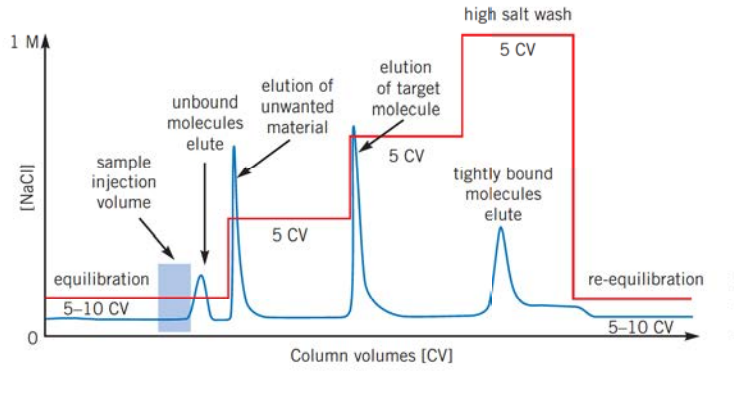


Fig. 10b. Typical IEX separation using step elution (25–30 column volumes).

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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.

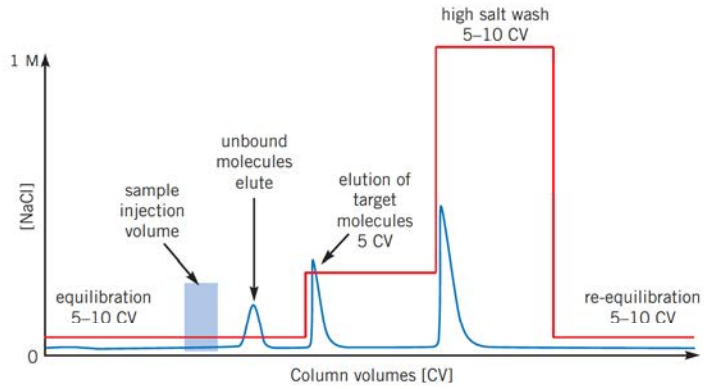


Fig. 10c. Typical IEX separation using a step elution to separate groups of proteins with very different charge properties (20–35 column volumes).

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.

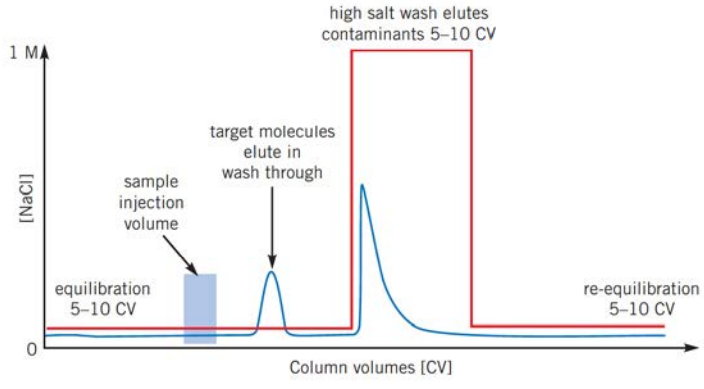


Fig. 10d. Contaminant removal: target protein(s) elute in the wash through, contaminants bind to the column.

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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.

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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*.

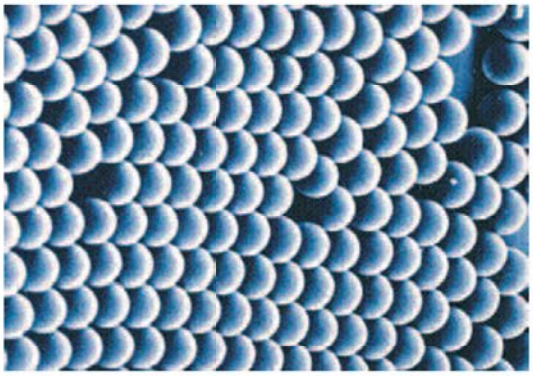


Fig. 11. Electron micrograph of MonoBeads showing spherical, monodispersed particles.

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.

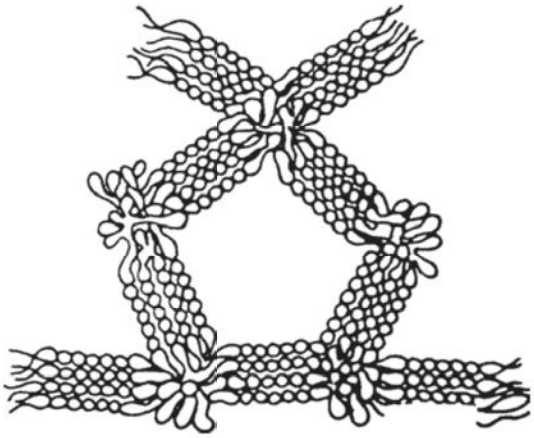


Fig. 12. Structure of cross-linked agarose media (Sepharose).

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.

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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).

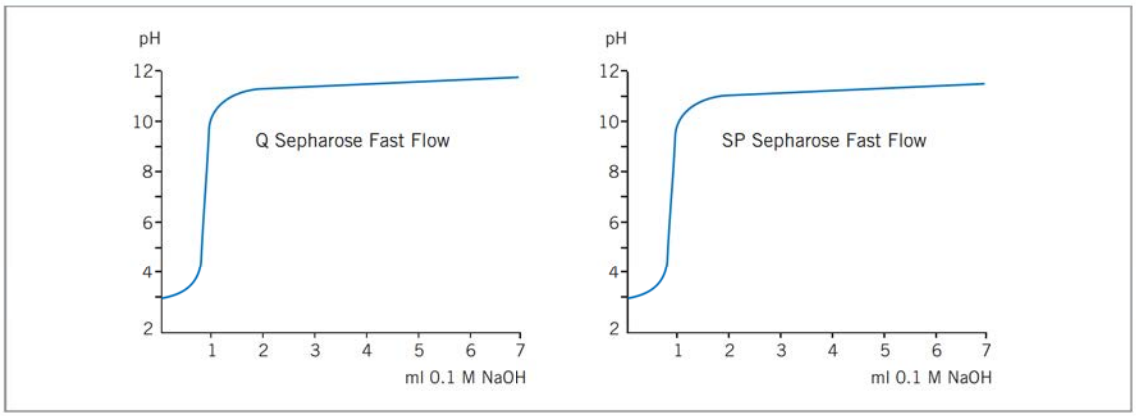


Fig. 13. Titration curves show the ion exchange capacity of strong ion exchangers Q and S. Approximately 5 ml of Q or S Sepharose Fast Flow are equilibrated in 1 M KCl and titrated with 0. 1 M NaOH.

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.

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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).

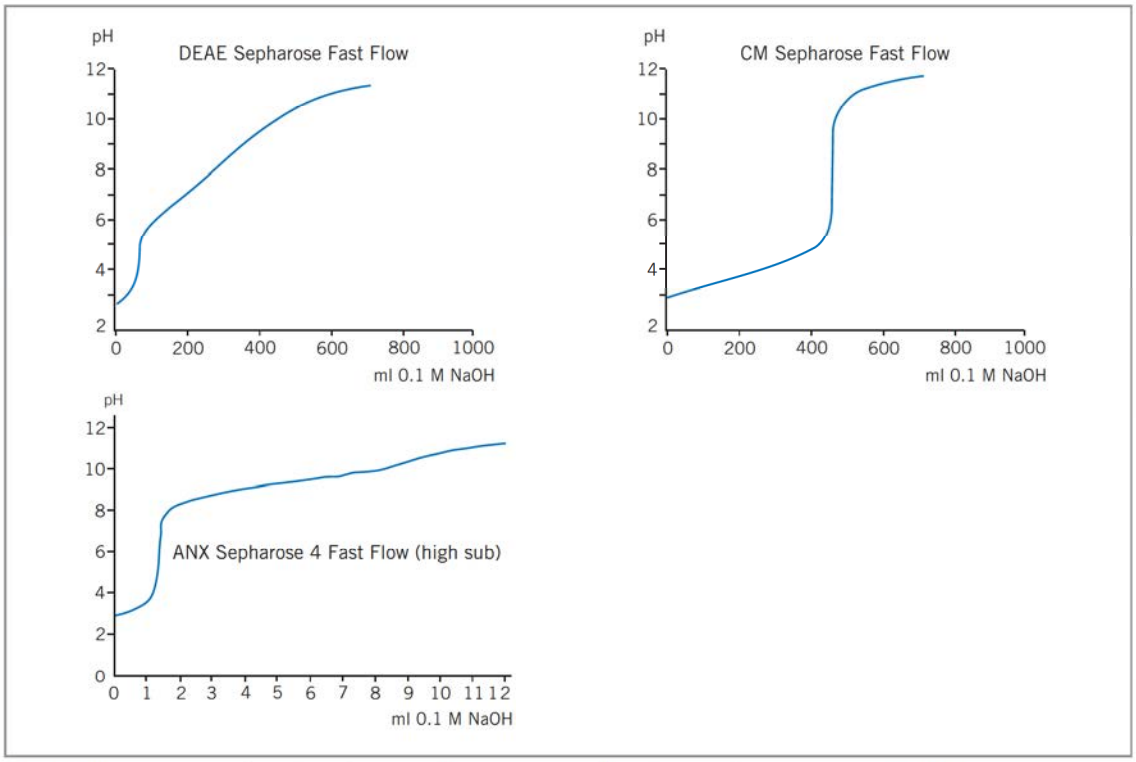


Fig. 14. Titration curves show how the ion exchange capacity of weak ion exchangers varies with pH.

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.

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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%.

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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.

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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.

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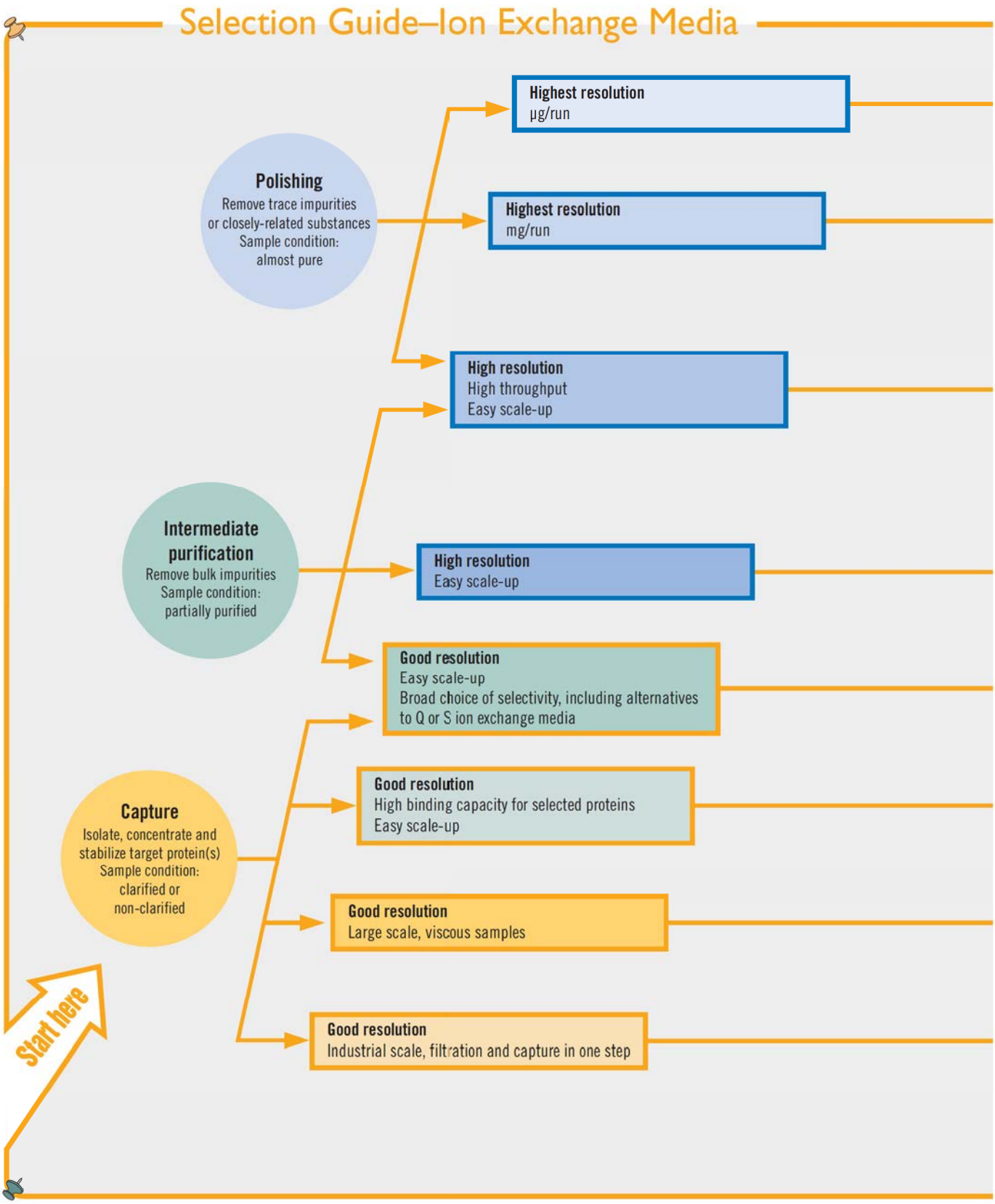
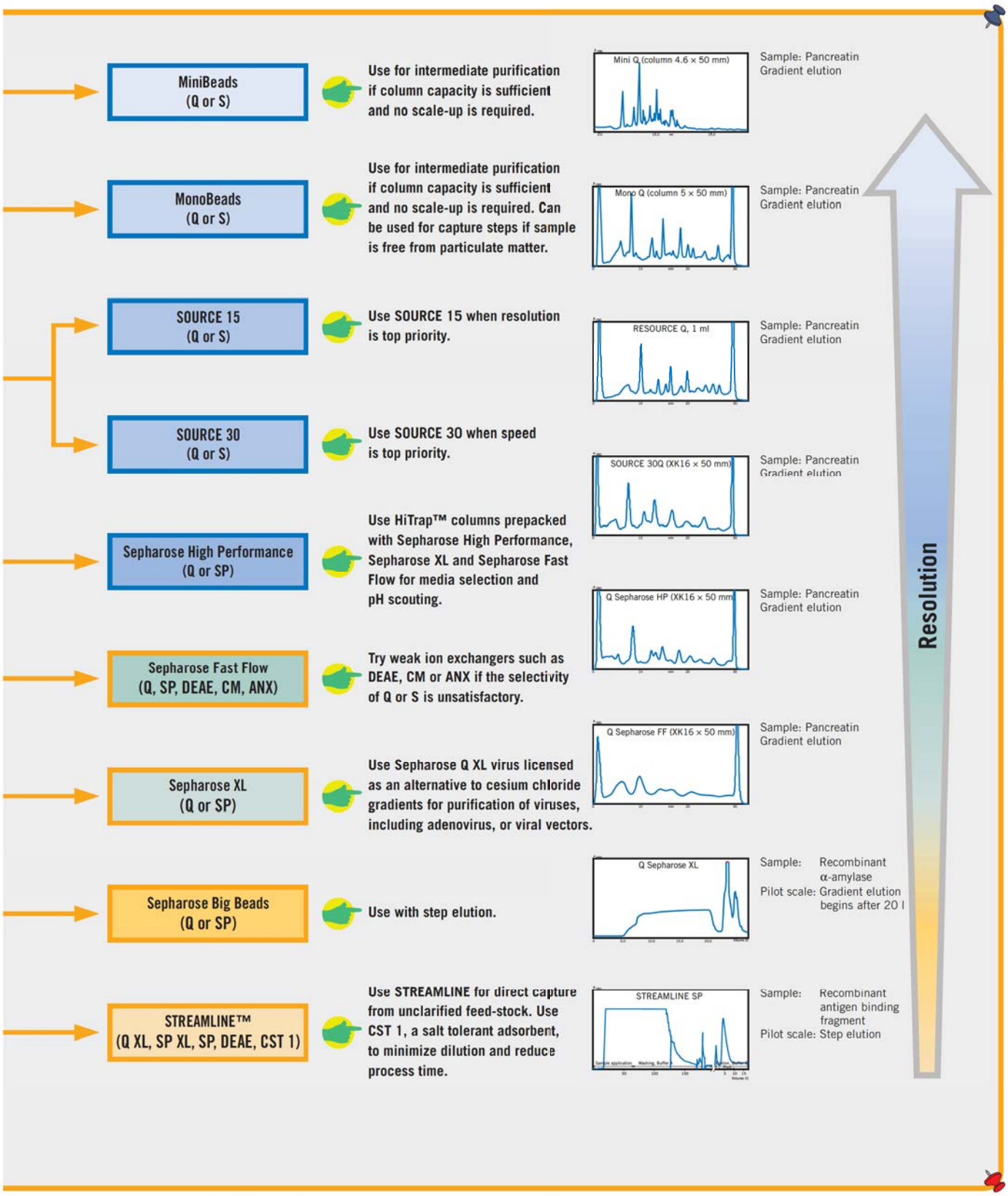


Fig. 15.

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.

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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*.

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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.

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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.

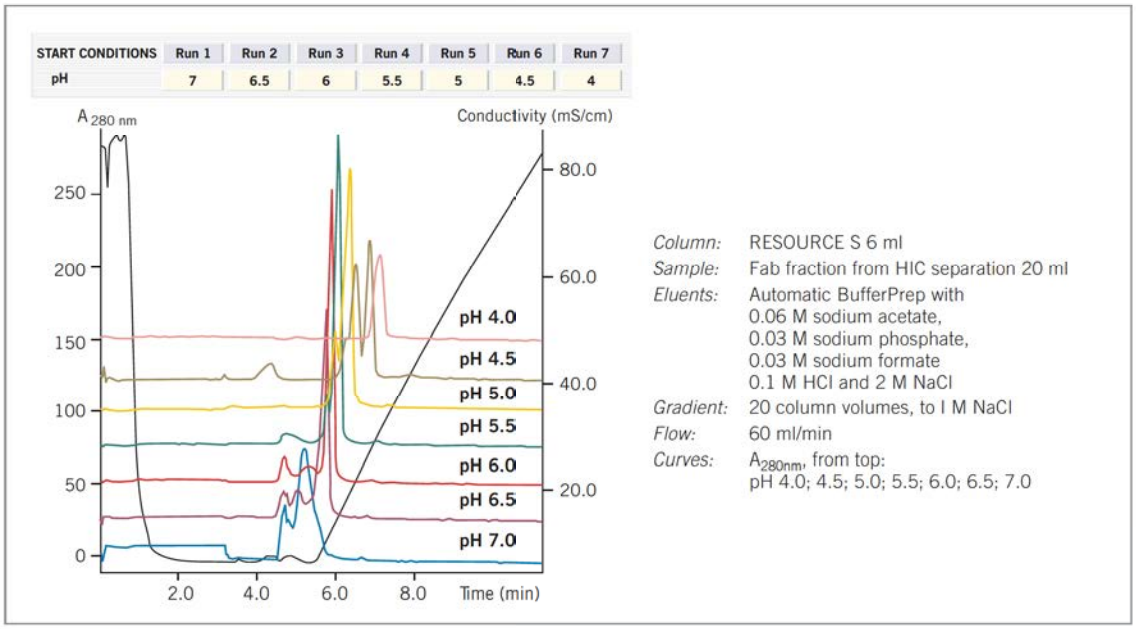


Fig. 17. Automatic pH scouting on ÄKTAexplorer™.

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.

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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.

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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.

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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

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(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.

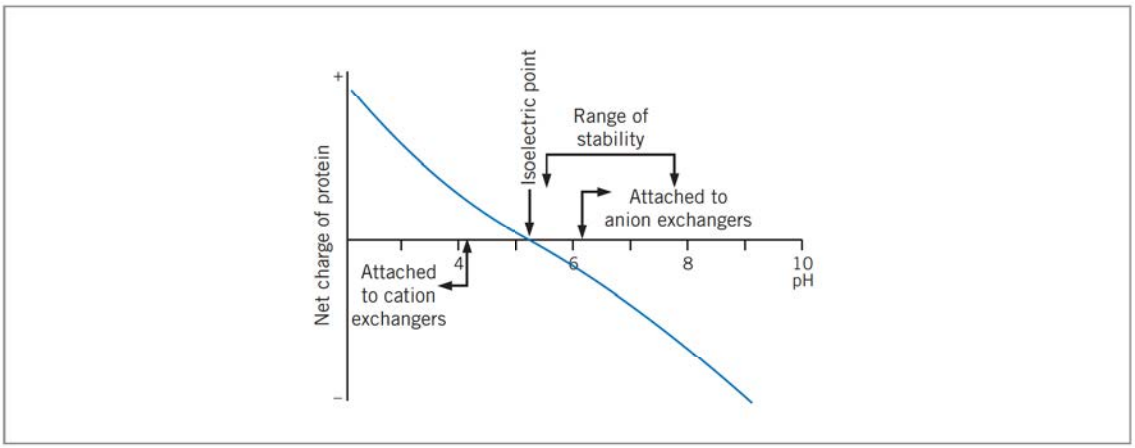


Fig. 18. Considerations when selecting a suitable IEX medium.

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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.

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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 ± 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.

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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.

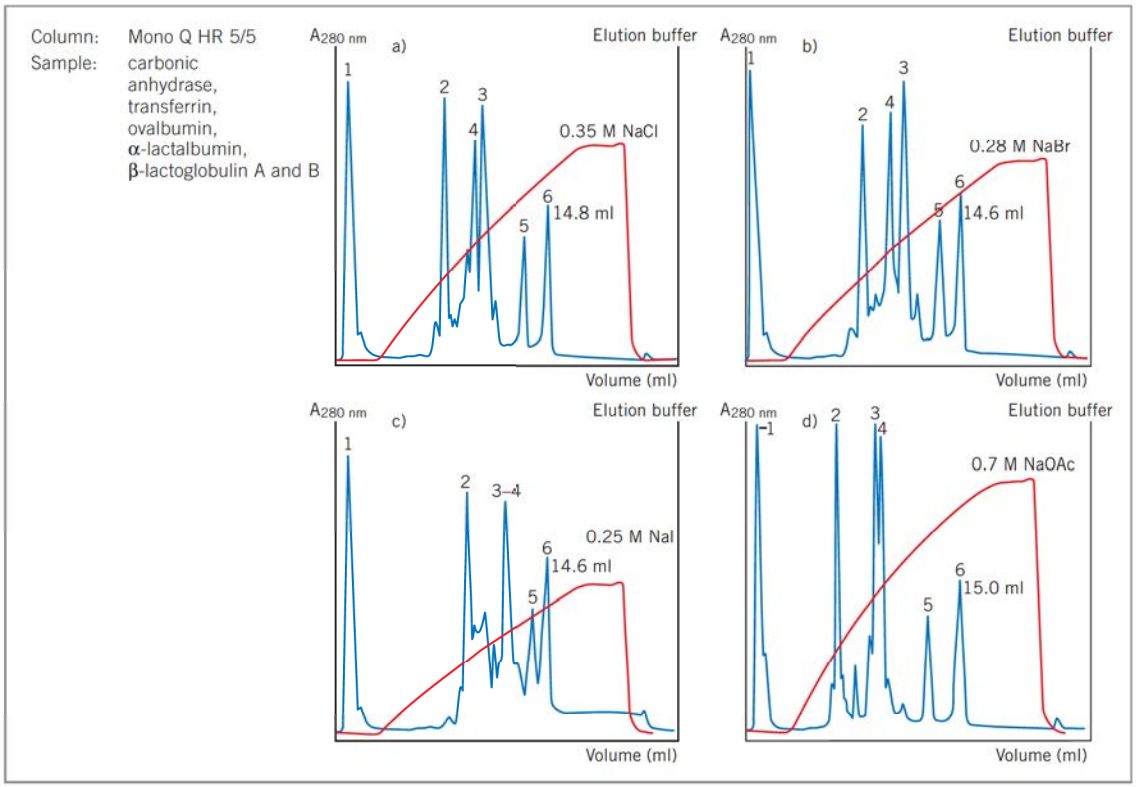


Fig. 19. Effect of counter-ions on selectivity and resolution (Mono Q HR 5/5 now available as Mono Q 5/50 GL). Note the variation in elution order of peaks 3 and 4.

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.

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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.

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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.

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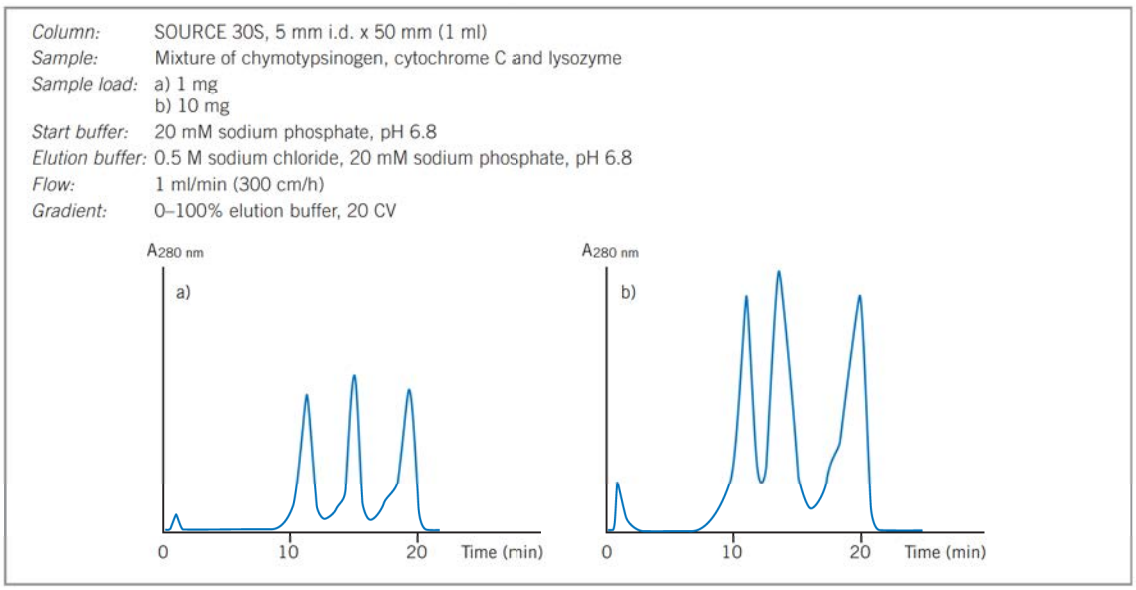


Fig. 20. The influence of increasing sample load on resolution.

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.

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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).

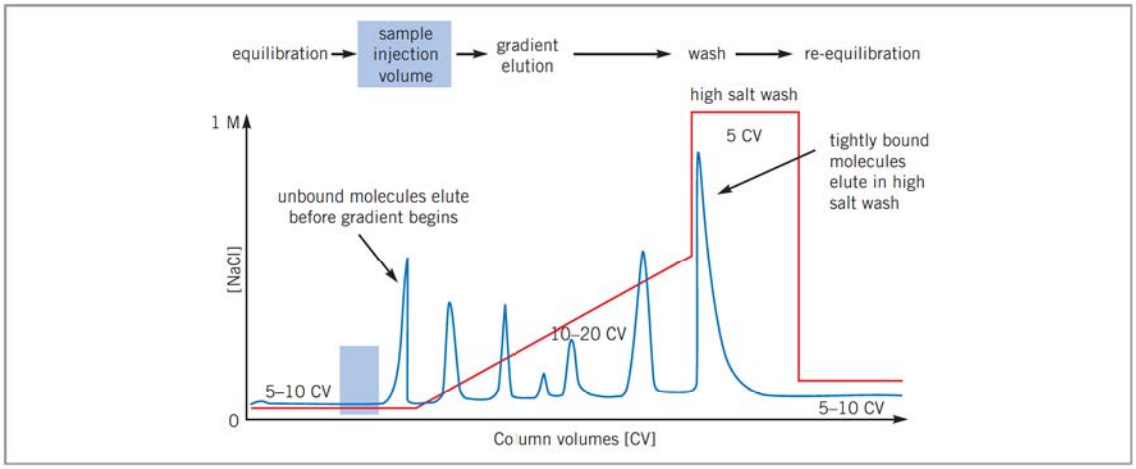


Fig. 21. Typical IEX separation using linear gradient elution. The UV (protein) and conductivity (salt) traces show the elution of protein peaks and the changes in salt concentration during elution.

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.

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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.

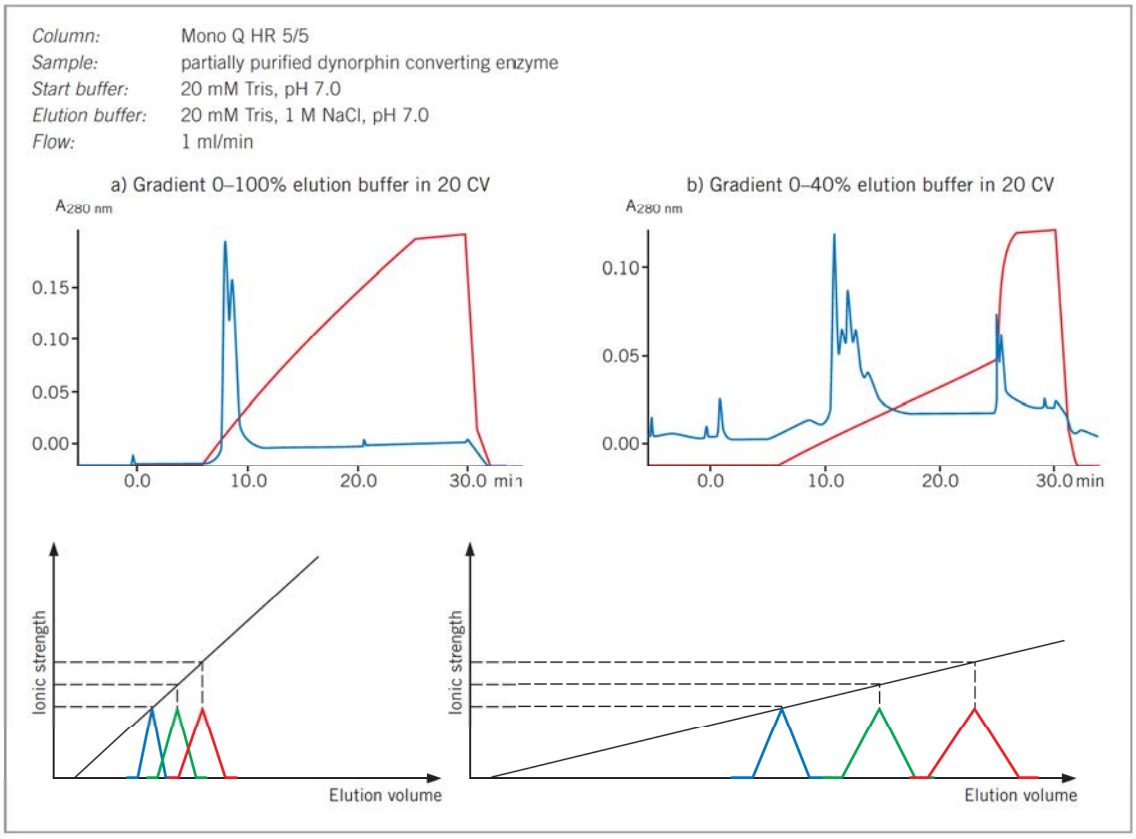


Fig. 22. Effect of gradient slope on resolution, in theory and in practice.

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.

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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.

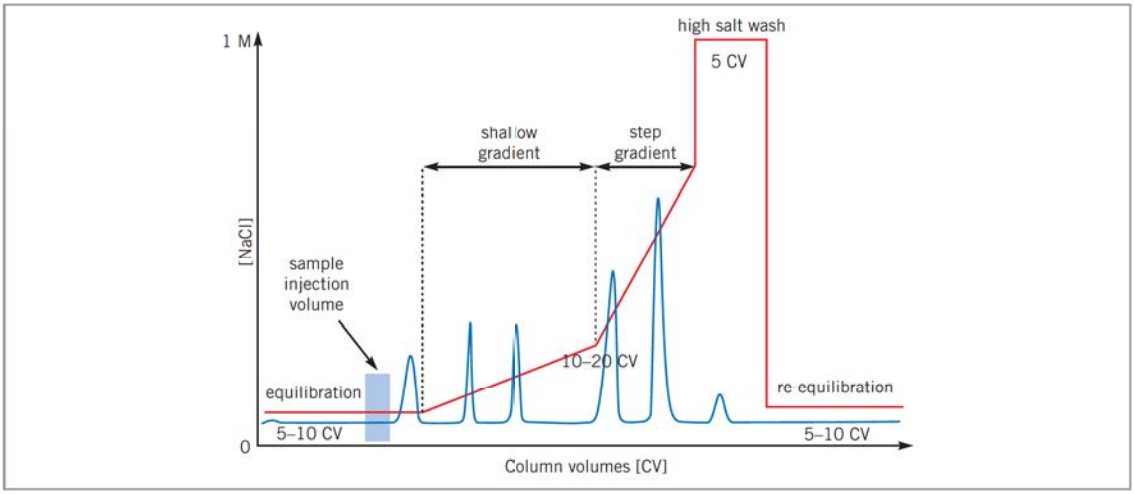


Fig. 23. Complex gradient profiles can reduce total separation time for certain separations.

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.

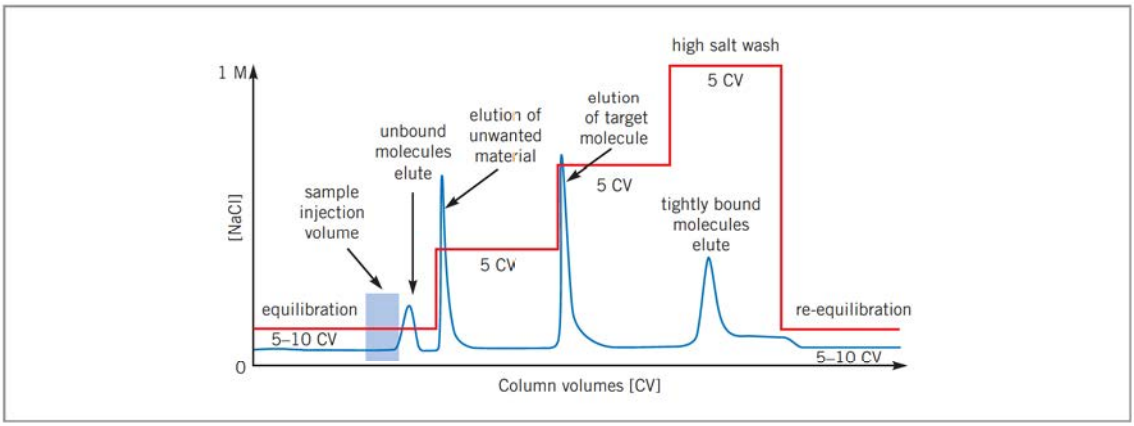


Fig. 24. Typical IEX separation using step elution. The UV (protein) and conductivity (salt) traces show the elution of protein peaks and the changes in salt concentration during elution.

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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.

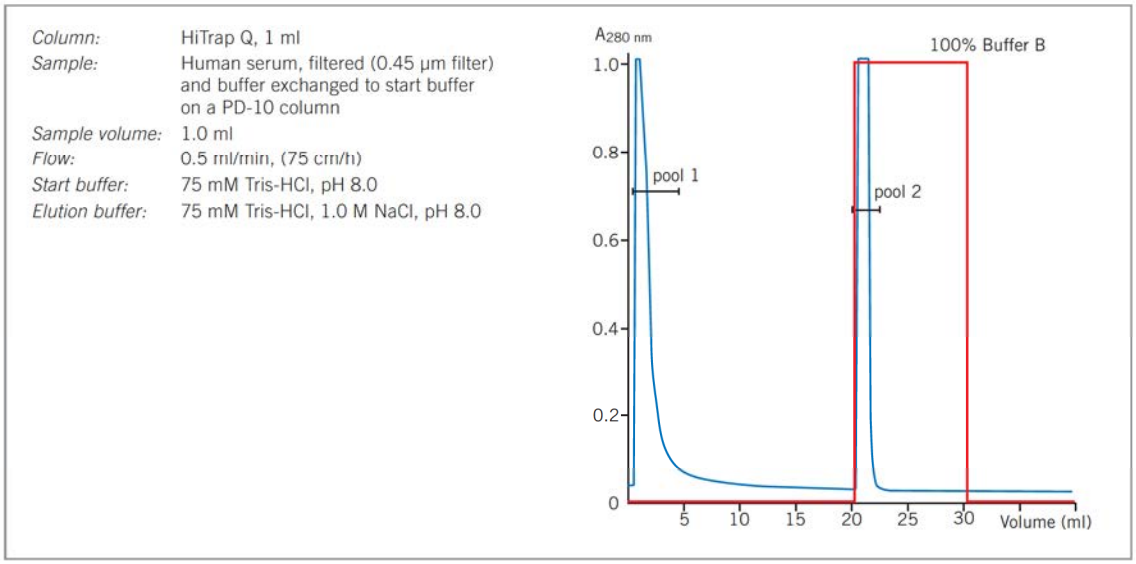


Fig. 25. Group separation of serum proteins on HiTrap Q HP.

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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.

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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.

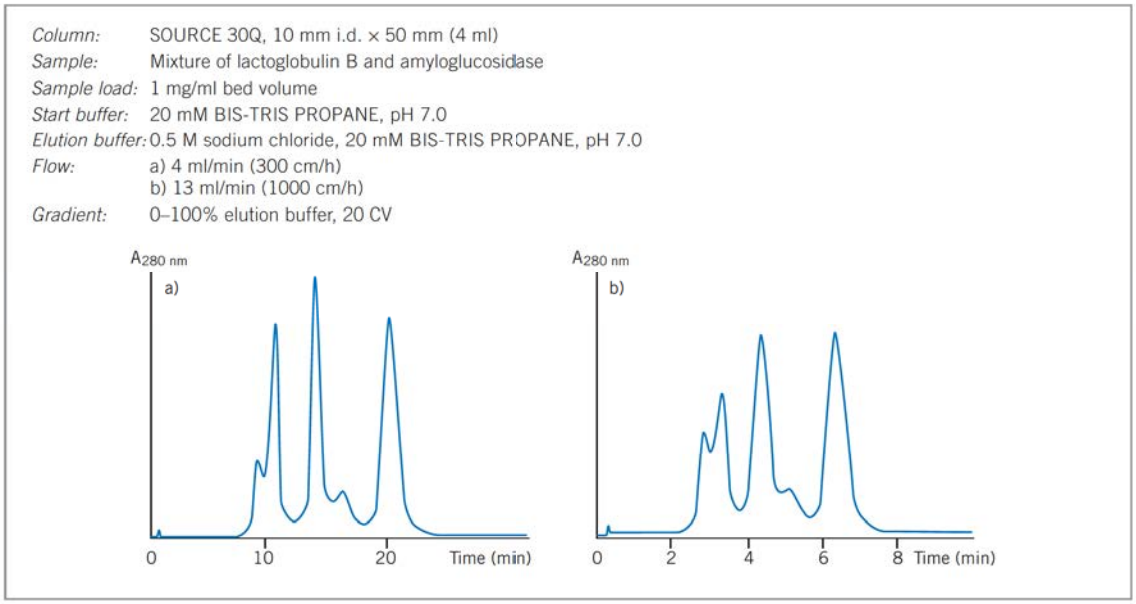


Fig. 26. Influence of increasing flow rate on resolution.

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.

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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.

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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.

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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.

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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.

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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.

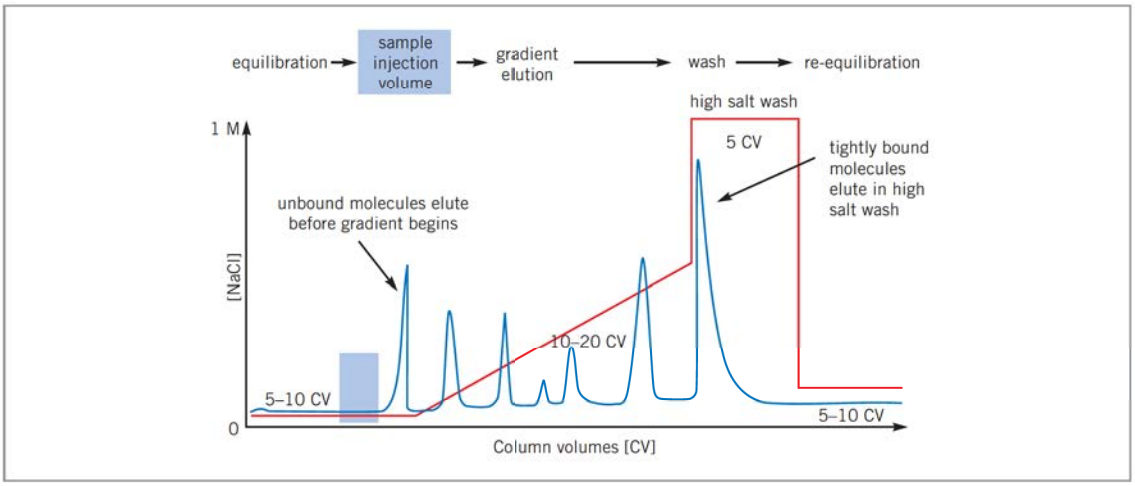


If an increase in back pressure is observed, either on the pressure monitor or by seeing the surface of the medium move downwards, check that the problem is actually caused by the column before starting the cleaning procedure. Disconnect one piece of equipment at a time (starting at the fraction collector), 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.

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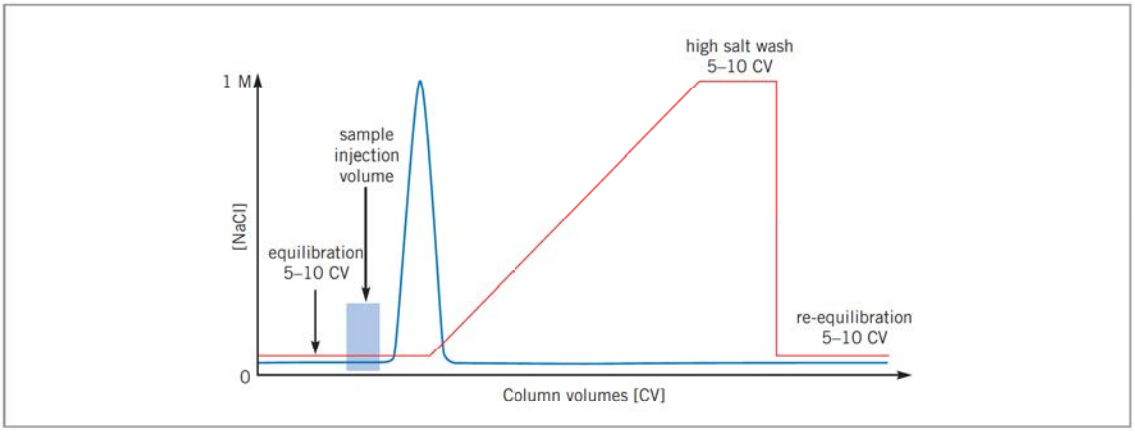
Troubleshooting

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.

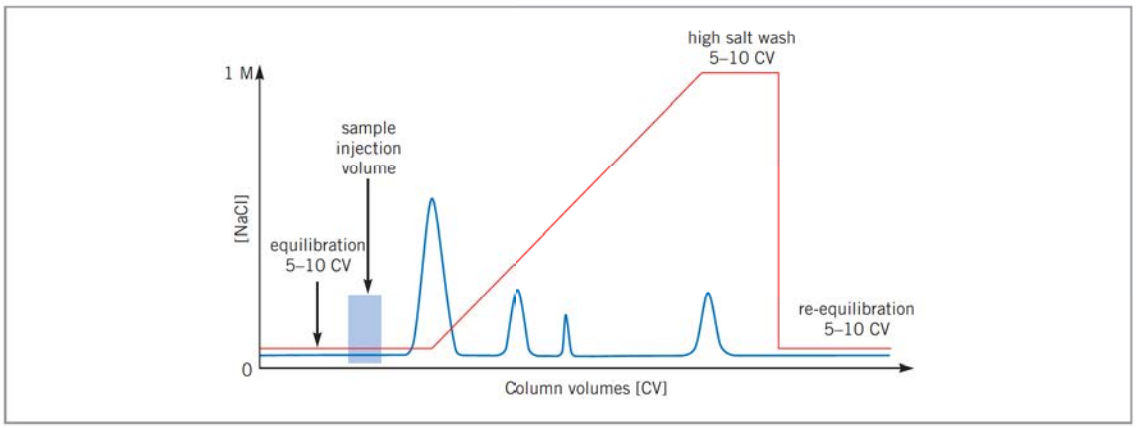
Sample elutes before salt gradient begins



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.

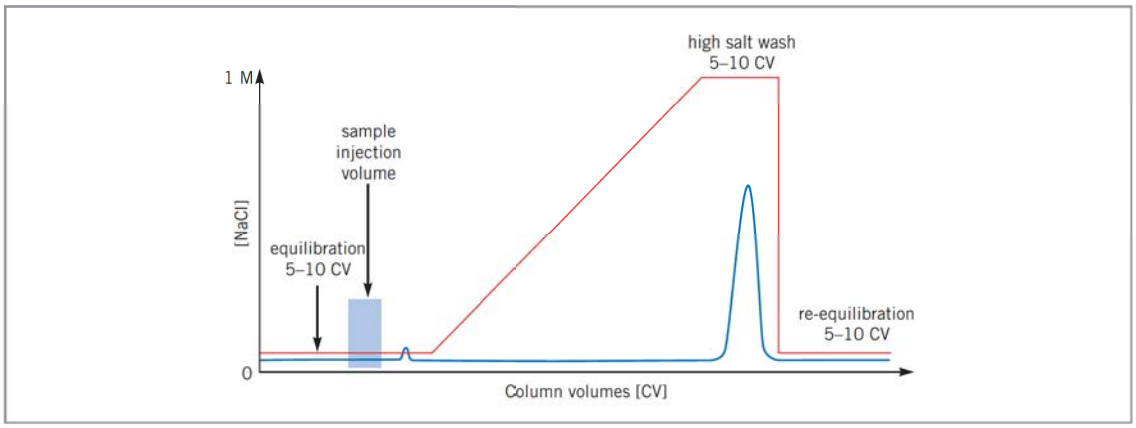
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Sample still eluting when gradient begins



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.

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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.

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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.

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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.*

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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.

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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.

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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*

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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.*

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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

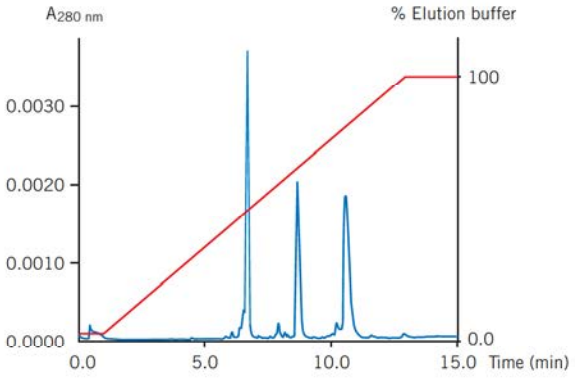


Fig. 28. Separation of a protein mixture on Mini S 4.6/50.

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)

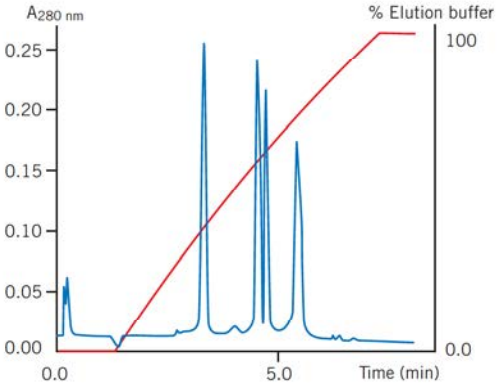


Fig. 29. Mini S PC 3.2/3 gives fast, high resolution separation.

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

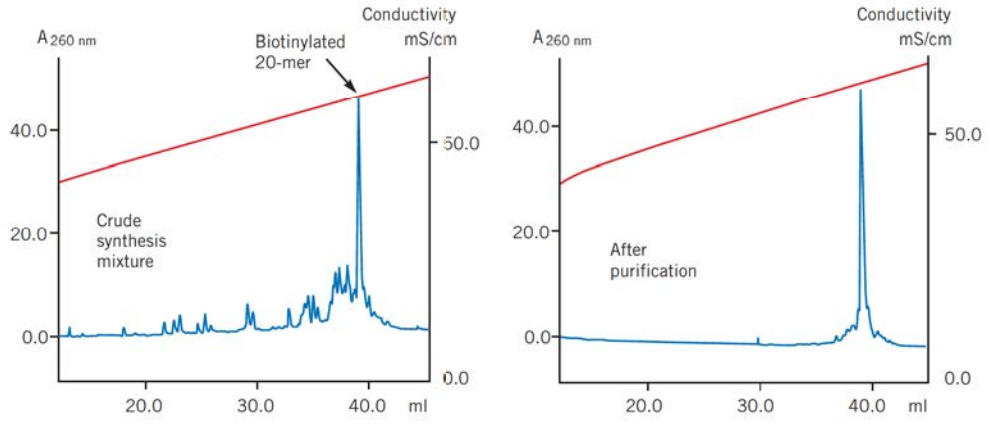


Fig. 30. Purity check of 5'-biotinylated synthetic oligonucleotide 20-mer on Mini Q 4.6/50 PE before and after purification on a RESOURCE RPC column.

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Long term reproducibility

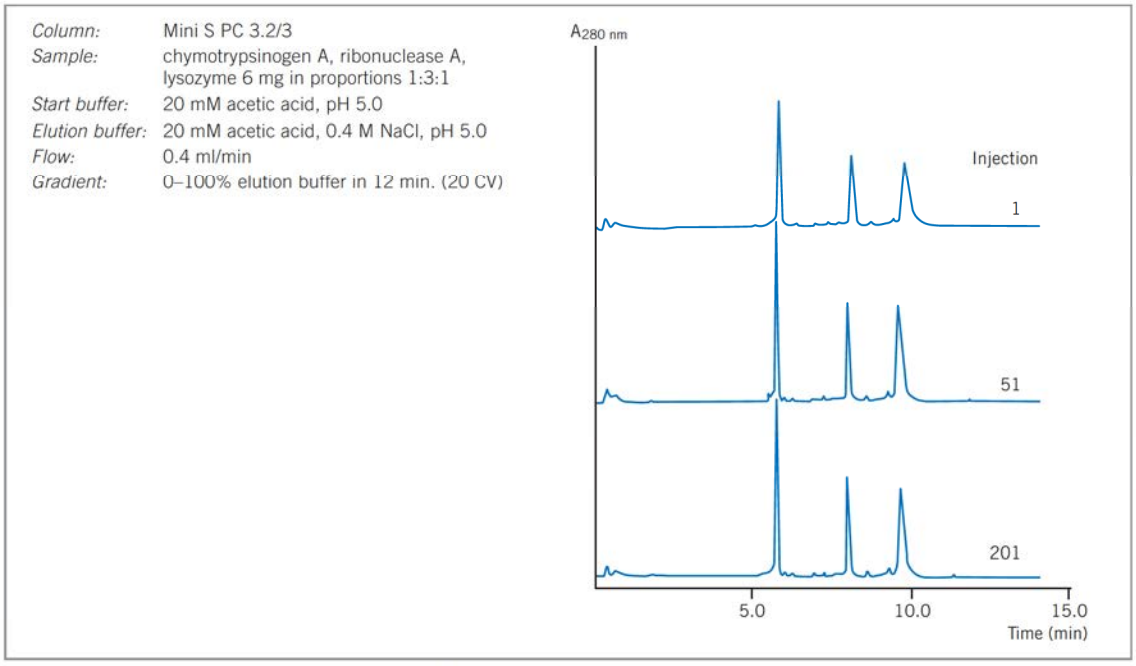


Fig. 31. Chromatograms from the 1st, 5th and 201st separation of a series run on the same Mini S PC 3.2/3 column. The same consistent reproducibility has been confirmed on Mini Q PC 3.2/3 (data not shown).

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.

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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.

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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.

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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.

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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.

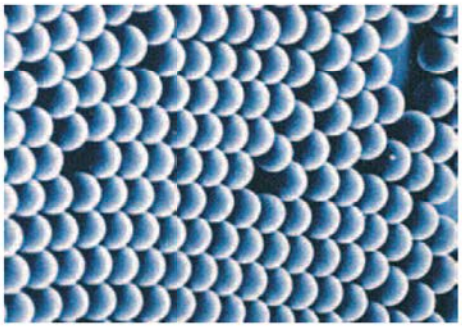


Fig. 32. Electron micrograph of MonoBeads showing their distinct monodispersity.

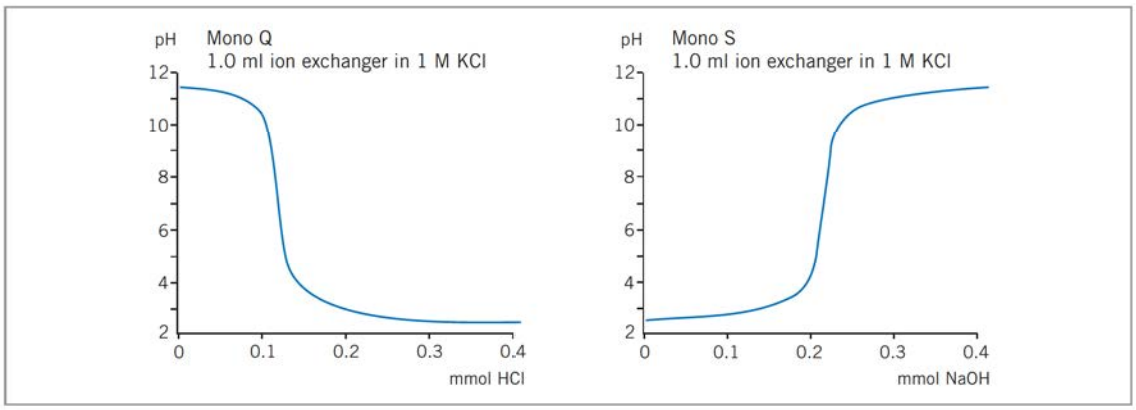


Fig. 33. Titration curves for Mono Q and Mono S™. Binding capacity remains constant over a broad pH working range.

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

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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.*

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Purification examples

Two step purification using complementary selectivities

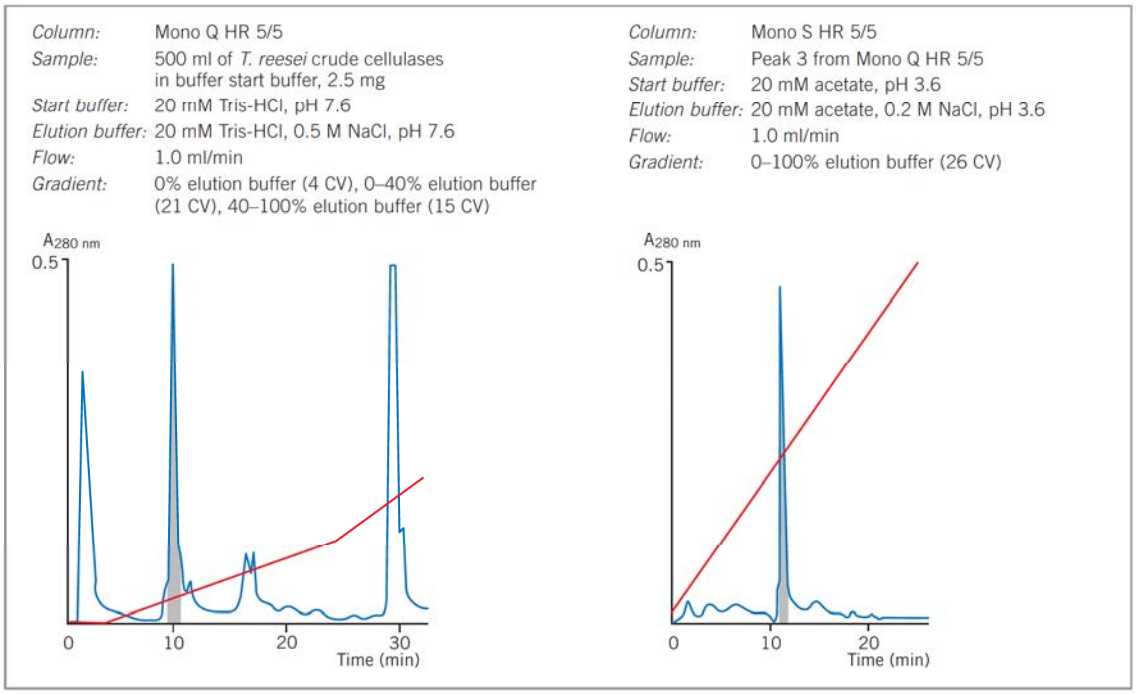


Fig. 35. Purification of cellulose on Mono Q and Mono S HR 5/5 columns (now available as Mono Q 5/50 GL and Mono S 5/50 GL).

High resolution, polishing step

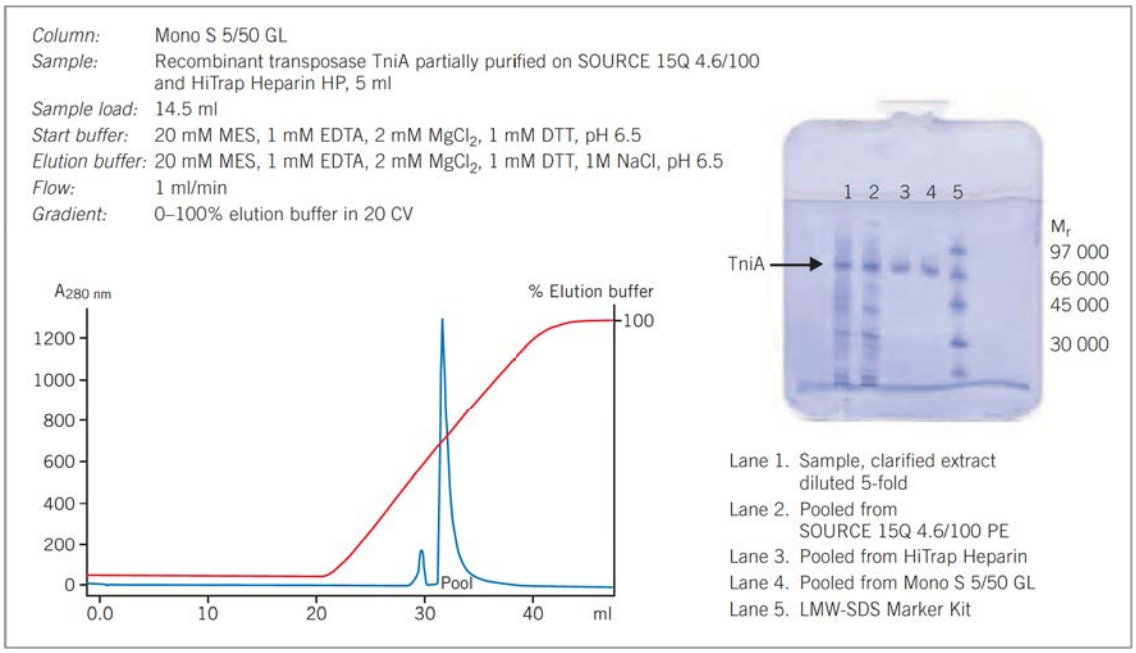


Fig. 36. Final polishing step in purification of a DNA binding protein, transposase TniA. Two well-resolved peaks after separation on Mono S 5/50 GL (a). SDS-PAGE analysis shows fractions from each of the three steps used in this protocol (b), PhastSystem™ using SDS-PAGE PhastGel™ Homogenous – 12.5 and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

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Long term reproducibility

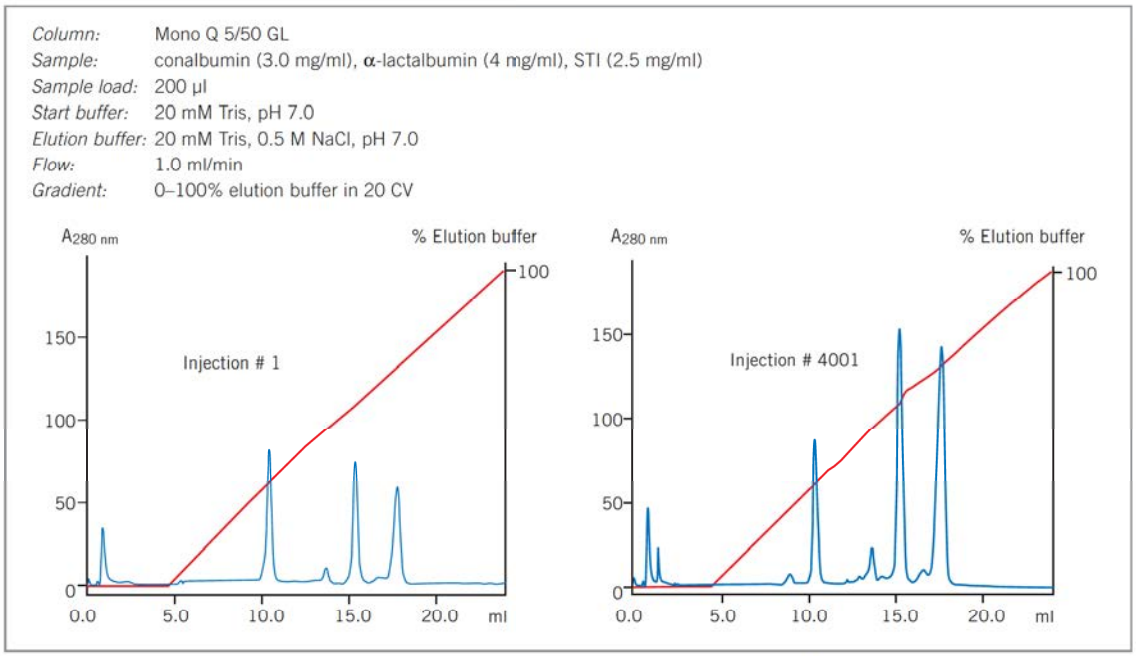


Fig. 37. Chromatograms illustrating run to run reproducibility for Mono Q 5/50 GL (Tricorn). Runs 1, 1000 and 2000 are shown.

Separation in organic solvents

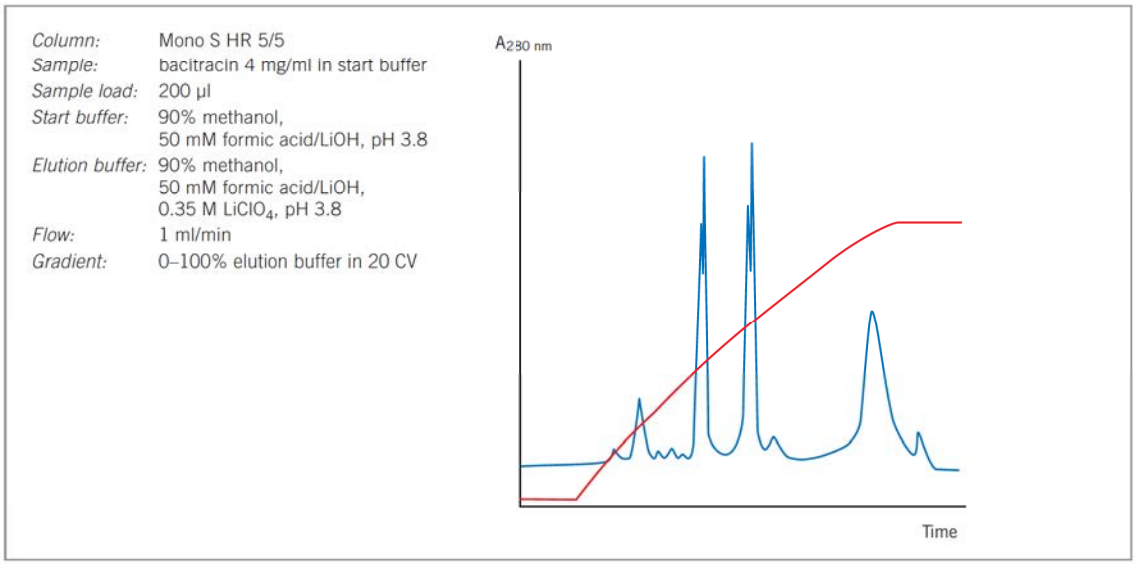


Fig. 38. Separation of the peptide bacitracin on Mono S HR 5/5 (now available as Mono S 5/50 GL).

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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.

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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.

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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.

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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™.

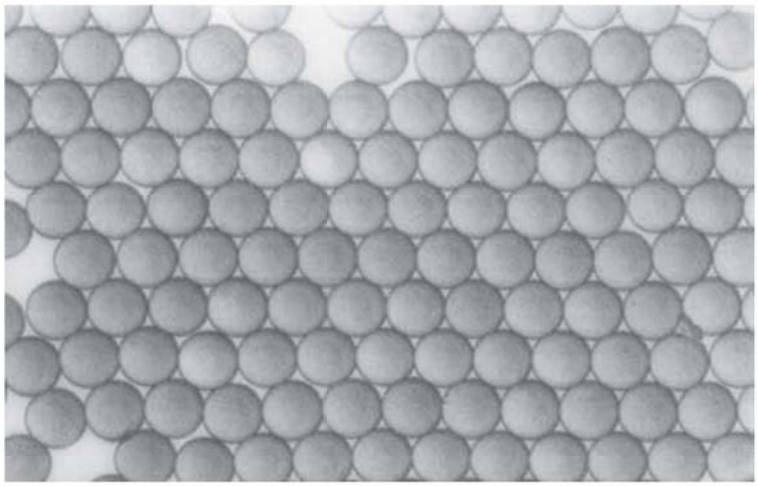


Fig. 39. Uniform size distribution of SOURCE monodispersed particles.

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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.*

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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

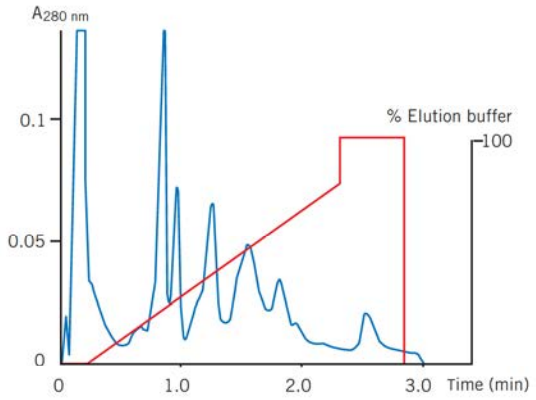


Fig. 41. Separation of pancreatin on RESOURCE Q, 1 ml within 3 minutes.

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

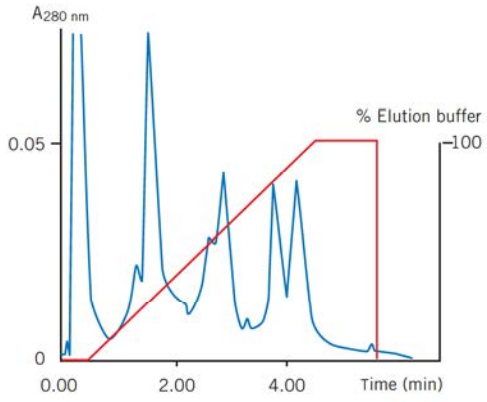


Fig. 42. Separation of snake venom on RESOURCE S, 1 ml within 4 minutes.

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Scaling up: resolution maintained

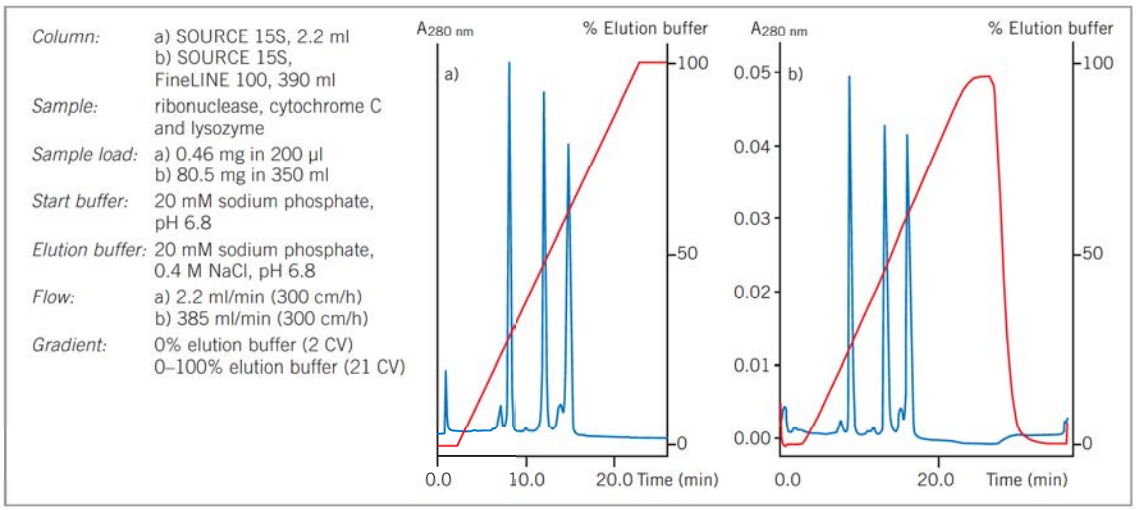


Fig. 43. Separation of proteins scaled up from a 2.2 ml column to a 390 ml column.

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.

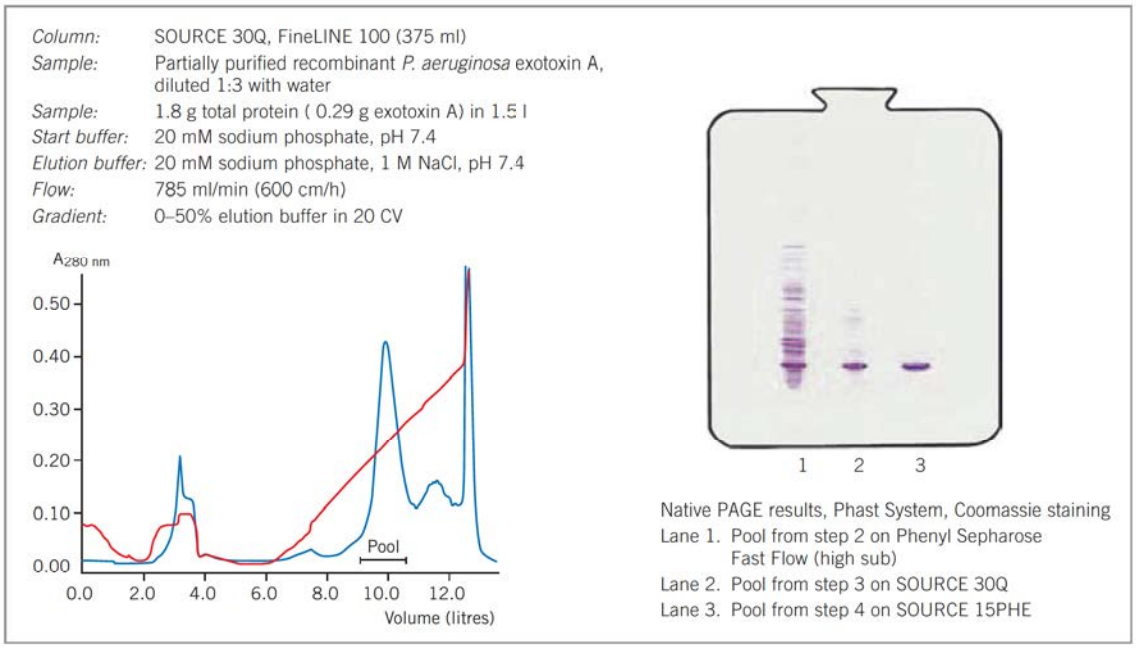


Fig. 44. Intermediate purification of recombinant *P. aeruginosa* exotoxin A on SOURCE 30Q.

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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.

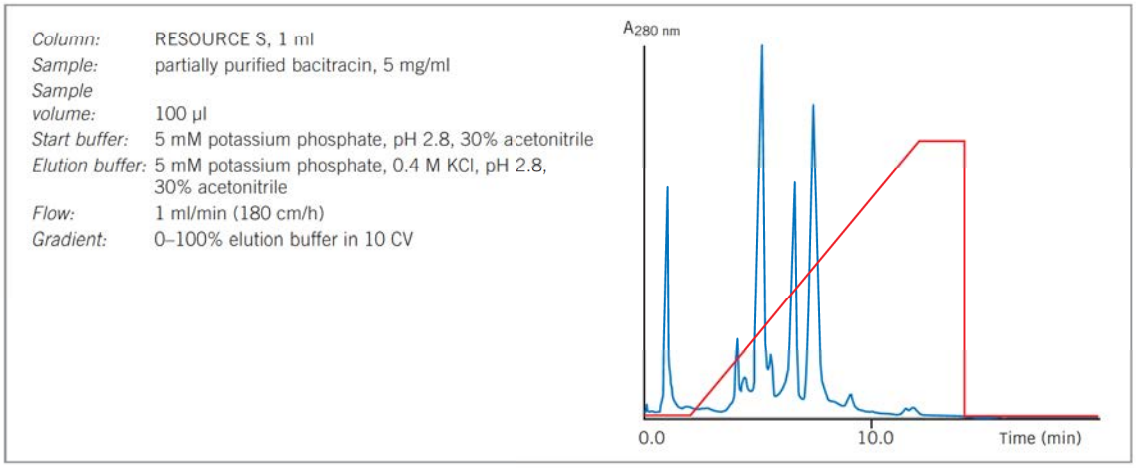


Fig. 45. Intermediate purification of the peptide bacitracin from *Bacillus subtilis* on RESOURCE Q, 1 ml.

Method optimization

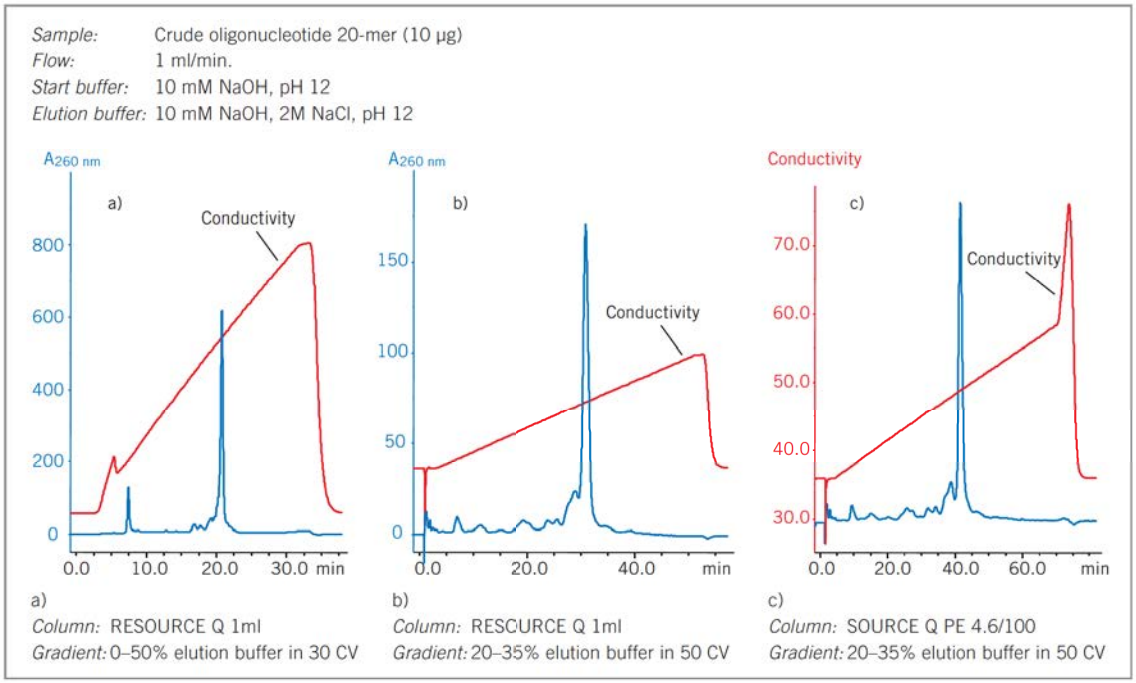


Fig. 46. Manipulation of gradient slope and shape to maximize resolution. Initial purification of a 20 mer oligonucleotide was optimized on RESOURCE Q 1 ml and transferred to SOURCE Q PE 4.6/100 to further increase resolution by increasing bed height to 10 cm.

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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.

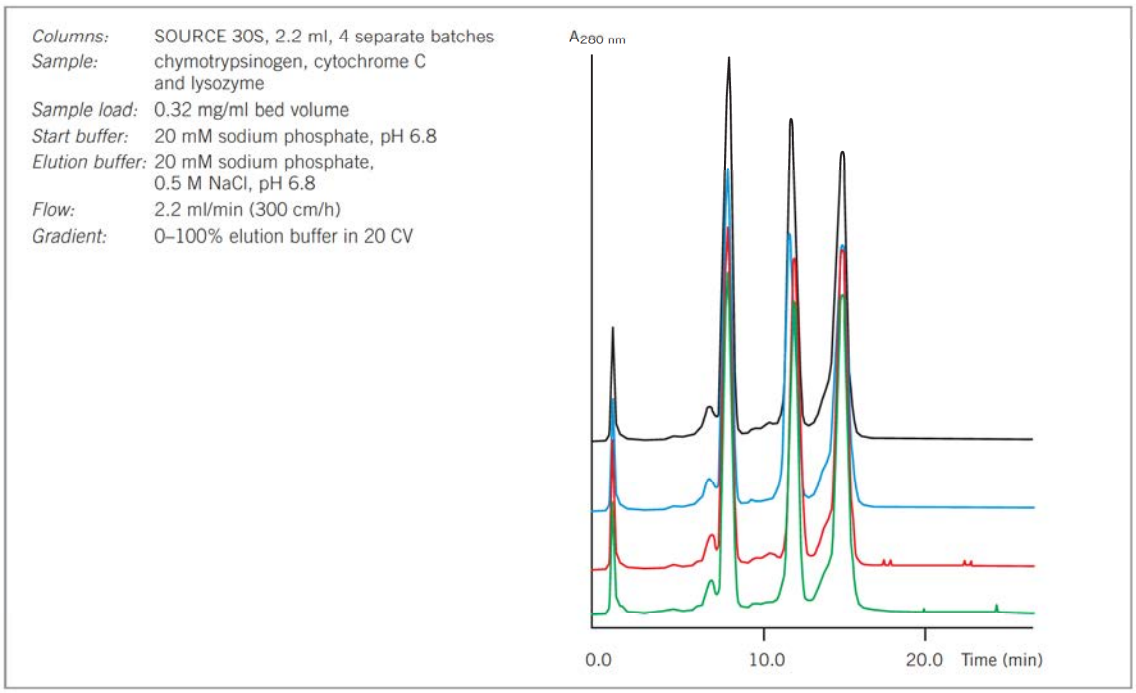


Fig. 47. Selectivity tests on 4 production batches of SOURCE 30S.

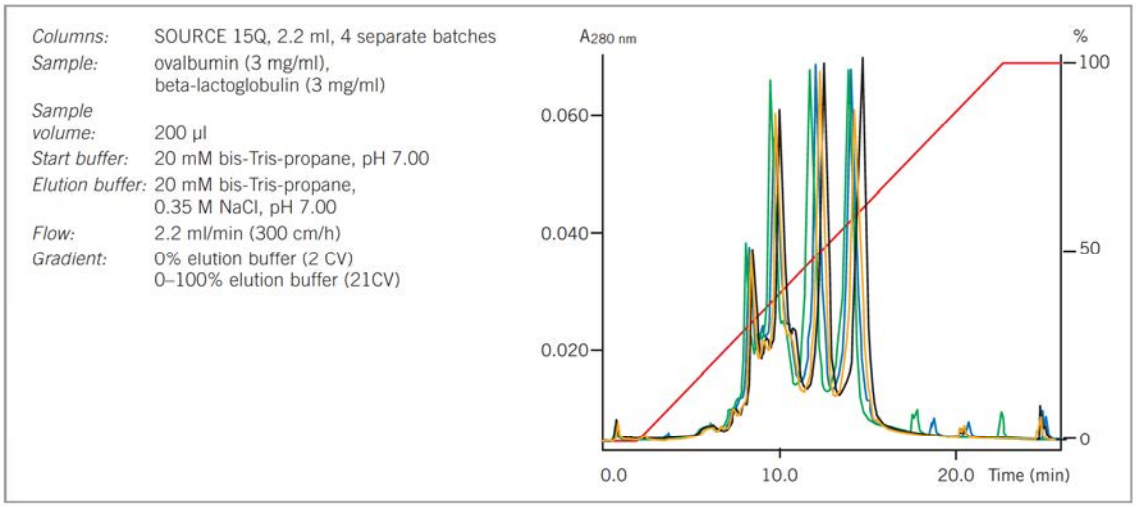


Fig. 48. Selectivity tests on 4 production batches of SOURCE 15Q.

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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.

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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.

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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.

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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.

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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.

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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.

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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)

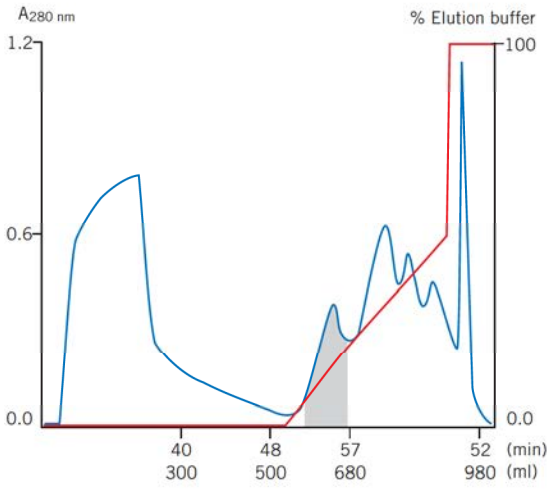


Fig. 50. Intermediate step from a purification protocol for α2-macroglobulin. An affinity step on Blue Sepharose was used to remove albumin, the major contaminant, before IEX.

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

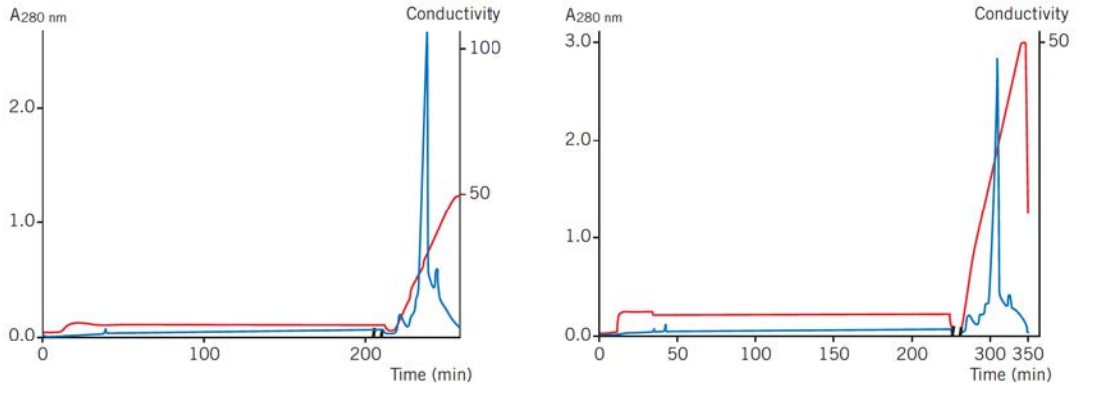


Fig. 51. Elution pattern, purity and yield are maintained when scaling-up from a HiLoad column to a BPG column.

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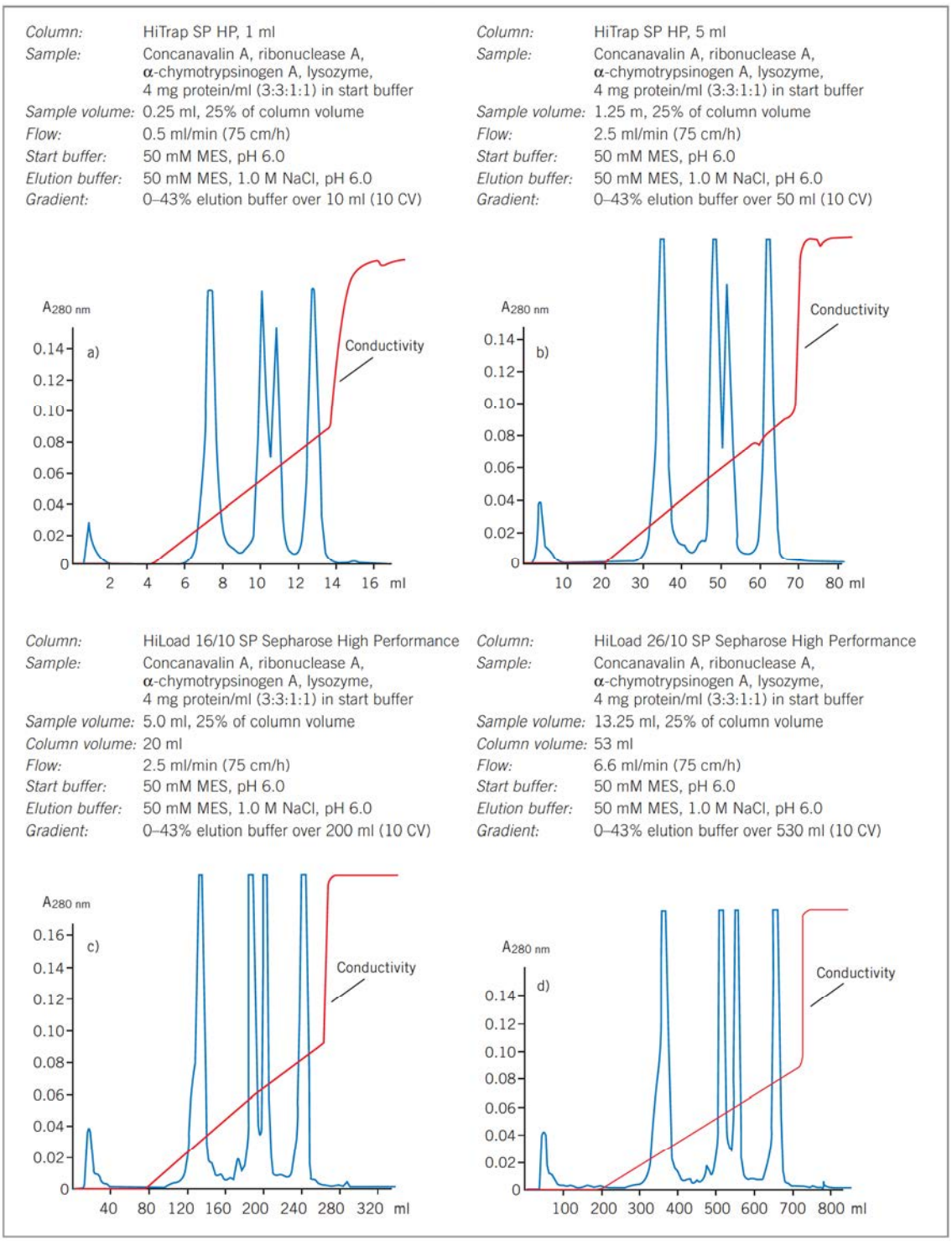


Fig. 52. Reproducibility maintained as purification is scaled up from a 1 ml HiTrap column through to a 53 ml HiLoad 26/10 SP Sepharose High Performance column.

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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.

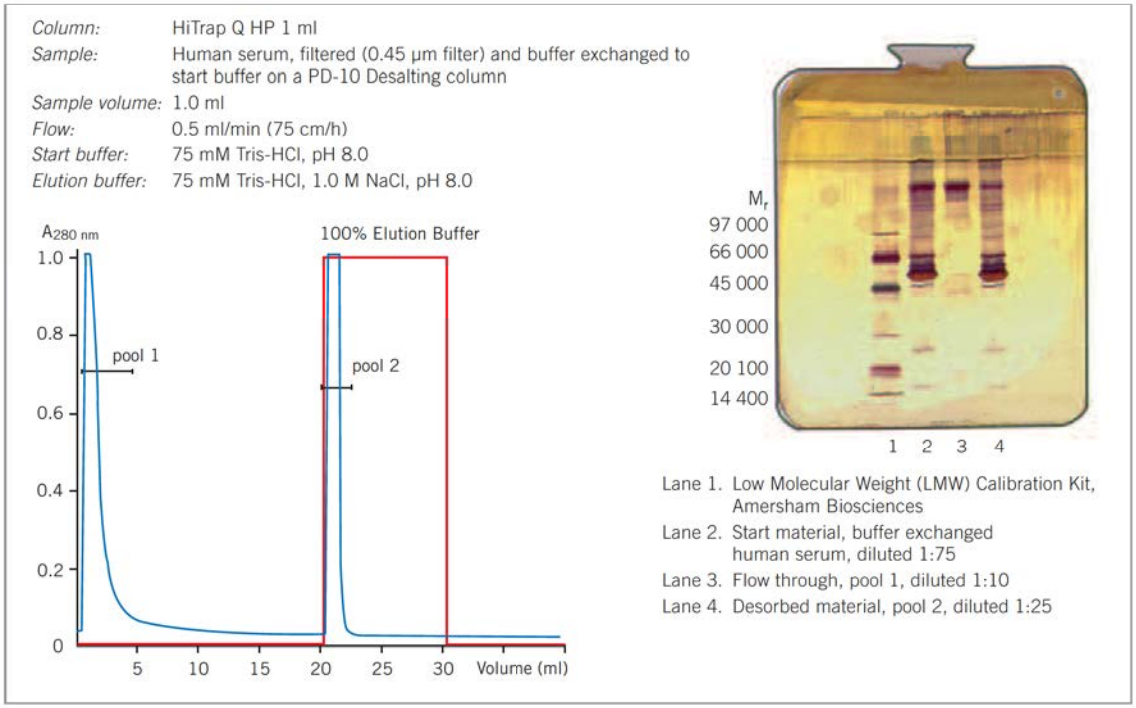


Fig. 53. Separation of IgG from human serum proteins on HiTrap Q HP, 1 ml, using one step elution. Analysis by SDS-PAGE (Phast System, PhastGel 10–15, silver staining).

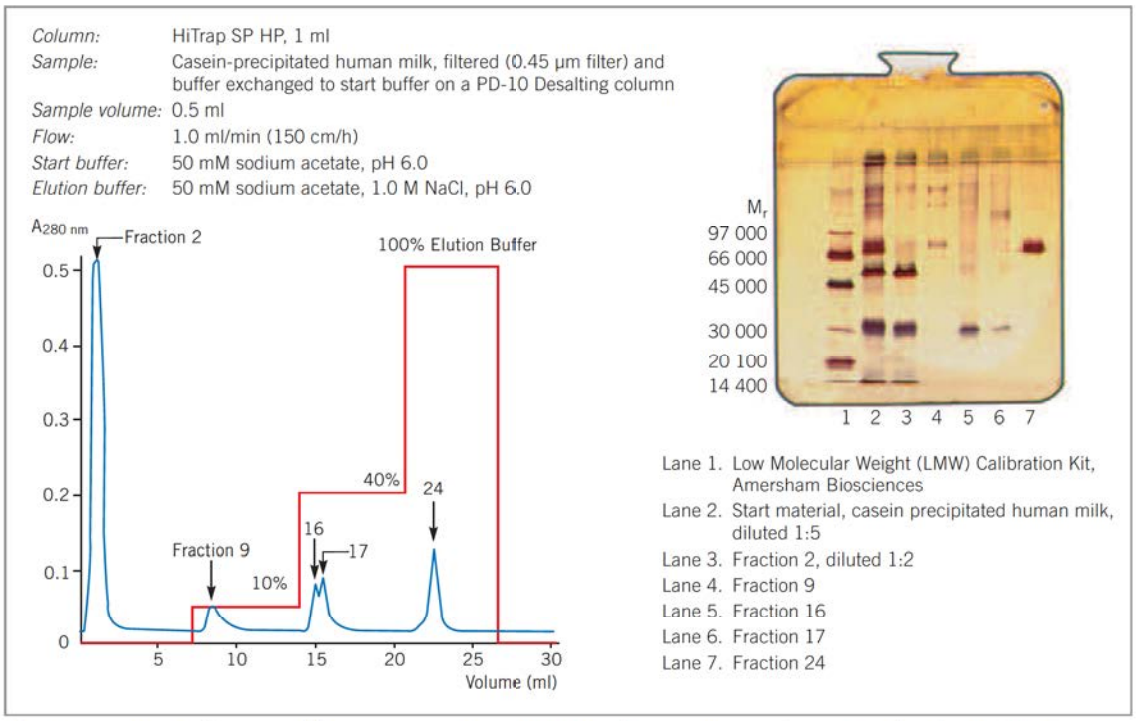


Fig. 54. Separation of human milk proteins on HiTrap SP HP, 1 ml, using one step elution. Analysis by SDS-PAGE (Phast System, PhastGel 10–15, silver staining).

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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.

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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.

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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.

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