

# Hydrophobic Interaction and Reversed Phase Chromatography

Principles and Methods



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# Hydrophobic Interaction and Reversed Phase Chromatography

Principles and Methods

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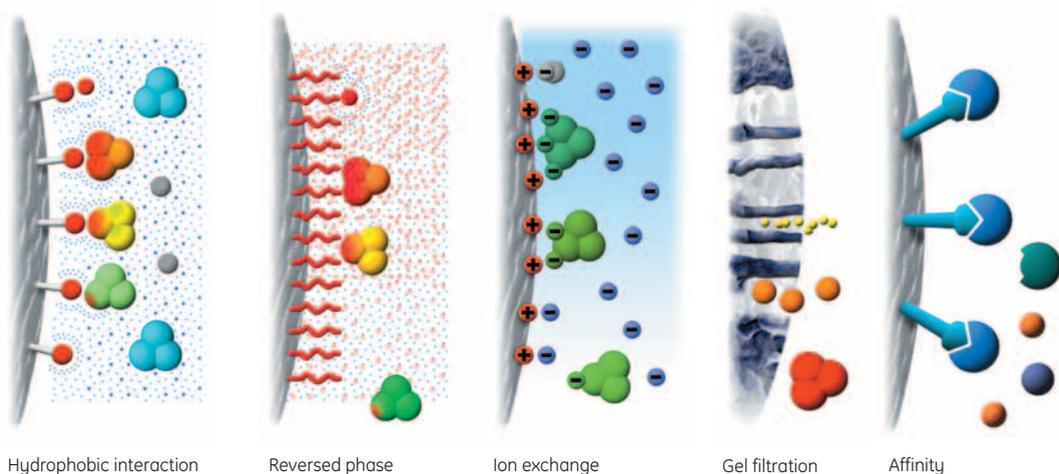
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# Introduction

Biomolecules are purified using chromatography techniques that separate them according to differences in their specific properties, as shown in Figure 1. Hydrophobic interaction chromatography (HIC) separates biomolecules, under relatively mild conditions, according to differences in their hydrophobicity.

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



**Fig 1.** Separation principles in chromatographic purification.

HIC is widely used in protein purification as a complement to other techniques that separate according to charge, size or biospecific recognition. The technique is an ideal next step when samples have been subjected to ammonium sulfate precipitation (frequently used for initial sample concentration and clean-up) or after separation by ion exchange chromatography. In both situations the sample contains a high salt concentration and can be applied directly to the HIC column with little or no additional preparation. The elevated salt level enhances the interaction between the hydrophobic components of the sample and the chromatography medium. During separation, samples are purified and eluted in smaller volumes, thereby concentrating the sample so that it can go directly to gel filtration or, after a buffer exchange, to an ion exchange separation. HIC can be used for capture, intermediate purification or polishing steps in a purification protocol. Media from GE Healthcare can be used for small-scale separations in the laboratory through to production of kilogram quantities of product.

This handbook describes both theoretical and practical aspects of the technique, the media available and how to select them, together with application examples and detailed instructions for the most commonly performed procedures. Practical information, with many tips and hints drawn from over 40 years of experience in chromatography purification, guides beginners and experts toward obtaining the best possible results from the latest chromatography media.

Chapter 5 focuses on RPC, a technique that provides the highest-resolution separations and is well suited for analytical work, for example, in combination with mass spectrometry for identification and characterization or when checking product purity. RPC can also be used as a final polishing step in a purification strategy, but care must be taken to ensure that the presence of organic solvents does not compromise recovery of biological activity or tertiary structure.

GE Healthcare offers a wide variety of prepacked columns and ready-to-use chromatography media and also provides a range of handbooks. The different techniques are covered in these handbooks, which ensure that purification becomes a simple and efficient procedure at any scale and in any laboratory.

## Symbols



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



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



highlights troubleshooting advice to help analyze and resolve difficulties.



highlights chemicals, buffers and equipment.



provides outline of experimental protocol.

## Common abbreviations

### In chromatography

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

AC: affinity chromatography

CF: chromatofocusing

CIPP: Capture, Intermediate Purification and Polishing

CV: column volume

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

HIC: hydrophobic interaction chromatography

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

MPa: megaPascal

$M_r$ : relative molecular weight

N/m: column efficiency expressed as theoretical plates per meter

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

psi: pounds per square inch

RPC: reversed phase chromatography

$R_s$ : resolution, the degree of separation between peaks

SDS: sodium dodecyl sulfate

### Abbreviations found in product names

CIP: cleaning in place

FF: Fast Flow

HMW: high molecular weight

HP: High Performance

i.d.: inner diameter

LMW: low molecular weight

PE: PEEK

ST: column manufactured in stainless steel

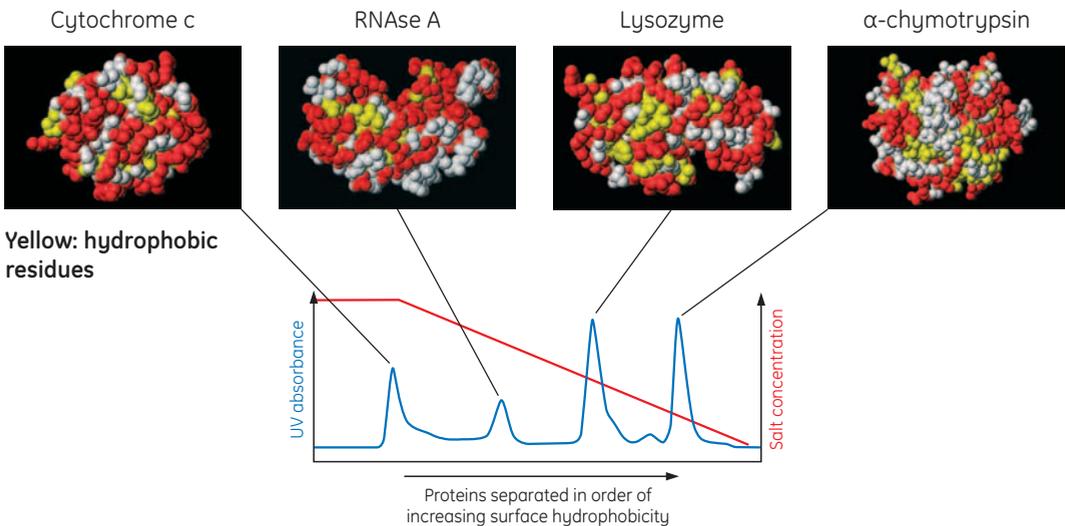
# Chapter 1

## Principles of hydrophobic interaction chromatography

This chapter provides a brief introduction to hydrophobic interaction chromatography (HIC), focusing mainly on the basic principles applied to achieve a separation. Practical aspects of performing a separation are covered in Chapter 2.

HIC separates proteins according to differences in their surface hydrophobicity by utilizing a reversible interaction between these proteins and the hydrophobic surface of a HIC medium. There is no universally accepted theory on the mechanisms involved in HIC, even though a number of suggestions can be found in the scientific literature.

Figure 2 shows how standard proteins with different degrees of surface hydrophobicity can be separated. The interaction between hydrophobic proteins and a HIC medium is influenced significantly by the presence of certain salts in the running buffer. A high salt concentration enhances the interaction while lowering the salt concentration weakens the interaction. In this example, all three proteins interact with the hydrophobic surface of the HIC medium, but, as the ionic strength of the buffer is reduced, the interaction is reversed and the protein with the lowest degree of hydrophobicity is eluted first. The most hydrophobic protein elutes last, requiring a greater reduction in salt concentration to reverse the interaction.



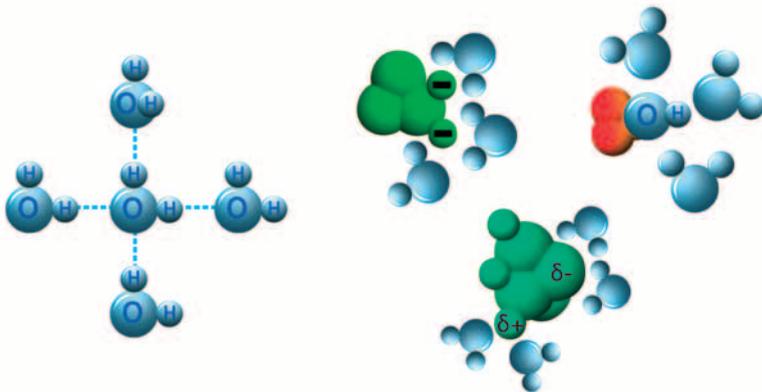
**Column:** Phenyl Sepharose High Performance packed in Tricorn 10/100 column  
**Sample:** Cytochrome c, RNase A, lysozyme,  $\alpha$ -chymotrypsin  
**Start buffer:** 1.7 M ammonium sulfate, 0.02 M Tris-HCl, pH 7.5  
**Elution buffer:** 0.02 M Tris-HCl, pH 7.5  
**Gradient:** 0–100% elution buffer in 10 CV  
**Flow:** 1 ml/min, 76 cm/h

**Fig 2.** Proteins are separated according to differences in their surface hydrophobicity (yellow indicates hydrophobic and red hydrophilic amino acid residues), as shown in this separation of standard proteins on Phenyl Sepharose High Performance.

# Hydrophobic interaction chromatography in theory

## **The role of water**

Water is a good solvent for polar substances, but a poor solvent for non-polar substances. In liquid water a majority of the water molecules occur in clusters due to hydrogen bonding between themselves (Figure 3). Although the half-life of water clusters is very short, the net effect is a very strong cohesion between the water molecules, reflected, for example, by a high boiling point.



**Fig 3.** The solubilizing properties of water reside in its ability to interact with dipoles and to form hydrogen bonds.

At an air-water interface, water molecules arrange themselves into a strong shell of highly ordered structure. Here, the possibility to form hydrogen bonds is no longer in balance, but is dominated by the liquid side of the interface. This gives rise to an ordered structure that manifests itself as a strong surface tension. Anything that influences the stability of the water shell also affects the surface tension.

When a hydrophobic substance such as a protein or hydrophobic ligand is immersed in water something analogous to the surface tension phenomenon happens. The water molecules cannot “wet” the surface of the hydrophobic substance. Instead they form a highly ordered shell around the substance, due to their inability to form hydrogen bonds in all directions.

Minimizing the extent of this shell leads to a decrease in the number of ordered water molecules, that is, a *thermodynamically more favorable situation in which entropy increases*. In order to gain entropy, hydrophobic substances are forced to merge to minimize the total area of such shells. Thus *hydrophobic interaction depends on the behavior of the water molecules rather than on direct attraction between the hydrophobic molecules* (Figure 3).

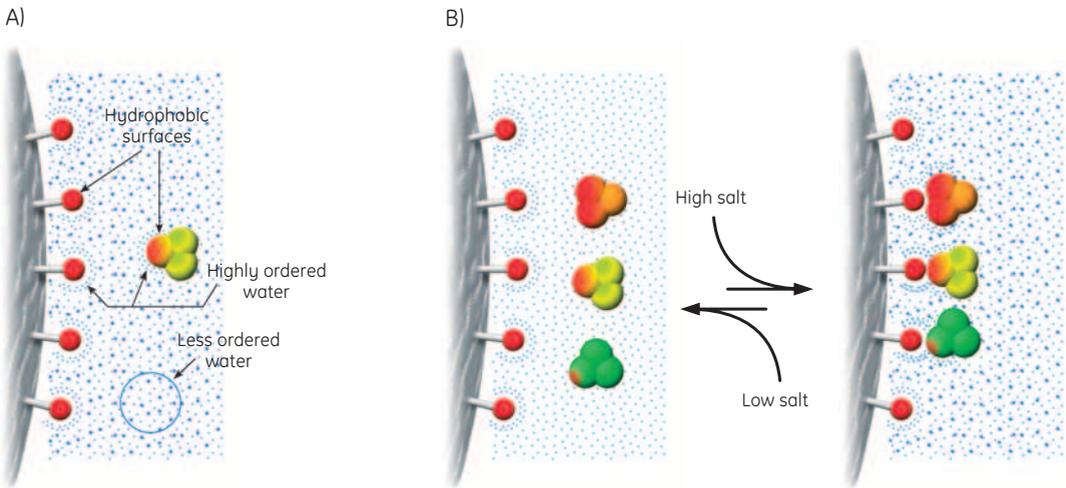
## **Protein structure**

The three-dimensional structure of a protein is a result of intra-molecular interactions as well as interactions with the surrounding solvent. In the case of readily soluble proteins, this solvent is water, and hydrophobic side chains are therefore typically driven to the interior of the protein. The final structure is a result of a thermodynamic compromise that best suits the surrounding solution so that, although hydrophobic amino acid residues are most frequently buried in the interior of globular proteins, some are exposed, resulting in hydrophobic patches on the protein surface.

Since proteins carry both hydrophilic and hydrophobic areas on their surfaces they may precipitate at high concentrations of certain salts, mainly caused by *enforced hydrophobic interaction*. Changes in ionic strength, the presence of organic solvents, temperature and pH (especially at the isoelectric point, pI, when there is no net surface charge) can all affect protein structure and solubility and, consequently, the interaction with other hydrophobic surfaces, such as those in HIC media.

## Reversible interactions

The hydrophobic ligands on HIC media can interact with the hydrophobic surfaces of proteins. In pure water any hydrophobic effect is too weak to cause interaction between ligand and proteins or between the proteins themselves. However, certain salts enhance hydrophobic interactions, and adding such salts brings about binding (adsorption) to HIC media. For selective elution (desorption), the salt concentration is lowered gradually and the sample components elute in order of hydrophobicity (Figure 4 B).



**Fig 4.** A) Highly ordered water shells surround the hydrophobic surfaces of ligands and proteins. Hydrophobic substances are forced to merge to minimize the total area of such shells (maximize entropy). Salts enhance the hydrophobic interaction. B) The equilibrium of the hydrophobic interaction is controlled predominantly by the salt concentration.

The final result of a HIC separation is based therefore on interplay between the prevalence and distribution of surface-exposed hydrophobic amino acid residues, the hydrophobicity of the medium, the nature and composition of the sample, and the type and concentration of salt used in the buffers.

## Steps in a HIC separation

HIC media are composed of *ligands* containing alkyl or aryl groups coupled to an inert *matrix* of spherical particles. The matrix is porous, in order to provide a high internal surface area, while the ligand plays a significant role in the final hydrophobicity of the medium. The medium is packed into a column to form a *packed bed*. For more details on column packing, refer to Appendix 2. The bed is then equilibrated with buffer that fills the pores of the matrix and the space in between the particles. Figure 5 illustrates the separation process that follows.

Interaction between the protein and the medium is promoted by moderately high salt concentrations, typically 1–2 M ammonium sulfate or 3 M NaCl. The type of salt and the concentration required in the start buffer are selected to ensure that the proteins of interest bind to the medium and that other less hydrophobic proteins and impurities pass directly through the column.

-  Binding conditions are a key factor in any HIC separation. It is at this stage where the final selectivity, resolution and binding capacity for the target protein(s) can be significantly influenced. Samples should be in the same salt conditions as the start buffer, but there is rarely any need to perform a buffer exchange as buffer ions and pH play less important roles. The pH can be adjusted directly if necessary.

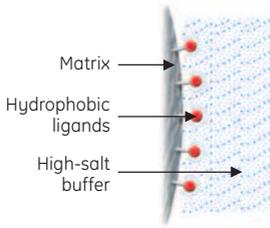
- Before optimizing binding conditions, it is important to check the “stability window” of the target protein(s) at different salt concentrations since many proteins may precipitate in raised salt concentrations. Precipitation of the target protein may make separation impossible or, at best, significantly reduce yield. The simplest approach to determine a stability window can be to observe the sample in a test tube at different salt concentrations and monitor protein activity left in the supernatant.

When sample loading is completed and the column has been washed so that all non-bound proteins have passed through (i.e., the UV signal has returned to baseline), conditions are altered to begin elution. Proteins are eluted by *decreasing the salt concentration* in the elution buffer. As the level of salt decreases those proteins with the lowest hydrophobicity begin to elute from the column. By controlling changes in salt concentration using gradients, proteins are eluted differentially in a purified, concentrated form. Those proteins with the highest degree of hydrophobicity will be most strongly retained and will be eluted last.

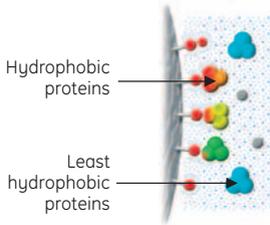
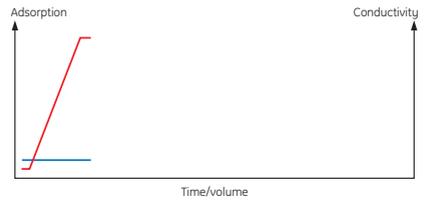
A wash step in a salt-free buffer removes most tightly bound proteins at the end of an elution. If the hydrophobicity of the medium and the proteins in the sample have been judged correctly, all proteins will be eluted by this stage. The column is then re-equilibrated in start buffer before applying more sample in the next run.

- Occasionally the hydrophobic interaction is so tight that harsher conditions may be required to remove all bound material, for example, 0.5–1.0 M NaOH, 70% ethanol or 30% isopropanol. These wash steps must be followed by a water or salt-free buffer wash before re-equilibrating the column with a high-salt start buffer, see also Column cleaning, Appendix 8.

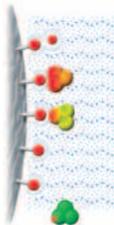
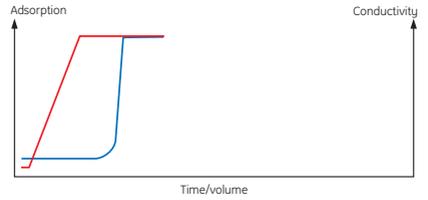
Alternatively, conditions can be chosen to maximize the binding of hydrophobic contaminants and allow the target protein(s) to pass through the column thus *removing contaminants*.



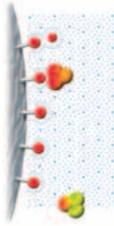
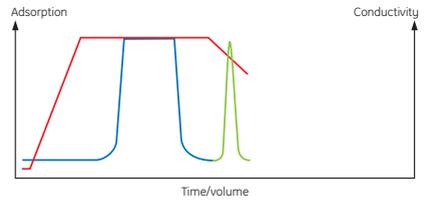
**Equilibration**  
 HIC medium equilibrated with high-salt start buffer.



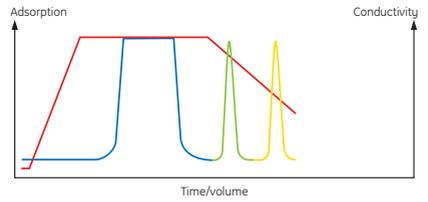
**Sample application**  
 Start buffer causes hydrophobic proteins bind to hydrophobic ligands on the medium, becoming concentrated on the column. Proteins with insufficient hydrophobicity elute during or just after sample application.



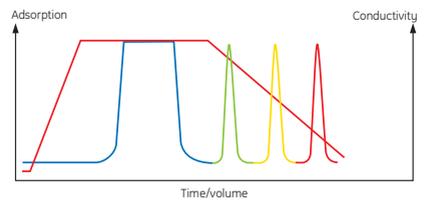
**Elution 1**  
 Decreasing salt content (using a linear gradient) causes hydrophobic proteins to elute: the least hydrophobic proteins elute first.



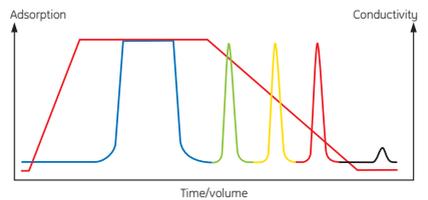
**Elution 2**  
 Further decreases in salt displace the more hydrophobic proteins (more tightly bound).



**Elution 3**



**Wash**  
 Final "salt-free" wash removes any hydrophobically bound proteins before re-equilibration.



**Fig 5.** Steps in a HIC separation.

## Resolution

The resolution of a HIC separation is a combination of the degree of separation between the peaks eluted from the column (selectivity), 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 and flow rates; all these are covered in detail in Chapter 2, Hydrophobic interaction chromatography 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 6.

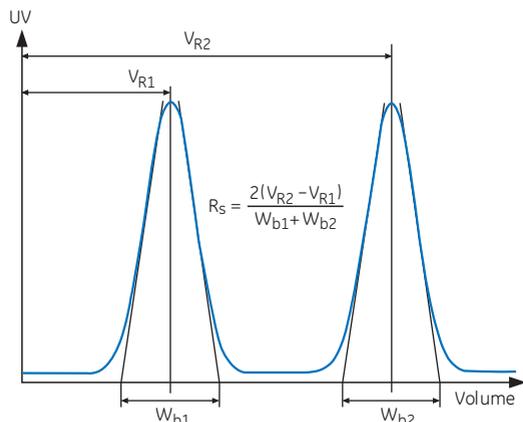


Fig 6. Determination of the resolution ( $R_s$ ) between two peaks.

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 7) 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  $R_s > 1.5$ . At this value, peak purity is 100%.

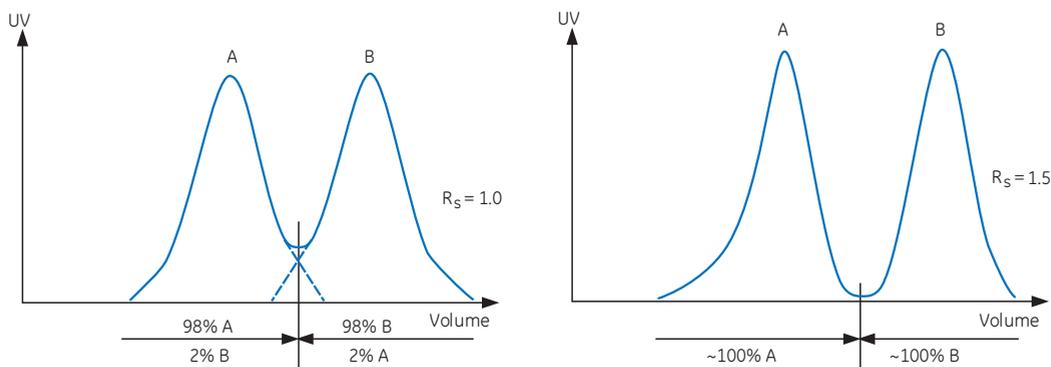


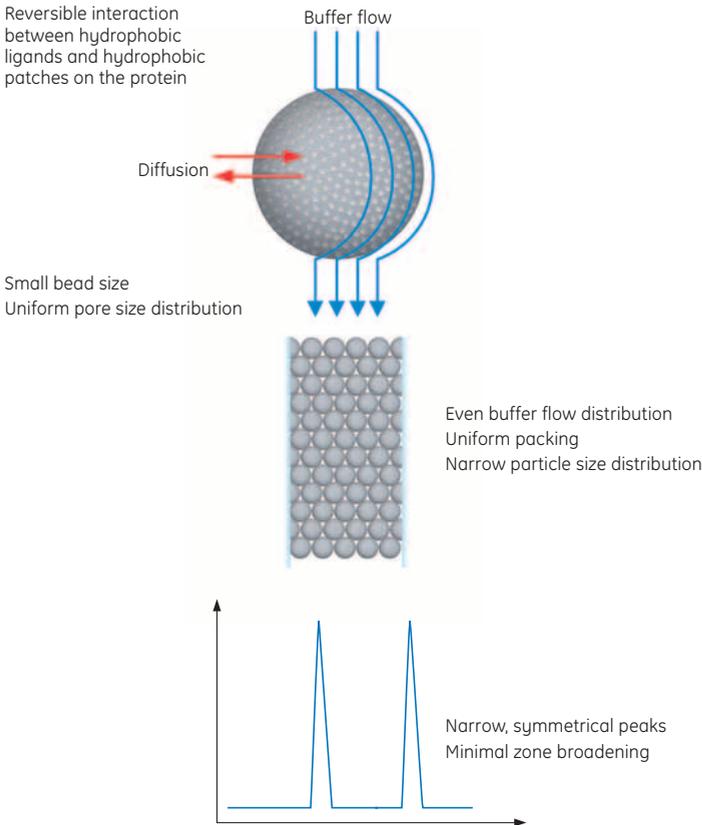
Fig 7. Separation results with different resolutions.



A single, well-resolved peak is not necessarily a pure substance, but may represent a series of components that could not be separated under the chosen elution conditions. Further purification may be required using alternative chromatography media. Refer to Chapter 4 for advice on purification strategies.

## Efficiency

Column efficiency (the ability to elute narrow, symmetrical peaks from a packed bed) relates to the *zone broadening* that occurs on the column and is frequently stated in terms of the number of theoretical plates (see Appendix 2 for determination of column efficiency). One of the main causes of zone broadening is longitudinal diffusion of the solute molecules (proteins). 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 contain air bubbles, will lead to channeling (uneven passage of buffer through the column), zone broadening and hence loss of resolution. Figure 8 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, in a well-packed column.



**Fig 8.** Factors that affect column efficiency.

Although resolution in terms of efficiency can be improved by decreasing the particle size of the matrix, using smaller particles often creates an increase in back pressure so that flow rates need to be decreased, lengthening the run time. Hence it is preferable to match the medium with the requirements for the purification (speed, resolution, purity, etc.). The viscosity of large volumes of highly concentrated sample may reduce resolution when running columns packed with small particles. Samples may need to be diluted or larger particles should be used.

## Selectivity

Good selectivity (the degree of separation between peaks) is a more important factor than high efficiency in determining resolution (Figure 9).

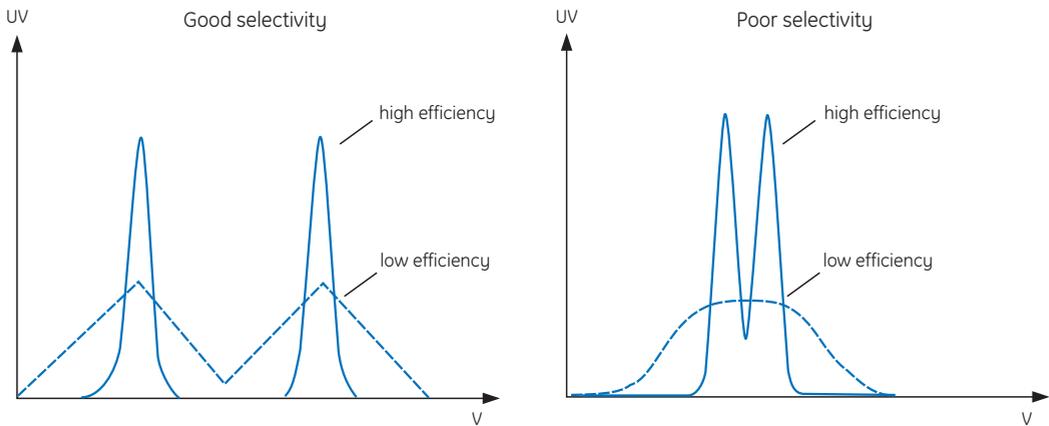


Fig 9. Effect of selectivity and efficiency on resolution.

In HIC, selectivity depends largely on the nature of the ligand and its degree of substitution on the matrix, the nature of the matrix, the nature of the target protein, the type of salt and the concentration of salt used for binding. Establishing a balance between these properties leads to a well-resolved, highly selective HIC separation.

### Selectivity and binding capacity

Although HIC media are described according to the type of ligand and, sometimes, ligand density, the actual amount of protein that can bind to a HIC medium, under defined experimental conditions, is of more practical relevance. 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. The *dynamic capacity* of a HIC medium is dependent on the properties of the medium, the protein being purified and the experimental conditions such as salt concentration, flow rate, temperature and, to a lesser extent, pH.



The properties of the ligand, target protein, salt and salt concentration play such a significant role in determining the final selectivity and binding capacity of a HIC medium that, unlike techniques such as ion exchange or affinity chromatography where 'standard proteins' can be used as a guideline to predict selectivity and capacity, these parameters must be determined and optimized by experimentation.

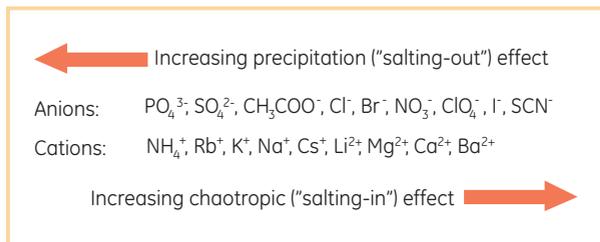
### Selectivity and salt selection

When using HIC media the ability of a particular salt to promote hydrophobic interaction depends on the ionic species present and their concentration.

Protein precipitation has the same driving force as seen when hydrophobic proteins interact with a hydrophobic medium and is therefore enhanced by increasing the ionic strength (concentration) of the surrounding buffer.

The elution/precipitation strength of an ion is described by the Hofmeister series (Figure 10). Small, highly charged ions are strong precipitators (anti-chaotropic) whereas organic acids and bases have a more stabilizing effect (chaotropic) on the presence of proteins in solution. The term chaotropic refers to the ability of the ion to produce order or chaos in the water structure. Salts of calcium and magnesium are

not the strong precipitators that might be expected from the Hofmeister series since these ions may bind to specific sites on the protein surface.



**Fig 10.** Hofmeister series: showing the effect of some anions and cations on protein precipitation.

Sodium, potassium or ammonium sulfates produce relatively high precipitation. It is these salts that effectively *promote* hydrophobic interaction and have a stabilizing influence on protein structure. In practice sodium, potassium or ammonium sulfates effectively promote ligand-protein interactions in HIC and have a stabilizing influence on protein structure. Hence the most commonly used salts are  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{NaCl}$ ,  $\text{KCl}$  and  $\text{CH}_3\text{COONH}_4$ .



**Fig 11.** Relative effects of some salts on protein precipitation.

The amount of protein that will bind to HIC media increases almost linearly up to a specific salt concentration. The amount bound then continues to increase in an exponential manner at higher salt concentrations. If the protein is unstable or its stability is unknown, it is preferable to perform protein binding in the region where the amount of bound protein increases linearly with the salt concentration.

## Selectivity and the properties of a HIC medium

While ligands contribute significantly to the degree of hydrophobicity of a medium, the matrix can also influence the final selectivity. Chromatography media for hydrophobic interaction are made from porous matrices, chosen for their physical stability, their chemical resistance to stringent cleaning conditions and their low level of non-specific interaction.

### Matrix

- An optimal balance between porosity and particle size offers a large surface area covered by ligands and so ensures a high binding capacity. High porosity with an open pore structure is an advantage when separating large biomolecules.
- 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 HIC media use either polymeric or agarose-based matrices to fulfill the requirements for chemical and physical stability, high binding capacity and different particle sizes (Table 1).

**Table 1.** Matrices used for HIC media.

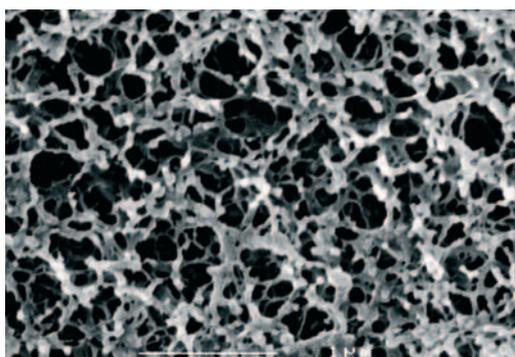
Matrix	Form	Mean particle size
SOURCE 15	Polystyrene/divinyl benzene	15 $\mu\text{m}$
Sepharose High Performance	Agarose 6%	34 $\mu\text{m}$
Sepharose 6 Fast Flow	Agarose 6%	90 $\mu\text{m}$
Sepharose 4 Fast Flow	Agarose 4%	90 $\mu\text{m}$

A SOURCE™ matrix is made from polystyrene with divinyl benzene to produce highly spherical (monodispersed), small (15  $\mu\text{m}$ ), porous particles (Figure 12) that facilitate high-resolution separations at high flow rates.



**Fig 12.** Electron micrograph of SOURCE showing spherical, monodispersed particles.

Sepharose™ matrices are based on hydrophilic chains of agarose, arranged in bundles and with different degrees of intra-chain cross-linking (Figure 13), to give a range of rigid, macroporous matrices with good capacity and low non-specific binding. The most suitable matrix can be selected according to the degree of resolution, binding capacity and flow rates required. For example, gradient elution on Sepharose High Performance (34  $\mu\text{m}$ ) will give a high-resolution separation whereas the larger particles of Sepharose Fast Flow (90  $\mu\text{m}$ ) are best suited for high capacity, step elution at high flow rate.

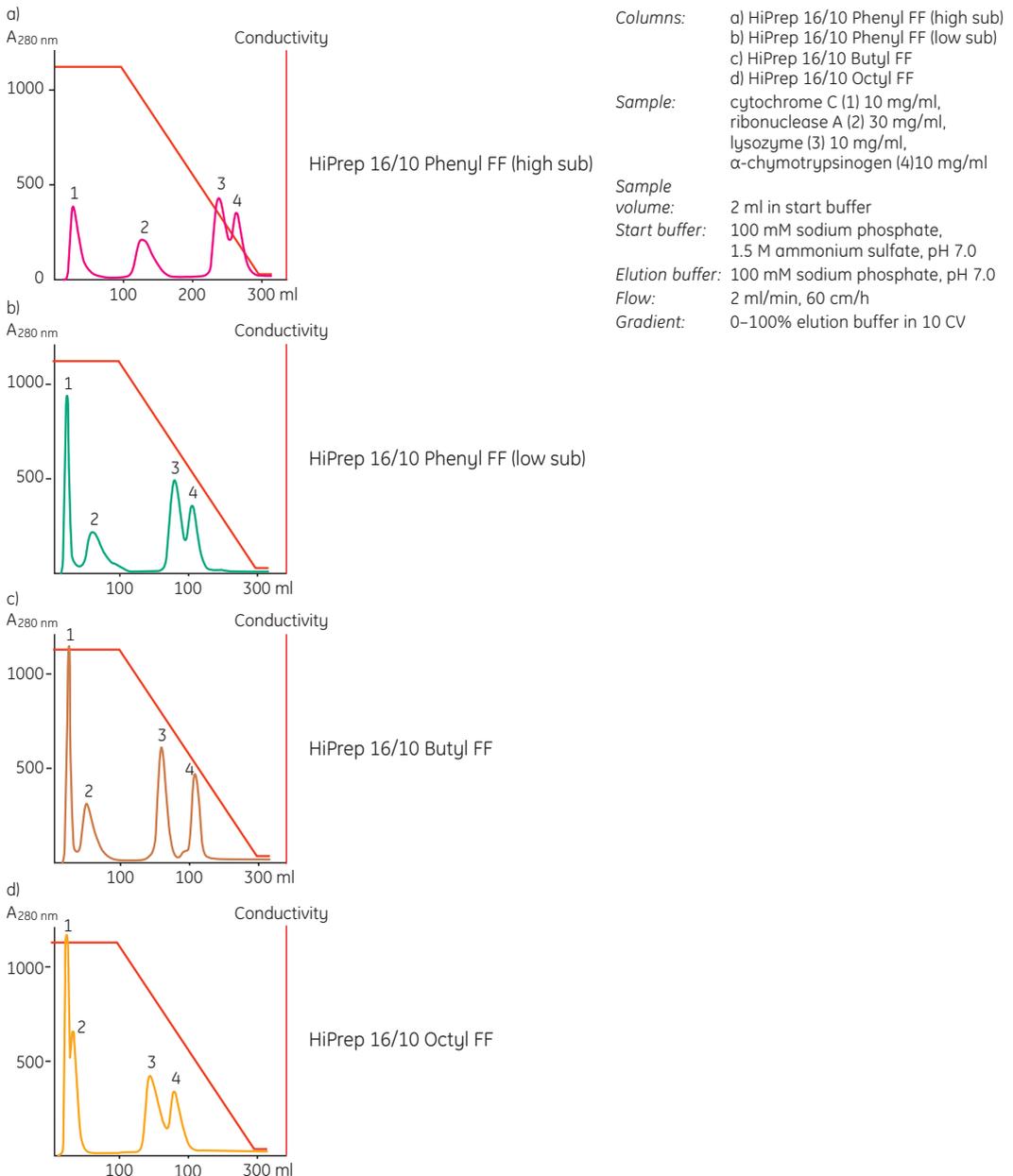


**Fig 13.** Structure of cross-linked agarose media (Sepharose).

Different matrices for HIC media have been used over the years, and references to these will still be found in scientific literature, for example, Sepharose CL-4B substituted with phenyl or octyl ligands. However, more recently developed matrices offer greater physical and chemical stability. To benefit from significantly faster separations and improved performance, it may be worth testing for selectivity on new media and re-optimizing old protocols.

## Ligands and degree of substitution

The ligand and the degree of ligand substitution on a chromatography matrix also contribute to the final hydrophobicity of the medium and hence to the selectivity. Figure 14 shows an example of a protein mixture separated on the same Sepharose Fast Flow matrix, but with four different ligand conditions: phenyl (high substitution), phenyl (low substitution), butyl and octyl.



**Fig 14.** Different ligands and differences in ligand density influence selectivity of a HIC medium.

The binding capacity of HIC media increases with increased ligand density up to a certain level. Simultaneously, the strength of the interaction increases, which may lead to difficulties in elution of bound components. Selecting a medium substituted with the same ligand, but at a lower ligand density, for example, Phenyl Sepharose 6 Fast Flow (low sub) instead of Phenyl Sepharose 6 Fast Flow (high sub) may solve the problem. Figure 14a and 14b show how the difference in ligand density between two media can influence selectivity.

The most common hydrophobic ligands are shown in Table 2. In general, HIC media fall into two groups, depending on their interactions with sample components. Straight alkyl chains (butyl, octyl, ether, isopropyl) show a "pure" hydrophobic character, while aryl ligands (phenyl) show a mixed-mode behavior in which both aromatic and hydrophobic interactions, as well as lack of charge, play a role in the final chromatographic properties.

**Table 2.** Ligands substituted on HIC media.

Phenyl	$\text{—O—}$ 
Butyl-S	$\text{—S—(CH}_2\text{)}_3\text{—CH}_3$
Butyl	$\text{—O—(CH}_2\text{)}_3\text{—CH}_3$
Octyl	$\text{—O—(CH}_2\text{)}_7\text{—CH}_3$
Ether	$\text{—O—CH}_2\text{—CHOH—CH}_2\text{—OH}$
Isopropyl	$\text{—O—CH—(CH}_3\text{)}_2$

 If the protein of interest does not bind under high salt conditions, use a more hydrophobic medium. If the protein of interest binds so strongly that non-polar additives are required for elution, decrease the salt concentration in the start buffer or use a less hydrophobic medium.

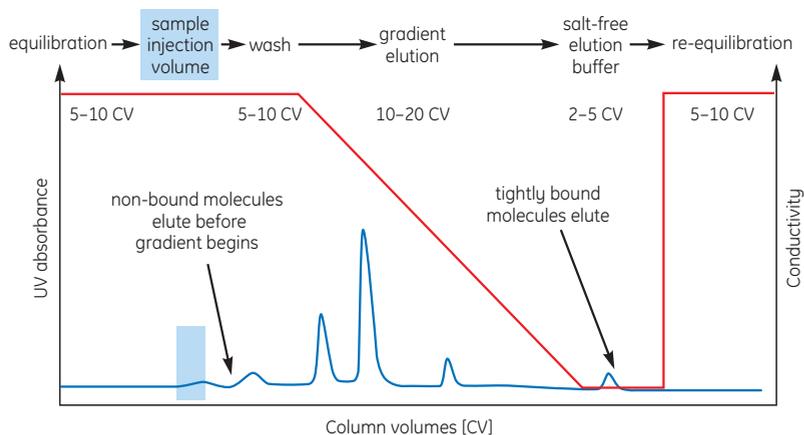
## Selectivity and elution

Figures 15 and 16 illustrate the most common forms of HIC separation in which proteins are eluted by decreasing the salt content of a buffer 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.

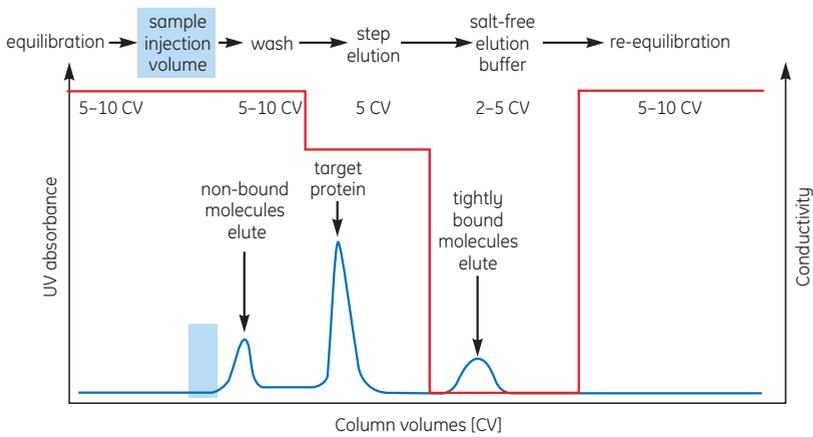
Using column volumes to describe a separation profile facilitates method development and transfer of methods to columns of different dimensions when scaling up.

*Gradient elution* (Figure 15) 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 15.** Typical high-resolution, HIC separation using linear gradient elution.

Step elution (Figure 16) is used in several ways. When a HIC 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.



**Fig 16.** Typical HIC separation using step elution.

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

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. Care must be taken to ensure that the binding capacity of the column is sufficient to bind all contaminants.



# Chapter 2

## Hydrophobic interaction chromatography in practice

### Introduction

This chapter includes practical advice on how to control experimental conditions for a successful separation and guidelines for selection of the most appropriate medium or prepacked column for each application. Additional application examples and product-related information are found in Chapter 3.

#### Media selection

The origin and differences between modern HIC media are explained in Chapter 1. Overall performance of a HIC medium (selectivity, resolution and binding capacity) is influenced by many parameters: protein properties, ligand type, degree of ligand substitution, concentration and type of salt used during sample application, presence of detergents and, to a lesser extent, temperature, pH and type of matrix. Understanding the role and importance of each parameter ensures that every separation can be performed with the required resolution, throughput and speed. With so many parameters to consider, it is recommended to prioritize separation development steps as follows:

1. Screen to select medium with correct selectivity\*.
2. Select type and concentration of salt to be used during binding.
3. Optimize gradient elution to maximize resolution and/or recovery.
4. Try additives and/or adjusting pH, if resolution or recovery is not ideal.

\* If more than one medium appears suitable, and since selectivity is the most important parameter when choosing a HIC medium, reselect according to the intended final scale of purification and purpose of the separation (capture, intermediate purification or polishing, see below). The choice of matrix will often depend on the availability of a medium with the correct selectivity.



Keep temperature constant throughout all separations.

### **HIC in a purification strategy (CIPP)**

To ensure efficient, reproducible protein purification giving the required degree of purity, it is beneficial to develop a multi-step process using the strategy of Capture, Intermediate Purification and Polishing (CIPP), shown in Figure 17 and described in more detail in Chapter 4.

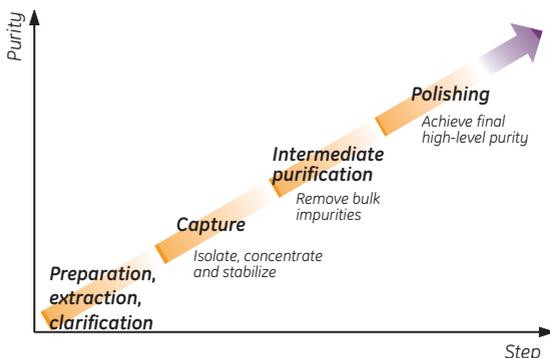


Fig 17. Strategy for protein purification.

CIPP is used in the pharmaceutical industry and in the research laboratory for faster method development, a shorter time to pure product and good economy.

HIC can be used for capture, intermediate purification or polishing steps. Since samples should be in a higher salt concentration to promote hydrophobic interaction, HIC is well suited for capture steps after sample clean-up by ammonium sulfate precipitation or for intermediate steps directly after an ion exchange separation. In both situations, the sample is already in a high salt solution and, apart from the addition of more salt, no further preparation is required. Since a HIC separation will concentrate the protein of interest into a reduced volume, fractions can also be transferred directly to gel filtration. HIC can be used with step-wise elution for a rapid capture step or with gradient elution to achieve the highest resolution in a polishing step.

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

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 as follows:

- Sepharose Fast Flow (90  $\mu\text{m}$  particle size) – capture or intermediate purification steps that require good resolution (flows up to 300 cm/h).
-  Use Sepharose High Performance (34  $\mu\text{m}$  particle size) HIC media for capture or scale-up when selectivity is satisfactory, high resolution is a priority and if lower flow rates (to compensate for a higher back pressure) are acceptable.
-  Use SOURCE (15  $\mu\text{m}$  particle size) if the required selectivity is not available in a medium of larger particle size.

## 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 as follows:

- Sepharose High Performance (34  $\mu\text{m}$  particle size) – for intermediate purification steps that require high resolution (flows up to 150 cm/h).
- Sepharose Fast Flow (90  $\mu\text{m}$  particle size) – intermediate purification steps that require good resolution (flows up to 300 cm/h) or when required selectivity is not available on a High Performance matrix.
-  Try SOURCE 15 (15  $\mu\text{m}$  particle size) – if the required selectivity is not available in a medium of larger particle size.

## Polishing

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

- SOURCE 15 (15  $\mu\text{m}$  particle size) – polishing in laboratory or large-scale applications that require high resolution and high throughput (flows up to 1800 cm/h can be used for re-equilibration and cleaning steps).
-  Try Sepharose High Performance (34  $\mu\text{m}$  particle size) if SOURCE media do not offer the required selectivity.
-  Try Sepharose Fast Flow (90  $\mu\text{m}$  particle size) if the required selectivity is not available in a medium of smaller particle size.

Figure 18 gives a selection guide for the media and prepacked columns currently available for hydrophobic interaction chromatography. Custom-designed media are available for large-scale industrial separations.

## Practical considerations for HIC separation

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

1. Equilibrate column with 5–10 column volumes of start buffer or until UV baseline and conductivity are stable.
2. Adjust the sample to the chosen salt concentration (and pH if necessary). Filter and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable, that is, when all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes, increasing the proportion of elution buffer until the salt concentration reaches a minimum, that is, salt-free buffer (100% elution buffer).

Alternatively, if gradient-making equipment is not available, elute bound proteins with up to 5 column volumes of elution buffer at a salt concentration lower than that in the start buffer. Repeat, lowering the salt content at each step until the target protein(s) has been eluted.

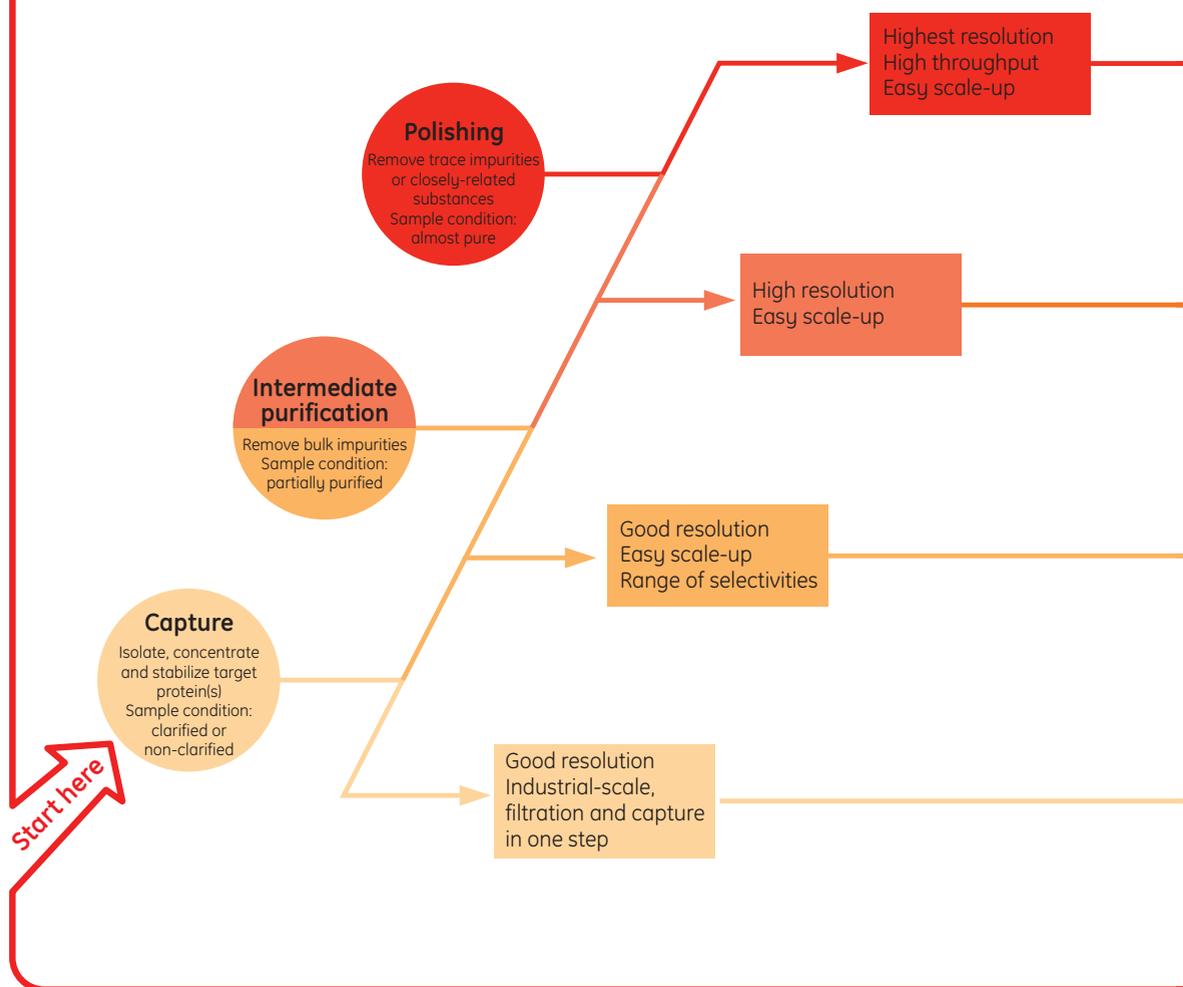
5. Wash with 2–5 column volumes of salt-free elution buffer to elute any remaining hydrophobically bound material.
6. Re-equilibrate with 5–10 column volumes of start buffer or until conductivity reaches the required value.

These steps are highlighted together with more detailed hints and advice throughout this section.

Buffer volumes 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, the gradient volume can be reduced if resolution can be maintained, and less buffer may be required for washing when separating reasonably clean samples.

-  Maintain sample, start and elution buffers, columns and chromatographic equipment at the same, constant temperature throughout a separation to ensure consistent, reproducible results.

## Selection Guide – Hydrophobic Interaction Chromatography Media



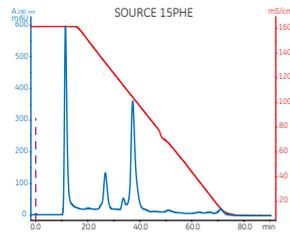
**Fig 18.** A typical purification strategy has three phases: Capture, Intermediate Purification and Polishing (CIPP). Each phase has a specific objective, dependent largely on the properties of the starting material. Select the appropriate HIC medium according to the objective of the purification step and the condition of the starting material.

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. STREAMLINE Phenyl is available only as a custom-designed product. For more information go to [www.gehealthcare.com/protein-purification](http://www.gehealthcare.com/protein-purification) to download the *Expanded Bed Adsorption Handbook* (Code no. 18-1124-26).

SOURCE 15  
(phenyl, isopropyl, ether)

Use RESOURCE HIC Test Kit to find suitable selectivity and optimize separation.

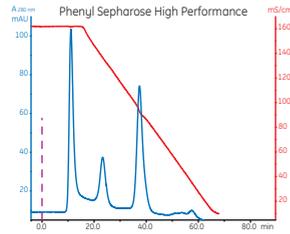
Use for intermediate purification or capture if no other medium offers suitable selectivity. Samples should be free from particulate matter.



Sepharose High Performance  
(phenyl, butyl\*)  
*\*available as a CDM product*

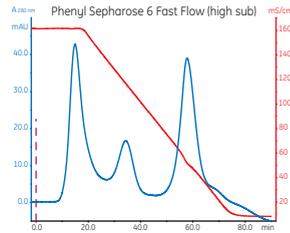
Use Sepharose High Performance for polishing if SOURCE media do not offer required selectivity.

Use HiTrap HIC Selection Kit to find suitable selectivity and optimize separation.



Sepharose Fast Flow  
(phenyl high sub,  
phenyl low sub, butyl,  
octyl, butyl-S)

Use Sepharose Fast Flow for polishing if media with smaller particle size do not offer required selectivity.



STREAMLINE  
(Phenyl)  
*\*available as a CDM product*

Use STREAMLINE for direct capture from unclarified feedstock.



## Screening for selectivity



**Fig 19.** HiTrap HIC Selection Kit and RESOURCE HIC Test Kit.

Time and sample can be saved in the early stages of development by using small (1 ml), prepacked columns such as those in the HiTrap™ HIC Selection Kit and RESOURCE™ HIC Test Kit. Media can be quickly and efficiently screened for the required selectivity. This approach is helpful since, even if the properties of the target protein(s) are known, the final selectivity, binding capacity and recovery depends largely upon the interaction of the medium with the specific protein of interest.

HiTrap columns are prepacked with hydrophobic interaction media based on Sepharose High Performance and Sepharose Fast Flow while RESOURCE HIC columns contain media based on SOURCE. All columns can be used for small-scale purification and are supplied with detailed protocols for use. The media in these test kits are available for large-scale production so that optimized methods can be easily transferred to the required scale of operation.

 Before starting any HIC separation, establish the “salt stability window” for the sample. For example, add increasing amounts of salt to the crude sample in order to establish the concentration at which precipitation occurs. Ensure that the sample is *below* this salt concentration when applied to a column in order to avoid precipitation. Refer to Appendix 1 for a guide to using ammonium sulfate for sample clean-up by precipitation. When possible, test for biological activity of the target protein to establish the concentration range over which activity can be maintained (remember that the sample may need to be desalted before an activity test).

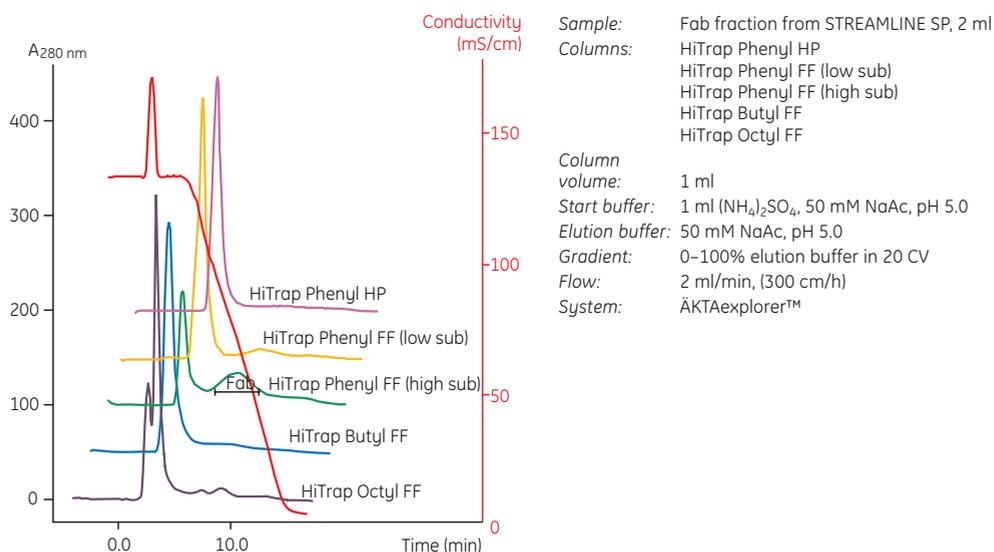
### ***Automated HIC media screening, method development and optimization***

Users of ÄKTAdesign™ chromatography systems can select suitable method templates and program the system to automatically perform separations using a range of columns and a range of buffer conditions.

1. Sample preparation: having established the salt stability window, begin with the *highest* salt concentration that retains biological activity and does not cause precipitation problems. Adjust the sample to the salt concentration of the start buffer to promote hydrophobic interaction. Add salt from a high concentration stock solution to avoid the risk of precipitation due to high salt concentrations that may occur locally if salt is added as a solid. Adjust the pH of the sample directly. Since HIC is not very sensitive to pH conditions, a complete buffer exchange is unnecessary.
2. Prepare start and elution buffers: 50 mM phosphate, pH 7.0. Add ammonium sulfate (up to 2 M, according to salt stability window) to the start buffer.
3. Wash each column in salt-free elution buffer before equilibrating in a high-salt start buffer. This avoids the risk of salt precipitation when columns have been stored in 20% ethanol.
4. Scout for optimum selectivity, applying sample to each column under a range of salt concentrations. Collect eluate throughout the run. Choose a medium in which the target protein(s) elute within the gradient.

- Scout for the lowest salt concentration in the start buffer that maximizes binding capacity for the target protein, maintains or improves resolution and minimizes the risk of contamination from other bound proteins.
- Optimize for the steepest gradient that gives acceptable resolution.
- Optimize for the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
- Optimize 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 transferring to a step elution when optimized separation conditions have been established. Sample loads can often be increased when using a step elution.



**Fig 20.** Media screening on different HIC media prepacked in HiTrap 1 ml columns.

Figure 20 shows an example of media screening on different HIC media prepacked in HiTrap 1 ml columns. Buffer pH was kept at pH 5.0 to minimize the need for sample conditioning after capture. Phenyl Sepharose 6 Fast Flow (high sub) was selected since the medium showed excellent selectivity for the target protein. The protein was eluted within the gradient and separated from the bulk contaminants. Conditions were then optimized so that a step elution could be used to maximize throughput and concentration of the target protein before scaling up. Figure 47 on page 74 shows the optimized elution scheme and subsequent scale-up.

## Manual media screening, method development and optimization

HiTrap columns can be used with a syringe or peristaltic pump for manual media screening, method development and method optimization. However, using a syringe limits the degree to which a HIC separation can be developed since the separation mechanism is not a simple “on/off” process and requires some degree of gradient elution to achieve a satisfactory separation.

The methods here are optimized for use with 1 ml HiTrap columns and should be adjusted if other column volumes are used. Note that flow rates may need to be reduced due to the viscosity of sample or buffers.

## Screening for selectivity using HiTrap HIC Selection Kit

1. Sample preparation: having established the salt stability window, begin with the *highest* salt concentration that retains biological activity and does not cause precipitation problems. Adjust the sample to the salt concentration of the start buffer to promote hydrophobic interaction. Add salt from a high concentration stock solution to avoid the risk of precipitation due to high salt concentrations that may occur locally if salt is added as a solid. Adjust the pH of the sample directly. Since HIC is not very sensitive to pH conditions, a complete buffer exchange is not necessary.
2. Prepare start and elution buffers: 50 mM phosphate, pH 7.0. Add ammonium sulfate (up to 2 M, according to salt stability window) to the start buffer.
3. Wash the column(s) with 5–10 ml salt-free elution buffer at 1 ml/min. This avoids the risk of salt precipitation when columns have been stored in 20% ethanol.
4. Equilibrate the column(s) with 5–10 ml start buffer at 1 ml/min.
5. Apply a known amount of the sample at 1 ml/min. Collect eluate.
6. Wash at 1 ml/min with at least 5 ml of start buffer or until no material appears in the eluent. Collect eluate.
7. Elute bound material with elution buffer at 1 ml/min (3–5 ml is usually sufficient, but other volumes may be required dependent on the exact experimental conditions). Collect eluate.
8. Analyze all eluates (e.g., by an activity assay) and determine purity and the amount bound to the column.
9. Select the medium to which the target protein(s) binds and can be eluted. If running a gradient, select the medium that gives the best selectivity and resolution.

## Screening for binding (salt) conditions

1. Using the selected medium and buffer from the previous protocol, set up a series of start buffers at the same pH, but with reduced concentrations of ammonium sulfate in each buffer (e.g., from 1.5 M, 1.0 M, 0.5 M with a lowest concentration of 0.05 M).
2. Repeat steps 2–7 from the previous protocol for each salt concentration.
3. Determine the salt concentration that permits binding of the target protein(s) while contaminants either wash through or remain bound to the column. Determine the lowest salt concentration required to achieve complete elution of the target protein.

## Optimization of gradient, flow rate and sample loading

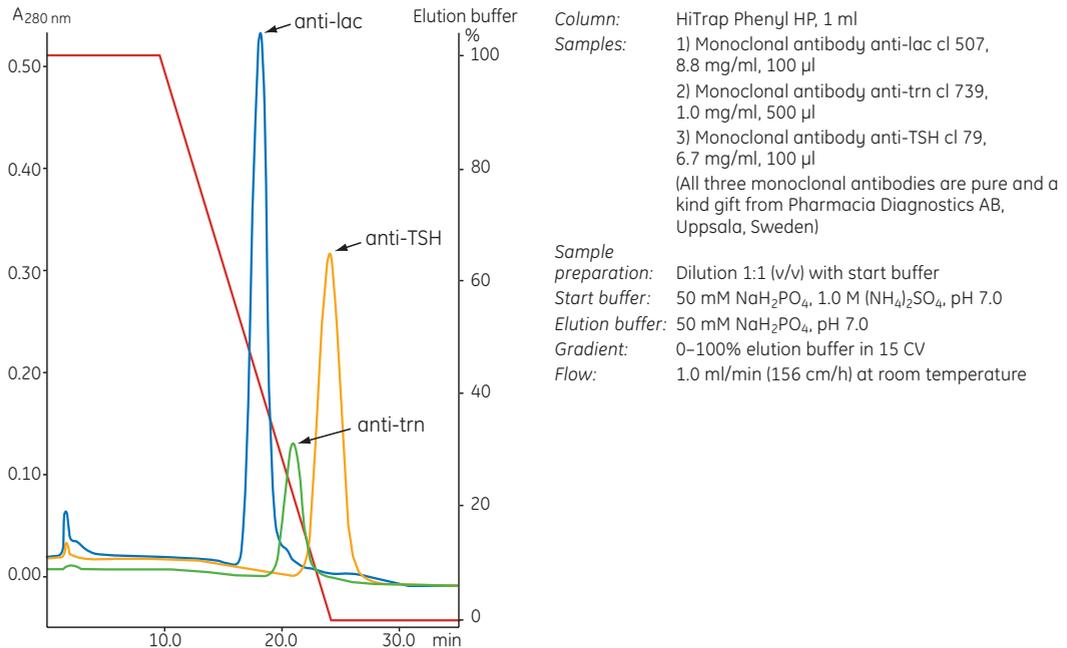
1. If gradient making equipment is available, determine the steepest gradient that gives acceptable resolution.
2. Begin with a gradient of 10 column volumes. Start from the salt concentration required to bind the target protein and go down to the lowest salt concentration required for elution, based on the values determined when screening. Alternatively, begin with a gradient of 0–50% elution buffer and a gradient volume of 10–20 column volumes.
3. To save time, determine the highest flow rate that maintains resolution and minimizes separation time. Check recommended flow rates for the specific medium.
4. 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.



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

## Sample properties and choice of ligand

The type of ligand and the nature of the target protein are highly significant parameters in determining the selectivity of a HIC medium. Consequently, the most suitable ligand must be determined empirically through screening experiments (see Screening for selectivity, page 28), preferably using the target protein. Proteins that could be assumed to have very similar properties can interact quite differently under identical experimental conditions in a HIC separation, as demonstrated by the behavior of three monoclonal antibodies in Figure 21.



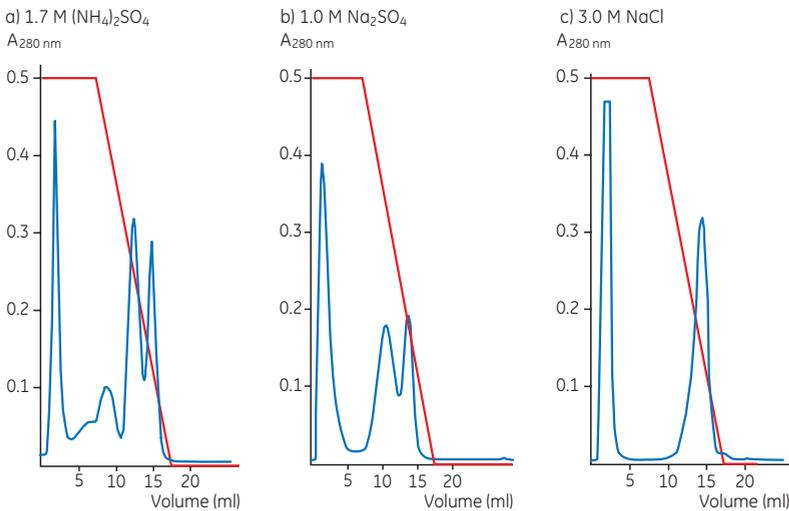
**Fig 21.** Three monoclonal antibodies interact differently under identical running conditions using a phenyl ligand. A suitable ligand must be determined empirically.

## Salt selection and buffer preparation

### Salts

In HIC the binding process is more selective than the elution process, so it is essential to optimize the conditions of the start buffer. The correct choice of salt and salt concentration are the most important parameters that influence capacity and final selectivity. The objective is to optimize conditions to achieve the required selectivity to bind the target protein(s) and ensure that the majority of impurities pass through the column.

The influence of different salts on hydrophobic interaction is explained in Chapter 1. In practice sodium, potassium or ammonium sulfates effectively promote ligand-protein interactions in HIC and have a stabilizing influence on protein structure. Hence the most commonly used salts are  $(\text{NH}_4)_2\text{SO}_4$ ,  $\text{Na}_2\text{SO}_4$ ,  $\text{NaCl}$ ,  $\text{KCl}$  and  $\text{CH}_3\text{COONH}_4$ . Figure 22 shows an example of how different salts can affect selectivity. Here the best resolution of four standard proteins was obtained using 1.7 M ammonium sulfate in the start buffer.



Column: HiTrap Butyl FF, 1 ml  
Sample: 1 mg/ml cytochrome C, 1 mg/ml lysozyme, 1 mg/ml  $\alpha$ -chymotrypsinogen, 3 mg ribonuclease A  
Sample loading: 6 mg in 1 ml start buffer  
Flow: 0.5 ml/min, 75 cm/h  
Start buffers: a) 0.1 M sodium phosphate, 1.7 M ammonium sulfate, pH 7.0  
b) 0.1 M sodium phosphate, 1.0 M sodium sulfate, pH 7.0  
c) 0.1 M sodium phosphate, 3 M  $\text{NaCl}$ , pH 7.0  
Elution buffer: 0.1 M sodium phosphate, pH 7.0  
Gradient: 0%–100% elution buffer in 10 CV

**Fig 22.** Different salts influence selectivity: elution takes place in order of increasing elution volumes: cytochrome C, lysozyme, ribonuclease A,  $\alpha$ -chymotrypsinogen.

As with media selection, the choice of salt for a HIC separation can be a matter of trial and error since each salt differs in its ability to promote hydrophobic interactions. As the concentration of a salt increases, the amount of protein bound will increase almost linearly up to a specific salt concentration and continue to increase in an exponential manner at higher concentrations.



At a given concentration, ammonium sulfate often gives the best resolution when compared to other salts and can be used at concentrations up to 2 M.



Concentrations up to 3 M are usually required when using sodium chloride.

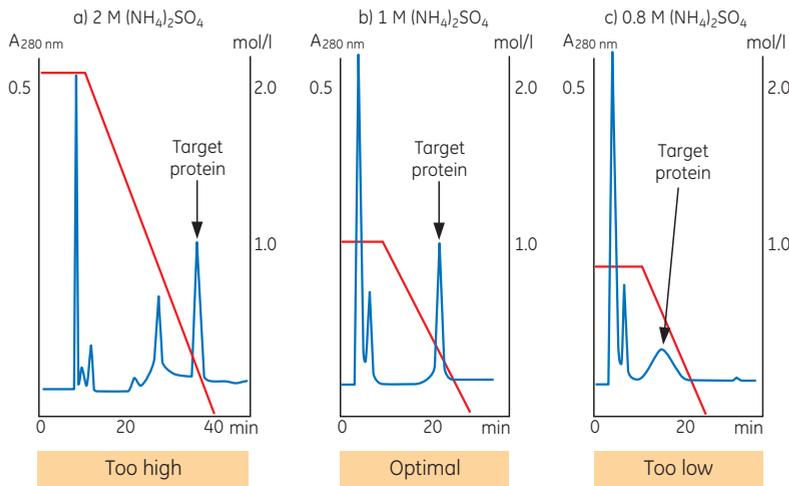


Sodium sulfate is a very good salting-out agent, but problems with protein solubility may exclude its use at high concentrations.



Ammonium sulfate is not recommended for working at pH values above 8.0.

## Salt concentration



**Fig 23.** Salt concentration in the start buffer influences selectivity and resolution.

Figure 23 shows the influence of salt concentration on selectivity and resolution. In this example the target protein is the last peak to elute. The selectivity of the medium is satisfactory since the protein elutes within the gradient and is well resolved from contaminants. In (a), the target protein elutes in a sharp zone, but late in the gradient. Lowering the initial salt concentration (b) gives similar resolution, but ensures that contaminants that bound during earlier runs (when a higher salt concentration was used) now elute during the initial wash step. Only the target protein is bound, reducing the risk of a contaminant co-eluting with the target protein and increasing the capacity of the column for the target protein. A run performed at even lower initial salt concentration (c) shows good selectivity but poor efficiency for the target protein. The sample is not bound strongly enough during sample application, resulting in significant peak broadening during elution.



If the target molecule elutes too late or not at all and switching to a different medium is not possible, try binding in 50% less salt.



Some proteins begin to precipitate at high salt levels. The salt concentration in the start buffer may need to be reduced to prevent precipitation during the run. Loading the sample repetitively in small amounts can also help to avoid losing yield due to precipitation.

## Buffer ions and pH

Selection of buffering ions is not critical for hydrophobic interaction. Phosphate buffers are most commonly used.

The pH chosen must be compatible with protein stability and activity, and it is advisable to check for optimum pH conditions for each specific application. However, between pH 5–8.5, pH values have very little significance on the final selectivity and resolution of a HIC separation. An increase in pH weakens hydrophobic interactions and retention of proteins changes more drastically at pH values above 8.5 or below 5.0.

-  Check for stability at the pH and salt concentrations used during the separation, especially if recovery of biological activity is a priority. Avoid extreme changes in pH or other conditions that may cause inactivation or even precipitation.
-  Use a buffer concentration, typically 20–50 mM, that is sufficient to maintain buffering capacity and pH during sample application and changes in salt concentration.
-  Transfer the purified protein into a volatile buffer if the product is to be lyophilized. Table 3 lists suitable volatile buffer systems.

**Table 3.** Volatile buffer systems.

pH-range	Buffer system	pK <sub>a</sub> -values for buffering ions <sup>1</sup>
3.3–4.3	Formic acid	3.75
3.3–4.3; 4.8–5.8	Formic acid / Pyridin	3.75; 5.25
3.3–4.3; 8.8–9.8	Formic acid / Ammonia	3.75; 9.25
3.3–4.3; 9.3–10.3	Formic acid / Trimethylamine	3.75; 9.81
4.3–5.8	Acetic acid / Pyridin	4.75; 5.25
4.3–5.3; 7.2–8.2	Acetic acid / N-ethylmorpholine	4.75; 7.72
4.3–5.3; 8.8–9.8	Acetic acid / Ammonia	4.75; 9.25
4.3–5.3; 9.3–10.3	Acetic acid / Trimethylamine	4.75; 9.81
5.9–6.9; 8.8–9.8	Hydrogen carbonate / Ammonia	6.35; 9.25

<sup>1</sup> Ref: *Handbook of chemistry and physics, 83rd edition, CRC, 2002–2003.*

-  Prepare buffers at the same temperature at which they will be used to ensure the correct pH.
-  Filter buffers and samples after all salts and additives have been included. Use high-quality water and chemicals. Use 1 µm filters for media with particle sizes above 90 µm, 0.45 µm filters for 34 µm particles, and 0.22 µm filters for particles below 15 µm or when sterile or extra-clean samples are required. To avoid formation of air bubbles in a packed column and to ensure reproducible results, the column and buffers should be at the same temperature when preparing for a run.
-  For samples with unknown hydrophobic properties, try the following:

start buffer: 1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0

elution buffer: 50 mM sodium phosphate, pH 7.0

## Buffer additives

Additives can be used to improve selectivity and resolution, for example when a protein binds too strongly to a HIC medium. However, if used at high concentrations, there is a risk of inactivating and/or denaturing the target protein. Additives can influence a separation by improving protein solubility, modifying protein conformation and promoting elution of bound proteins. Water-miscible alcohols, detergents, and chaotropic salts (limited use) are the most widely used additives in HIC separations. Typical additives are shown in Table 4.

**Table 4.** Additives used to improve HIC separations.

Additive type	Typical additives	Effect
Alcohols	Up to 10% ethanol Up to 30% isopropanol Up to 10% glycerol 20–80 v/v % ethylene glycol	Alter buffer polarity. Decrease the surface tension of water thus weakening the interaction and causing dissociation. Non-polar regions compete with the proteins for the hydrophobic ligands, causing dissociation.
Detergents	From 0.1% up to 1% v/v Triton X-100	As above.
Chaotropic salts	MgCl <sub>2</sub> CaCl <sub>2</sub> KI NaCNS up to 8 M urea	Decrease the hydrophobic effect in solution thus weakening the interaction and causing dissociation. May also affect the conformation of the protein. Ca <sup>2+</sup> increases stability during purification of calcium-binding proteins; Mg <sup>2+</sup> decreases stability.



Run blank elution gradients with additives included in order to check their effect on the elution profile (that is, perform a run but do not load any sample).

## Column and media preparation

Equilibrate column with 5–10 column volumes of start buffer or until UV baseline and conductivity are stable.

Use prepacked columns 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/or preservatives before using any HIC medium. Wash columns with 10 column volumes of salt-free elution buffer before equilibrating with start buffer to avoid the risk of ethanol in the storage solution causing salt precipitation.

Appendix 2 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 with approximately 5-fold excess of the binding capacity required (total protein should be equivalent to 20% of the binding capacity) and a bed height up to 20 cm.

-  Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 2. Note that this does not apply to HiTrap columns.

## Sample preparation

Simple steps to clarify any sample before application to a column will avoid the risk of blockage, reduce the need for stringent washing procedures and avoid deterioration in column performance and increases in back pressure. Appendix 1 contains a detailed overview of sample preparation techniques.

Correct sample preparation is essential in order to achieve optimal separation. In HIC, the initial binding conditions also influence the final selectivity of the separation.

Before starting any separation, establish the “salt stability window” for the sample, for example, add increasing amounts of salt to the crude sample in order to establish the concentration at which precipitation occurs. Ensure that the sample is *below* this salt concentration when applied to the column in order to avoid blockage. Refer to Appendix 1 for a guide to using ammonium sulfate in precipitation experiments.

When possible, test for biological activity of the target protein to establish the concentration range over which activity can be maintained (remember that high salt concentrations may need to be reduced before assaying for activity). Having established the salt stability window, begin with the *highest* salt concentration that retains biological activity and does not cause precipitation problems. The salt content and pH of the sample should be the same as those of the start buffer to ensure optimal binding conditions.

HIC requires a minimum of sample preparation work. Binding takes place predominantly as a result of the high salt conditions and the technique is fairly insensitive to pH conditions. It is not necessary to exchange the sample buffer before applying sample to a HIC column; simply ensure that there is sufficient salt, and adjust the pH directly, if necessary. Add salt from a high concentration stock solution to avoid the risk of precipitation due to local, high salt concentrations when salt is added as a solid.

- 
 Samples must be clear and free from particulate matter, particularly when using media of particle sizes 34 µm or less. For small sample volumes, a syringe-tip filter of cellulose acetate or PVDF can be sufficient for sample filtration. Filter samples after the addition of salt and any other additives.
- 
 Ensure that sample is at the same temperature as buffers, columns and chromatographic equipment.
- 
 Use buffer exchange (see page 136) to remove chaotropic agents, such as guanidine hydrochloride or urea, that have been used for initial solubilization as they will inhibit hydrophobic interaction.
- 
 If sample begins to precipitate at the salt concentration needed in the start buffer, reduce the salt concentration and divide the sample into smaller aliquots before application. The concentration of the start buffer is unchanged.
- 
 Lipids or other very hydrophobic substances in the sample may interact with the medium, reducing binding capacity during the run and in subsequent runs. Using a slightly less hydrophobic medium (such as Butyl-S Sepharose 6 Fast Flow) as a “pre-column” is one alternative for removing these contaminants before the main separation.

### Concentration and viscosity

Viscosity varies with temperature and will increase at very high salt concentration. The solubility or viscosity of a sample may limit the quantity that can be applied to a column. High sample viscosity can cause 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. If dilution is not an option, using a lower salt concentration or a medium with a larger particle size may help to overcome viscosity problems. If high viscosity is caused by the presence of nucleic acid contaminants, see Appendix 1 for advice on their removal.
- 
 Samples should generally not exceed 50 mg/ml protein, but may vary according to the type of sample and the type of chromatography medium.

### Sample application

Adjust the sample to the chosen salt concentration (and pH if necessary). Filter and apply to the column.

Wash with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable, indicating that all unbound material has washed through the column.

For efficient binding, the sample should be at the same salt concentration as the start buffer. The sample volume can be relatively large without affecting the separation since sample will bind near the top of the column as long as application 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 and type of column, the type of HIC medium and the requirements for gradient accuracy during elution.

## Sample load

Sample load (mass) is of greater importance than sample volume. The amount of sample that can be applied to a column depends on the binding capacity of the medium and the degree of resolution required. Binding capacity is determined largely by the medium, protein properties and the binding conditions, size and shape of the molecules, pore size of the matrix and, to a lesser extent by flow rate, temperature and pH.

Sample load has a major influence on resolution since the width of the peaks is directly related to the amount of substance present. 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.

 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.

Capacity can be increased, for example, by decreasing flow rates or optimizing start conditions to favor binding of the target protein(s) and minimize binding of contaminants.

Capacity will decrease with increasing flow rates so that a balance must be found between achieving the maximum dynamic binding capacity and a fast separation, particularly when applying large sample volumes.

Capacity will also decrease for molecules of very large diameter or length, for example, protein complexes  $>M_r$  400 000, asymmetric proteins and DNA. These molecules are unable to penetrate the matrix pores, limiting their interaction primarily to the hydrophobic 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.

## Sample volume

As a binding technique, HIC is independent of sample volume as long as the salt content of the sample and the start buffer ensure adequate binding conditions.

## Temperature

It is generally agreed that the role played by temperature is highly complex. In practice this means that working at a constant temperature will improve reproducibility and that a separation developed at room temperature may not be reproducible under cold-room conditions or *vice versa*.

Figure 24 demonstrates the importance of having sample, start and elution buffers, columns and chromatographic equipment at the same temperature. Both separations were performed at room temperature (23°C) under identical conditions except for the sample temperature (23°C or 4°C). With all components at the same temperature, the target protein was bound and then eluted in the middle of the gradient. With the sample at 4°C, the target protein eluted in the flowthrough.

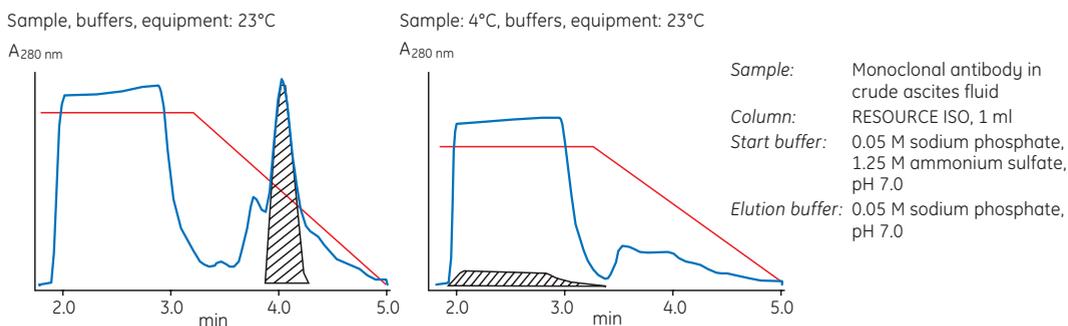


Fig 24. Influence of temperature on a HIC separation.

-  In most cases, increasing temperature enhances hydrophobic interactions so working at lower temperatures (typically below 10°C) can minimize aggregation caused by hydrophobic interactions between sample components. Lowering temperature can be used instead of adding detergents to improve solubility.
-  Ensure that sample, column, start and elution buffers are at the same temperature. Note that temperature will also affect the viscosity of sample and buffers.

## Elution

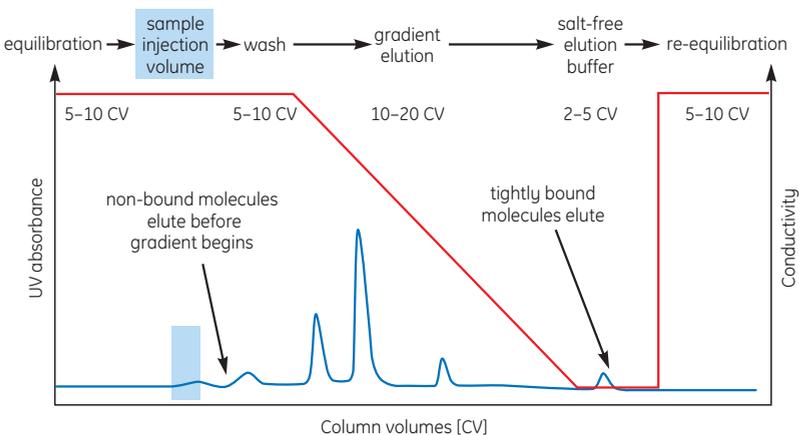
Bound proteins are eluted by controlled decreases in salt concentration, using a linear or step elution selected according to the aim of the separation:

- Linear gradient elution
    - high-resolution separation or analysis
    - determination of conditions for a step elution
    - optimized gradient elution at increased speed while retaining required resolution
  - Step elution
    - faster separation time, reduced buffer consumption
    - group separation
-  Fractions collected during a HIC separation are likely to contain relatively high salt concentrations that may interfere with assays or subsequent chromatographic steps. These fractions can be desalted before further processing (see Appendix 1, Sample preparation and, in particular, Buffer exchange and desalting, page 136 for details).

### Linear gradient elution

**Aim: high-resolution separation, media screening, screening for optimal salt conditions**

Begin elution using a gradient volume of 10–20 column volumes, increasing the proportion of elution buffer until the salt concentration reaches a minimum, that is, salt-free buffer (100%B).



**Fig 25.** Typical HIC 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 salt gradients, as shown in Figure 25, are most frequently used for elution. Always use a linear gradient 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). Decreasing the salt content of the running buffer weakens the hydrophobic interactions, and bound substances begin to elute. The elution buffer is usually the same buffer and pH as the start buffer, but without the high salt component.

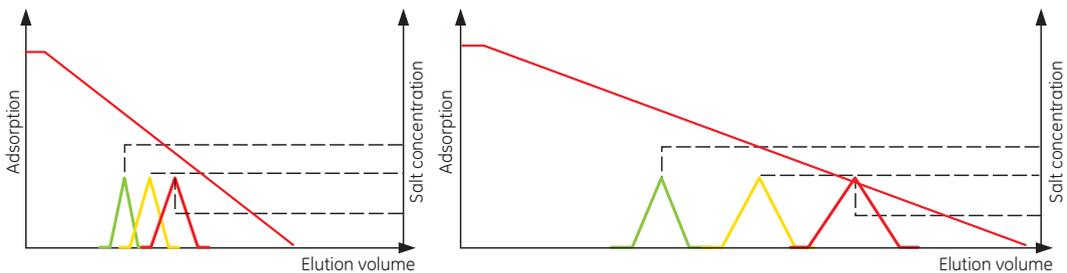


It is strongly recommended to use linear gradient elution during method development. Linear salt gradients are easy to prepare and very reproducible when generated by a suitable chromatography system. The results obtained serve as a base from which to optimize the separation.

Changes in gradient elution can alter the retention of hydrophobic proteins on a medium as follows:

- 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. The effects of changing gradient slope are shown in Figure 26.



**Fig 26.** Schematic chromatograms showing the effect of decreasing gradient slope.

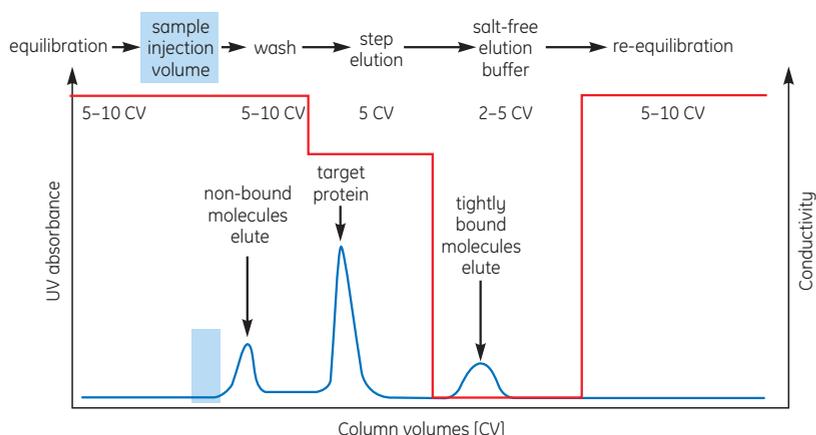


If gradient elution volumes are decreased, it may be necessary to decrease the sample load proportionally in order to maintain the same resolution. If sample load is increased (within the total binding capacity of the column), gradient volumes may need to be increased to maintain resolution.

Gradients are ideally formed using purpose-designed equipment, such as ÄKTAdesign systems that automatically control the mixing of solutions being supplied to a column. 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. The shortest flow path between a mixer and the top of a column will help to ensure accurate gradient formation.

## Step elution

Elute bound proteins with up to 5 column volumes of elution buffer at a salt concentration lower than that in the start buffer. Repeat, lowering the salt content at each step until the target protein(s) has been eluted.



**Fig 27.** Typical HIC separation using step elution. The UV (protein) and conductivity (salt) traces show the elution of protein peaks and the changes in salt concentration during elution.

As shown in Figure 27, step elution is performed by sequential addition of the elution buffer with decreasing salt concentration:

Step 1: salt concentration and volume of elution buffer are optimized to elute all compounds binding less strongly to the medium than the target protein(s). Note that the salt concentration and buffer volume should be large enough to elute the contaminating weaker binding substances, but must not exceed a level at which the peak of interest starts to co-elute.

Step 2: salt concentration is decreased to the point where the target protein(s) elutes. Note that the salt concentration should be low enough to elute the target protein(s) without excessive dilution, but must be kept above the level where more strongly bound contaminants start to co-elute.

Step 3: salt concentration is further decreased to elute all remaining contaminants. Water can also be used at this stage.

Step 4: column is re-equilibrated in start buffer in preparation for the next run.

*Aim of a step elution: reduced separation time, reduced buffer consumption*

When a HIC separation has been optimized using gradient elution, changing to a step elution can reduce the total number of column volumes used for a separation. This can speed up separation times and reduces buffer consumption while retaining the required purity level. Step elution of this type is often used for routine, large-scale separation.

*Aim of a step elution: group separation*

In a group separation target proteins or contaminants can be separated in one step elution. According to their hydrophobic properties, proteins or contaminants can be bound to the column, concentrated and rapidly separated. As in a normal step elution, conditions are chosen to maximize binding of the target proteins (or known contaminants) during sample application. The fraction of interest is either collected in the wash or eluted in an enriched, concentrated form. In either situation, only a single buffer change is required.

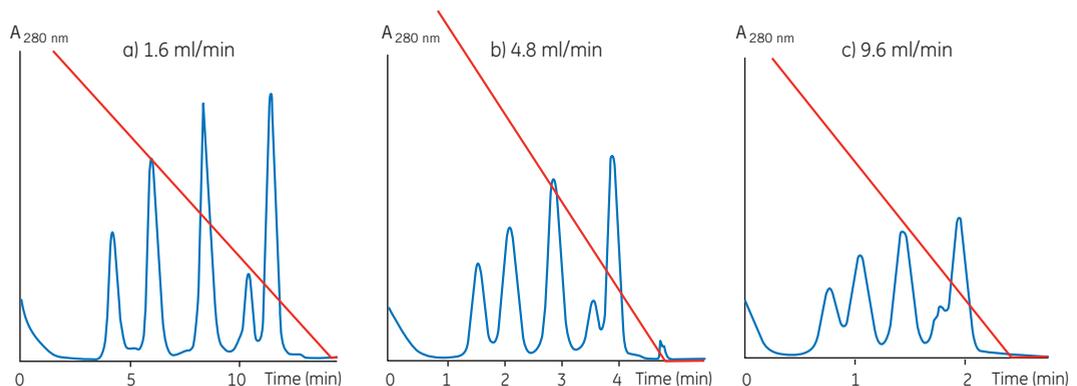
- Step-wise elution can be advantageous in small-scale applications since the target protein can be eluted in a more concentrated form if the eluting strength of the buffer is kept high enough to avoid co-elution with more strongly bound contaminants.
- Using a step elution with optimized salt concentration during sample application (to minimize binding of contaminants) often leads to a significant improvement in the purity level of the final product.

Step elution may offer a technically simple alternative if gradient-making equipment is unavailable. However, care must be taken in the design of the steps and the interpretation of results. Substances eluted by a sharp change in salt conditions may elute close together, giving a false peak that contains 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 salt concentration is introduced too early. For these reasons it is recommended to use a linear salt gradient when developing a new method. After characterizing the chromatographic behavior of the target protein(s) it is easier to use a step elution to increase resolution in the area where the peak of interest elutes.

## Flow rates

The maximum flow rate can be varied according to the stage of the separation. For example, lower flow rates allow time for binding or elution, higher flow rates could save time during equilibration, washing and re-equilibration. Flow rates are limited primarily by the rigidity of the media and by pressure specifications of the equipment.

Recommended flow rates for each HIC medium are given in Chapter 3. During separation, aim for 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 to save time. Alternatively, if peaks are well resolved it may be possible to load more sample and so benefit from higher capacity without significant loss of resolution. Figure 28 shows an example of how resolution can be maintained as flow rate is increased on a RESOURCE PHE column.



Column: RESOURCE PHE, 1 ml  
 Sample: Mixture of myoglobin, ribonuclease, lysozyme and chymotrypsinogen  
 Sample load: 0.38 mg  
 Start buffer: 2.0 M ammonium sulfate, 100 mM potassium phosphate, pH 7.0  
 Elution buffer: 100 mM potassium phosphate, pH 7.0  
 Flow:  
 a) 1.6 ml/min, (300 cm/h)  
 b) 4.8 ml/min, (900 cm/h),  
 c) 9.6 ml/min, (1800 cm/h)  
 Gradient: 20–100% B, 20 column volumes

**Fig 28.** Influence of increasing flow rate on resolution when separating a model protein mixture on RESOURCE PHE.

Flow rate is measured in simple volume terms, for example, 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 3). Results obtained at the same linear flow on different size columns will be comparable as far as the effects of flow rate are concerned.

-  Do not exceed the maximum recommended flow for the medium or prepacked column.
-  Higher flow rates and viscous buffers increase operating pressure (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 rigidity and excellent flow properties of media such as SOURCE and Sepharose High Performance.
-  Always pump 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.
-  When using a column that has been packed under normal laboratory conditions, always use a flow rate for separation that is less than the flow rate used for column packing in order to avoid shrinkage of the column bed by pressure increases that may occur when applying a sample. See Appendix 2 for column packing information.

## Wash and re-equilibration

Wash with 2–5 column volumes of salt-free elution buffer to elute any remaining hydrophobically bound material.

Most bound proteins are effectively eluted by simply washing the HIC medium with salt-free buffer solution. Include a salt-free 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.

-  Occasionally the hydrophobic interaction is so tight that harsher conditions may be required to remove all bound material, for example, 0.5–1.0 M NaOH, 70% ethanol or 30% isopropanol. These wash steps must be followed by a water or salt-free buffer wash before re-equilibrating the column with a high-salt start buffer.

Re-equilibrate with 5–10 column volumes of start buffer or until conductivity reaches the required value.

After washing, a re-equilibration step prepares the column for the next separation. Whenever possible, monitor conductivity to check that start conditions have been reached. The re-equilibration step can then be shortened or prolonged as necessary.

-  Appearance of a colored band at the top of the column, space between the upper adaptor and the bed surface, loss in resolution or a significant increase in back pressure indicates a need for cleaning. A general cleaning procedure for each HIC medium is given in Chapter 3 and Appendix 8 contains recommended procedures for removing severe contamination. In all cases, prevention is better than cure, and routine cleaning is recommended.

## Analysis of results and further steps

Analysis of results from the first separation will indicate if conditions can be improved to increase yield, achieve higher purity, speed up 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 6.

## 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, that is, column diameter
Linear flow (cm/h)	Volumetric flow rate (ml/min)
Sample concentration	Sample load
Gradient elution volume, that is, number of column volumes used for the gradient	

Using prepacked columns saves time and ensures reproducibility. For example, a separation can be developed on HiTrap HIC 1 ml or HiTrap HIC 5 ml columns and scaled up onto a HiPrep™ 16/10 HIC Sepharose Fast Flow (20 ml), HiLoad™ 16/10 or 26/10 Phenyl Sepharose HP (20 ml or 53 ml).

When scaling up a HIC 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 3) as used on the smaller column with the same ratio of gradient volume: column volume.

 Whenever possible develop the separation method on the medium that will be used at larger scale.

 For production-scale separations all the HIC media featured in this handbook (SOURCE, Sepharose High Performance and Sepharose Fast Flow) meet the needs for throughput and cleaning-in-place (CIP) at industrial scale.

 See Appendix 2 for column selection and column packing.

## Equipment selection

Appendix 3 provides a guide to the selection of systems for HIC.

-  Always rinse chromatography equipment, including valves and tubing, as thoroughly as possible after using HIC. The high salt concentrations can interfere with other separations, damage equipment and interfere with buffer flow.

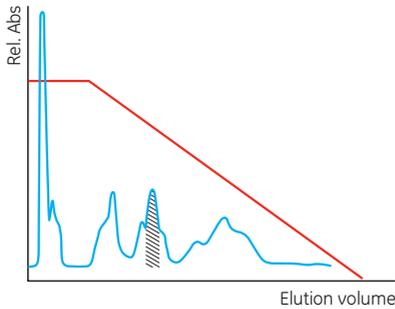
## Care of HIC media

A routine wash with 5–10 CV of distilled water between each separation should be sufficient to maintain the medium in good condition. However, when columns have been in use for some time, precipitated proteins or other contaminants may build up. The need for cleaning may be indicated by the appearance of a colored band at top of the column, space between the upper adaptor and the bed surface, loss in resolution or a significant increase in back pressure. A general cleaning procedure for each HIC medium is given in Chapter 3 and Appendix 8 also contains recommended procedures for removing severe contamination. In all cases, prevention is better than cure, and routine cleaning is recommended. See Appendix 1 for further details on sample preparation.

-  Always use filtered buffers, samples and cleaning solutions to reduce the need for additional column maintenance.
-  Always degas buffers and keep buffers, columns and samples at the same temperature to avoid the formation of air bubbles in the column and to ensure reproducible results.
-  If an increase in back pressure is observed, either on the pressure monitor or by the surface of the medium moving downward, check whether the problem is actually caused by the column before starting the cleaning procedure. Disconnect one piece of tubing at a time (starting at the fraction collector), start the pump, and check the pressure after each piece is disconnected. A blocked 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.
-  Store media and packed columns in 20% ethanol to prevent microbial growth.

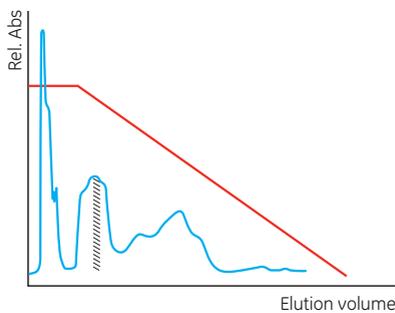
# Troubleshooting

## The ideal HIC separation: target protein is well resolved by gradient elution



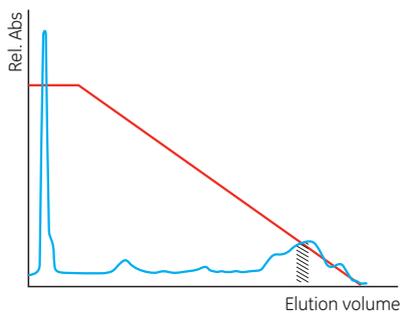
The rest of this section focuses on practical problems that may lead to a non-ideal HIC separation.

## Target protein is eluted early in the gradient. Poor resolution.



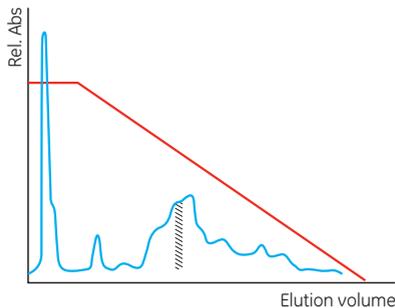
Repeat the separation at a higher salt concentration in the start buffer or with a salt of higher 'salting-out' strength (refer to the Hofmeister series on page 17). If no improvement in selectivity is obtained, try a medium with a different ligand or with a higher degree of ligand substitution, if available.

## Target protein is eluted near the end of the gradient. Poor resolution.



Repeat the separation with a lower salt concentration in the start buffer or using a salt with a lower salting-out strength (refer to the Hofmeister series on page 17). If no improvement in selectivity is obtained, try a medium with a different ligand or with a lower degree of ligand substitution, if available. A decrease in the initial salt concentration will weaken the binding, resulting in earlier elution of the protein. It may, however, not have a positive effect on selectivity, since the contaminants are eluted very close to, both before and after, the protein of interest.

## Target protein is eluted in the middle of the gradient. Poor resolution.



Optimize the gradient around the target protein; for example, use a segmented gradient with a shallower region around the target protein. Also consider the use of additives to improve resolution (see page 35). If resolution cannot be improved, use an alternative chromatography technique such as ion exchange for further purification.

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 or use a scavenger column of lower hydrophobicity, (see Appendix 1). Follow cleaning procedures, Appendix 8.
	Protein precipitation in the column.	Reduce salt concentration in start buffer. Follow cleaning procedures to remove precipitated proteins, Appendix 8.
	Sample is too viscous.	Dilute with buffer. Maintain protein concentration below 50 mg/ml.
	Lipoproteins or protein aggregates have precipitated.	Remove lipoproteins and aggregates during sample preparation (see Appendix 1).
Peak of interest is poorly resolved from other major peaks.	Microbial growth has occurred in the column.	Follow cleaning procedures, Appendix 8. Always filter buffers. Store in the presence of 20% ethanol to prevent microbial growth when not in use.
	Large mixing spaces at top of or after column.	Adjust top adaptor to surface of medium if necessary. Reduce all post-column volumes.
	Incorrect salt conditions.	Check binding conditions. Prepare new solutions.
	Sub-optimal elution conditions, e.g., gradient too steep, flow rate too high.	Alter elution conditions: use shallower gradient, reduce flow rate.
	Column is poorly packed.	Check column efficiency (see Appendix 2). Repack if needed. Use prepacked columns.
	Column overloaded with sample.	Decrease sample load.
Proteins do not bind or elute as expected.	Precipitation of proteins in the column.	Follow cleaning procedures, Appendix 8. Reduce salt concentration in buffer or apply sample in aliquots at lower salt concentration while maintaining salt concentration in buffer.
	Incorrect salt conditions.	Check conditions required. Prepare new solutions.
	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 has changed during storage.	Prepare fresh samples.
	Protein may be unstable or inactive in the elution buffer.	Determine the stability of the protein.
	Column equilibration incomplete.	Repeat or prolong the equilibration step until conductivity and pH are constant.
Proteins are forming aggregates and binding strongly to the medium.	Proteins are forming aggregates and binding strongly to the medium.	Use lower salt concentrations. Consider use of additives to reduce hydrophobic interactions, see Buffer additives page 35.
	Sample, buffer or temperature conditions are different from previous runs.	Check conditions.

*continues on following page*

<b>Situation</b>	<b>Cause</b>	<b>Remedy</b>
Protein elutes later than expected or not at all.	Salt concentration too high.	Decrease salt concentration in elution buffer.
	Hydrophobic interactions too strong.	Use medium with lower hydrophobicity or lower ligand density. Consider using an additive to reduce hydrophobic interaction, see Buffer additives page 35.
Protein elutes earlier than expected (during the wash phase).	Salt concentration of sample and buffer is too low.	Increase salt in sample and buffer.
	Column equilibration incomplete.	Repeat or prolong the equilibration step until conductivity is 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 2).
	Column overloaded with sample.	Decrease sample load and repeat.
	Column contaminated.	Clean using recommended procedures.
Peaks are tailing.	Column packing too loose.	Check column efficiency (see Appendix 2). Repack using a higher flow rate. Use prepacked columns.
Peaks have a leading edge.	Column packing compressed.	Check column efficiency (see Appendix 2). Repack using a lower flow rate. Use prepacked columns.
Medium/beads appears in eluent.	Column packing compressed.	Check column efficiency (see Appendix 2). 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 during equilibration.
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 salt conditions, adjust to improve sample solubility.
	Proteins not eluting.	Consider use of additives to reduce hydrophobic interactions, see Buffer additives page 35. Use less hydrophobic medium.

*continues on following page*

Situation	Cause	Remedy
Peaks too small.	Sample absorbs poorly at chosen wavelength.	If appropriate, check absorbance range on monitor. If satisfactory, use a different wavelength, e.g., 214 nm instead of 280 nm.
	Different assay conditions have been used before and after the chromatographic step.	Use same assay conditions for all assays.
	Excessive peak 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. Check purity of protein.
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.	Desalt/dialyze the original sample before measuring activity, as cell lysates/extracts often contain low molecular weight substances that can affect activity.
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.
	Sample is too viscous.	Dilute with buffer. Maintain protein concentration below 50 mg/ml.
	Sample not filtered properly.	Clean the column, filter the sample and repeat.
	Microbial growth.	Follow cleaning procedures, Appendix 8. Store in the presence of 20% ethanol to prevent microbial growth. Always filter buffers.
	Turbid sample.	Improve sample preparation (see Appendix 1).
	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. Reduce salt concentration of start buffer.
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 2).
Cracks in the bed.	Large air leak in column.	Check all connections for leaks. Repack the column if possible (see Appendix 2).
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 blank elution gradients.	Incomplete elution of previous sample.	Wash the column according to recommended methods.
Spikes in chromatogram.	Air bubble trapped in UV monitor flow cell.	Always use degassed buffers.
	Buffer impurities.	Use high-quality reagents.

# BioProcess Media - made for bioprocessing

Specific BioProcess™ Media have been designed for each chromatographic stage in a production process from Capture to Polishing. High flow rate, high capacity and high recovery contribute to the overall productivity of an industrial process. 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. GE Healthcare can assure future supplies of BioProcess Media, making them a safe investment for long-term production.

The media are produced following validated methods and tested under strict control to fulfill high performance specifications. A certificate of analysis is available with each order.

Regulatory Support Files 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.

All BioProcess Media have a chemical stability that allows 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.gehealthcare.com/protein-purification-bioprocess](http://www.gehealthcare.com/protein-purification-bioprocess)

## **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 GE Healthcare works in close collaboration with the user to design, manufacture, test and deliver media for large-scale purification.

In HIC the combination of all components (proteins, buffers and medium) is important for successful separation. A medium must exhibit the correct specificity with minimal differences between batches and a narrow specification range. Since the hydrophobic properties of the medium are determined by the combination of matrix, ligand and ligand density, these parameters can be altered in order to develop a Custom Designed Medium specifically suited to a particular HIC separation step. Only small differences in hydrophobicity can ensure the success of a specific application.

Examples of Custom Designed Media include:

- Butyl Sepharose High Performance was developed to provide a medium that was less hydrophobic than the standard Phenyl Sepharose High Performance for separations being developed on a High Performance matrix.
- Butyl Sepharose 6 Fast Flow was developed to fulfill a customer's requirement for increased productivity by increasing the rigidity of the matrix compared to Butyl Sepharose 4 Fast Flow.

CDM products are available on request. Please ask your local representative for further details.

## ***Custom Products***

GE Healthcare offers the largest selection of prepacked columns and bulk media available, encompassing most liquid chromatography techniques. However, special configurations, or a combination of column and medium not offered as a standard catalogue product, can be supplied by the Custom Products Group. The group can supply any chromatography medium packed in any column, ranging from HiTrap and Tricorn™ to FineLine™ and BPG™ 100 columns. Columns from GE Healthcare are designed to ensure the highest performance and to meet the demands of modern pharmaceutical manufacturing.

The Custom Products team works closely with each customer from the initial discussions to establish specific needs, column and media selection to final delivery of a product with certified quality. Each column is packed, tested, and certified under stringent ISO 9001 standards. A certificate and user instruction is supplied with each column, describing column performance. Customized pack sizes and media normally available only in prepacked columns can also be supplied.

Delivery time is 2–4 weeks, depending on specific media and column specifications. Please ask your local representative for more details about Custom Products.



# Chapter 3

## Media for hydrophobic interaction chromatography

### Introduction

The first commercially available hydrophobic interaction chromatography (HIC) media were introduced during the 1970s, using a matrix of Sepharose CL-4B substituted with phenyl or octyl ligands. References to the use of these earlier media for separation of biomolecules can still be found in older scientific literature.

More recently developed matrices offer improved capacity with greater physical and chemical stability, and much improved flow properties. Stringent cleaning conditions can be used when required, and there is no need for frequent column repacking. These media are designed to meet the cleaning-in-place requirements for large-scale industrial chromatography.

Chapter 1 describes how matrix characteristics determine chromatographic properties such as efficiency, capacity and recovery as well as chemical and physical stability and flow properties. This chapter describes commercially available media together with recommendations for use, purification examples, separation and cleaning protocols, and tips and hints for achieving the best performance.

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

### SOURCE: purification with high resolution and easy scale-up

 Use SOURCE media for polishing steps in laboratory or large-scale applications that require highest resolution. Use SOURCE media for capture or intermediate purification if a suitable selectivity is not available in a medium of larger particle size.

 Run SOURCE columns on systems such as ÄKTAdesign, FPLC™ System and HPLC. Appendix 3 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 hydrophobic ligands: phenyl, ether or isopropyl (Figure 29). The media demonstrate extreme chemical and physical stability. 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 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.

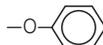
Separation methods can be easily scaled up from prepacked RESOURCE columns through to large-scale columns such as FineLINE.



#### Ligand

Ether (ETH)  $\text{—O—CH}_2\text{—CHOH—CH}_2\text{—OH}$

Isopropyl (ISO)  $\text{—O—CH—(CH}_3\text{)}_2$

Phenyl (PHE)  $\text{—O—}$  



Increasing ligand hydrophobicity

**Fig 29.** Ligands are coupled to monodispersed SOURCE particles via uncharged, chemically stable O-ether linkages.

## Purification options



**Fig 30.** HIC media based on a SOURCE matrix are available in prepacked columns and as media packs.

**Table 6.** HIC products based on SOURCE.

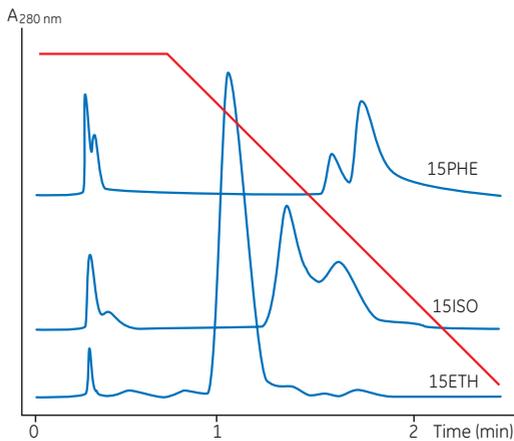
Product	Ligand*	Recommended working flow <sup>†</sup>	Maximum flow <sup>†</sup>	Maximum operating back pressure <sup>‡</sup> (MPa/psi) 1 MPa=10 bar
RESOURCE PHE, 1 ml	phenyl	1.0–10 ml/min	10 ml/min	1.5/220
SOURCE 15PHE 4.6/100 PE, 1.7 ml	phenyl	0.5–2.5 ml/min	5 ml/min	4/580
SOURCE 15PHE	phenyl	150–900 cm/h	1800 cm/h	0.5/72
RESOURCE ETH, 1 ml	ether	1.0–10 ml/min	10 ml/min	1.5/220
SOURCE 15ETH	ether	150–900 cm/h	1800 cm/h	0.5/72
RESOURCE ISO, 1 ml	isopropyl	1.0–10 ml/min	10 ml/min	1.5/220
SOURCE 15ISO	isopropyl	150–900 cm/h	1800 cm/h	0.5/72

\* The nature of the SOURCE matrix makes it impossible to define ligand density in the way that is used to compare Sepharose-based media.

<sup>†</sup> See Appendix 3 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa. Note that final working flow will depend also on factors such as column size and bed height, sample characteristics and loading conditions, the equipment used and the back pressure that the equipment can withstand.

<sup>‡</sup> Maximum operating back pressure refers to the pressure above which the medium begins to compress.

-  It is important to note that the binding capacity of a HIC medium is highly dependent on the properties of the target protein and contaminants, the selectivity of the medium and the binding conditions. Capacity must be determined empirically during media screening and method development.
-  Use the RESOURCE HIC Test Kit, comprising three prepacked RESOURCE 1 ml columns (ETH, ISO and PHE), to rapidly screen for the most suitable medium for a specific application. Select the medium that gives the best selectivity, resolution and loading capacity at the lowest salt concentration. Figure 31 shows an example of the differences in selectivity seen during media selection for an IgM purification.



Columns: SOURCE 15ETH, SOURCE 15ISO and SOURCE 15PHE (1 ml prepacked)  
 Sample: Purified monoclonal mouse IgM antibodies in phosphate-buffered saline  
 Sample volume: 20  $\mu$ l  
 Start buffer: 2.0 M ammonium sulfate, 100 mM sodium phosphate, pH 7.0  
 Elution buffer: 100 mM sodium phosphate, pH 7.0  
 Flow: 5 ml/min, (1500 cm/h)  
 Gradient: 20–100% B, 10 column volumes

Fig 31. Screening of HIC media for an antibody purification.

- Use prepacked RESOURCE columns for fast media selection, method scouting, group separations or sample concentration.
- Use SOURCE 15PHE 4.6/100 PE to improve resolution by increasing column length. Further optimization may be required. Use optimized conditions as the first step toward scaling up.

Table 7. Packing volumes and bed heights for SOURCE media.

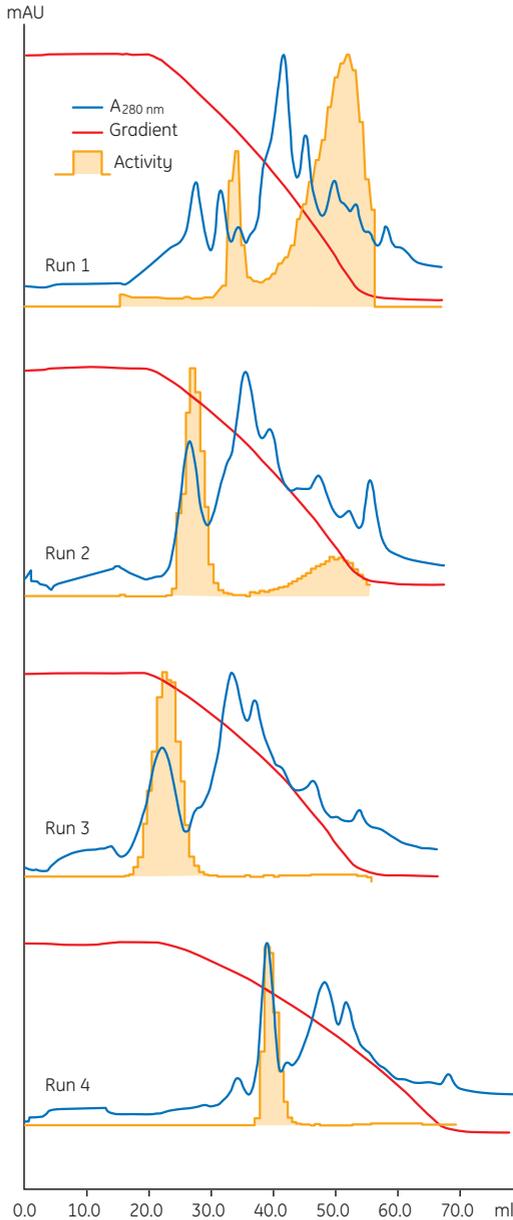
Empty glass column i.d./length (mm)	Volume	Bed height
Tricorn 5/150	up to 3 ml	up to 15 cm
Tricorn 5/200	up to 4 ml	up to 20 cm
Tricorn 10/100	up to 8 ml	up to 10 cm
Tricorn 10/150	up to 12 ml	up to 15 cm
Tricorn 10/200	up to 16 ml	up to 20 cm

Select a production column such as FineLINE for larger volumes.

# Purification examples

## Method optimization

Figure 32 shows examples of runs made while optimizing the intermediate purification step for a recombinant protein, tyrosine phosphatase. The protein was partially purified in an initial capture step, using ion exchange chromatography on Q Sepharose XL. The active fraction was isolated and applied to a SOURCE 15PHE 4.6/100 PE column for intermediate purification by HIC. Results show that increasing the ammonium sulfate concentration used during sample application and increasing the gradient volume used for elution had the most significant impact on resolution. Note that, after the first run, glycerol was included to reduce the strong binding of tyrosine phosphatase and facilitate elution (refer to page 35 for further details on additives used during HIC separations).

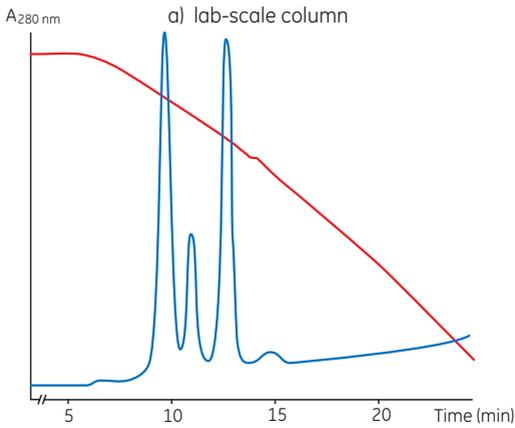


Purification protocol: CIPP	
Starting material:	Diluted <i>E. coli</i> supernatant
Capture:	Q Sepharose XL
Intermediate purification:	SOURCE 15PHE
Polishing:	Superdex 75

Column: SOURCE 15PHE 4.6/100 PE  
Sample: Recombinant protein tyrosine phosphatase, partially purified on HiTrap Q XL 5 ml  
Sample volume: 10 ml  
Start buffer (run 1): 1.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 25 mM Tris, 1 mM EDTA, 2 mM DTT, pH 7.5  
Elution buffer (run 1): 25 mM Tris, 1 mM EDTA, 2 mM DTT, pH 7.5  
Start buffer (run 2): 1.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 50 mM Tris, 1 mM EDTA, 2 mM DTT, pH 7.5  
Elution buffer (run 2): 50 mM Tris, 1 mM EDTA, 2 mM DTT, 10% glycerol, pH 7.5  
Start buffer (run 3): 1.5 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM MES, 1 mM EDTA, 2 mM DTT, pH 6.5  
Elution buffer (run 3): 20 mM MES, 1 mM EDTA, 2 mM DTT, 10% glycerol, pH 6.5  
Start buffer (run 4): 2 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 20 mM MES, 1 mM EDTA, 2 mM DTT, pH 6.5  
Elution buffer (run 4): 20 mM MES, 1 mM EDTA, 2 mM DTT, 10% glycerol, pH 6.5  
Flow : a) 1.5 ml/min (run 1)  
b) 1.0 ml/min (runs 2,3,4)  
Gradient: 0–100% elution buffer in 20 CV (runs 1,2,3)  
0–100% elution buffer in 27 CV (run 4)  
Activity measurement: p-nitrophenyl phosphate (pNPP) activity assay at 405 nm.

Fig 32. Optimization steps for intermediate purification of a recombinant protein.

## Scaling up



**Column:** SOURCE 15ISO  
a) 7.5 x 50 mm (2.2 ml)  
b) FineLINE 100, 100x50 mm (400 ml)

**Sample:** lactalbumin, chymotrypsinogen

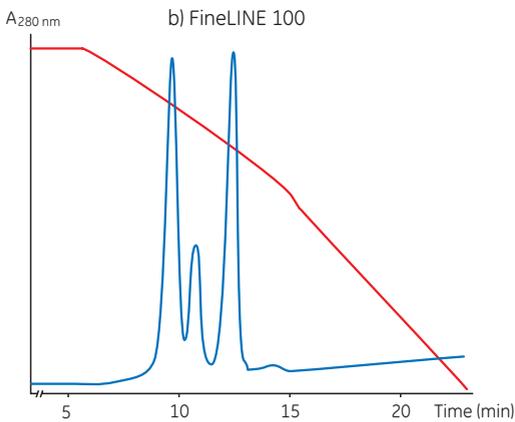
**Sample load:** 0.3 mg/ml medium

**Start buffer:** 100 mM potassium phosphate, 2.0 M ammonium sulfate, pH 7.0

**Elution buffer:** 100 mM potassium phosphate, pH 7.0

**Flow :** a) 2.2 ml/min (300 cm/h)  
b) 385 ml/min (300 cm/h)

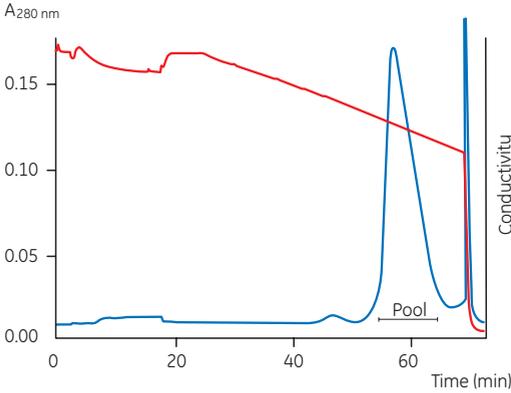
**Gradient:** 20–100% elution buffer, 20 CV



**Fig 33.** Reproducible results when scaling up on SOURCE 15ISO. Separation of a model protein mixture shows a 180-fold scale-up from a laboratory-scale column (a) to a FineLINE 100 production-scale column (b).

## Polishing

Figure 34 shows the use of SOURCE 15PHE as the final polishing step in a large-scale purification of a recombinant protein, rExotoxin A (PE553D), expressed in the periplasm of *rPseudomonas aeruginosa*. Ammonium sulfate (1.0 M) was added to partially purified protein before sample application. The bound exotoxin A was eluted using a linear gradient from 1.0 to 0.55 M ammonium sulfate over 15 column volumes. This step removed the remaining contaminant proteins, as shown by a single peak on reversed phase chromatography (Figure 35).



Purification protocol: CIPP

Starting material: Unclarified *E. coli* homogenate

Capture: STREAMLINE DEAE

Intermediate purification: Phenyl Sepharose 6 Fast Flow

Intermediate purification: SOURCE 30Q

Polishing: SOURCE PHE

Column: SOURCE 15PHE, 35 mm i.d. x 100 mm

Sample: 0.5 l/cycle was applied from previous SOURCE 30Q step, adjusted to 1.0 M ammonium sulfate

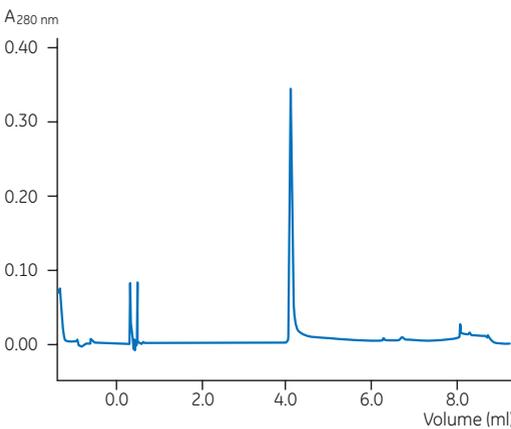
Start buffer: 50 mM phosphate, 1.0 M ammonium sulfate, pH 7.4

Elution buffer: 50 mM phosphate, pH 7.4

Gradient: 0–45% elution buffer, 15 CV

Flow: 200 cm/h

**Fig 34.** SOURCE 15PHE used as the final polishing step in a large-scale purification of a recombinant protein, rExotoxin A (PE553D).



Column:  $\mu$ RPC C2/C18, SC 2.1/10

Sample: Pooled fractions from SOURCE 15PHE

Sample volume: 50  $\mu$ l

Start buffer: 0.1% trifluoroacetic acid (TFA) in water

Elution buffer: 0.1% TFA in acetonitrile

Gradient: 25–75% elution buffer over 47 min

Flow: 150  $\mu$ l/min

**Fig 35.** Chromatographic analyses demonstrate purity after polishing step on SOURCE 15PHE.

## Performing a separation

Guidelines for the selection of media, buffer, salt concentration and pH, 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 and samples after all salts and additives have been included. Use high-quality water and chemicals. Filter solutions using 0.22 µm filters. To avoid formation of air bubbles in a packed column and to ensure reproducible results, the column and buffers should be at the same temperature when preparing for a run.
-  To avoid problems with precipitation, check the salt stability window of the sample components. Avoid working at concentrations near to the stability limit of the target protein. For samples with unknown hydrophobic properties, try the following:

start buffer: 1.5 M ammonium sulfate, 50 mM phosphate buffer, pH 7.0

elution buffer: 50 mM phosphate buffer, pH 7.0

-  Note that flow rates may need to be reduced due to the viscosity of the chosen start buffer, sample characteristics, loading requirements and the equipment used.

## First-time use or after long-term storage

- To remove ethanol, wash with 5 column volumes of distilled water or elution buffer.  
Flow: 2 ml/min, SOURCE 15PHE 4.6/100 PE  
4 ml/min, RESOURCE 1 ml  
200 cm/h for SOURCE packed in larger columns
- Wash with 5 column volumes of start buffer, same flow rate as in step 1.
- Perform a blank elution (i.e., perform a run but do not load any sample) to check conductivity profile.

## Separation by gradient elution

Flow: 2 ml/min, SOURCE 15PHE 4.6/100 PE  
4 ml/min, RESOURCE 1 ml  
200 cm/h for SOURCE packed in larger columns

Collect fractions throughout the separation.

- Equilibrate the column with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable.
- Adjust the sample to the chosen salt concentration (and pH if necessary). Filter and apply to the column.
- Wash with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable so that all unbound material has washed through the column.
- Begin elution using a gradient volume of 10–20 column volumes, increasing the proportion of elution buffer until the salt concentration reaches a minimum, that is, salt-free buffer.
- Wash with 2–5 column volumes of salt-free elution buffer to elute remaining hydrophobically bound material.
- Re-equilibrate with 5–10 column volumes of start buffer or until conductivity reaches the required value.

## Separation by step elution

Flow: 2 ml/min, SOURCE 15PHE 4.6/100 PE  
4 ml/min, RESOURCE 1 ml  
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 salt concentration (and pH if necessary). Filter and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable so that all unbound material has washed through the column.
4. Elute with 5 column volumes of elution buffer + salt at chosen concentration.
5. Repeat step 4 at lower salt concentrations until the target protein(s) has been eluted.
6. Wash with 2–5 column volumes of salt-free elution buffer to elute remaining hydrophobically bound material.
7. Re-equilibrate with 5–10 column volumes of start buffer or until conductivity reaches the required value.



Save time by using higher flow rates during the salt-free wash and re-equilibration steps, but do not exceed the maximum recommended flow for the medium.



Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 2.



Never leave columns or equipment in high-salt solutions.

## Cleaning

Correct preparation of samples and buffers and application of a salt-free buffer 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.



Whenever possible, reverse the direction of flow during cleaning so that contaminants do not pass through the entire column length. 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 such as precipitated proteins.

Flow: 0.2 ml/min, SOURCE 15PHE 4.6/100 PE  
1 ml/min, RESOURCE 1 ml  
40 cm/h, with a contact time of 1–2 hour, for SOURCE packed in larger columns  
Note that flow rates may need to be reduced due to the condition of the column or the viscosity of the sample, buffers or storage solutions.

1. Wash with at least 4 column volumes of 1 M NaOH.
2. Wash with at least 3 column volumes of water or until eluent pH is neutral.

- 3a. To start a new separation: re-equilibrate with at least 3 column volumes of start buffer or until the correct eluent pH is achieved.
- 3b. For storage: wash with at least 5 column volumes of storage solution. Allow UV baseline to stabilize before storing the column.
- To remove lipids, lipoproteins and very hydrophobic proteins, see Appendix 1.

## Media characteristics

Composition: rigid, monodisperse, polystyrene/divinyl benzene particles (15 µm) with an optimized pore size distribution. The base matrix is coupled to one of three hydrophobic ligands: ether, isopropyl or phenyl.

**Table 8.** Characteristics of SOURCE HIC media.

Products	Matrix	pH stability*	Mean particle size
SOURCE 15PHE	Polystyrene/divinyl	Long term: 2–12	15 µm (monosized)
SOURCE 15ETH	benzene, monodispersed	Short term: 1–14	
SOURCE 15ISO	particles		

\*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 GE Healthcare.

## Chemical stability

For daily use, HIC media based on SOURCE are stable in all common aqueous buffers, 1 M HCl, 2 M NaOH, 20% ethanol, 100% isopropanol, denaturing agents (6 M guanidine hydrochloride), 1 M acetic acid, 30% isopropanol, 30% acetonitrile and up to 2% SDS.

## 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. Ensure that the column is sealed well to avoid drying out. Store columns and unused media at 4°C to 30°C in 20% ethanol. Do not freeze.

Large-scale production columns are often stored in 0.01 M sodium hydroxide as an alternative to 20% ethanol.



To avoid the formation of air bubbles, ensure that columns, buffers and equipment are at the same temperature before use.

# Sepharose High Performance: purification with high resolution

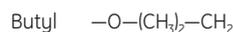
- Use Sepharose High Performance for intermediate or polishing steps that require high resolution (recommended flows up to 300 cm/h). Use when SOURCE media do not offer the required selectivity.
- Use Sepharose High Performance (34  $\mu\text{m}$  particle size) HIC media for capture or scale-up when selectivity is satisfactory, high resolution is a priority and if lower flow rates (to compensate for a higher back pressure) are acceptable.
- Run Sepharose High Performance columns on systems such as ÄKTAdesign, FPLC System and HPLC. Appendix 3 gives guidance on how to select the most suitable ÄKTAdesign system.

Sepharose High Performance is based on a matrix of particles (mean size 34  $\mu\text{m}$ ) made from 6% agarose and highly cross-linked for chemical and physical stability. The small particle size facilitates fast binding and dissociation even at high sample loads and flow rates. In combination with the correct selectivity, high-resolution separations can be achieved. Particle size and bed volumes remain stable, despite changes in salt concentration, to ensure fast separations at high flow rates. Ligands are coupled via a chemically stable O-ether linkage (Figure 36).

- Sepharose High Performance can be used for group separations, sample concentration or capture steps. However, these separations should be limited to reasonably clean samples to avoid the risk of blocking the column filter (34  $\mu\text{m}$  particles requires the use of finer column filters).

Reference lists highlighting the use of Phenyl Sepharose High Performance columns are available at [www.gehealthcare.com/protein-purification-labresearch](http://www.gehealthcare.com/protein-purification-labresearch)

## Ligand



**Fig 36.** Ligands are coupled to the Sepharose High Performance matrix via uncharged, chemically stable O-ether linkages.

## Purification options



**Fig 37.** Phenyl Sepharose High Performance is available prepacked in HiTrap or HiLoad columns and in media packs.

**Table 9.** HIC products based on Sepharose High Performance.

Product	Ligand density per ml medium <sup>‡</sup>	Recommended working flow*	Maximum flow*	Maximum operating back pressure <sup>†</sup> (MPa/psi) 1 MPa=10 bar
HiTrap Phenyl HP, 1 ml	25 µmol	up to 1 ml/min	4 ml/min	0.3/43
HiTrap Phenyl HP, 5 ml	25 µmol	up to 5 ml/min	20 ml/min	0.3/43
HiLoad 16/10 Phenyl Sepharose HP, 20 ml	25 µmol	up to 5 ml/min	5 ml/min	0.3/43
HiLoad 26/10 Phenyl Sepharose HP, 53 ml	25 µmol	up to 13 ml/min	13 ml/min	0.3/43
Phenyl Sepharose High Performance	25 µmol	30–150 cm/h	150 cm/h	0.5/72

Custom Designed Media, including Butyl Sepharose High Performance, are also available, see page 50.

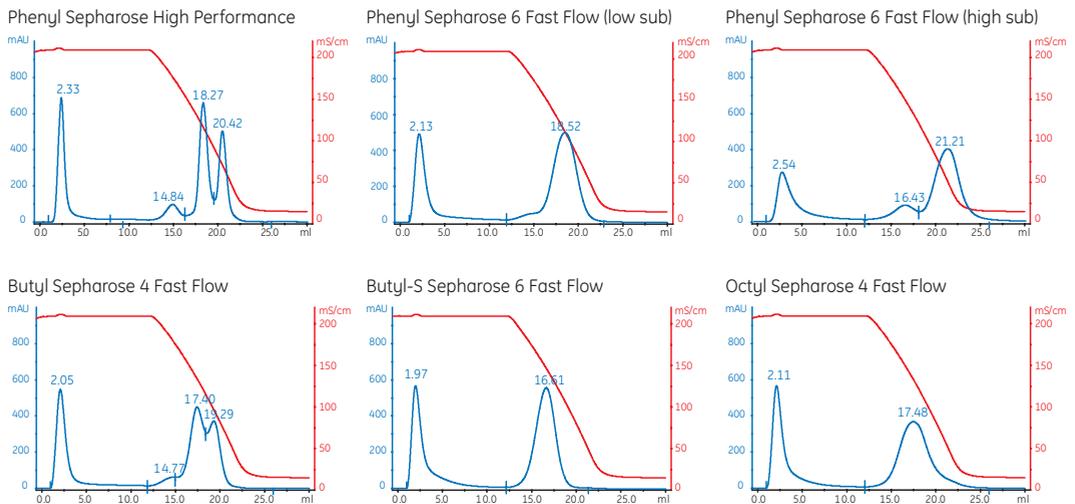
\* Recommendations are for separations at room temperature in aqueous buffers. See Appendix 3 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa. Note that final working flow will depend also on factors such as column size and bed height, sample characteristics and loading conditions, the equipment used and the back pressure that the equipment can withstand.

<sup>†</sup> Maximum operating back pressure refers to the pressure above which the medium begins to compress.

<sup>‡</sup> Refer to page 19 for more details about the influence of ligand density.

It is important to note that the binding capacity of a HIC medium is highly dependent on the properties of the target protein and contaminants, the selectivity of the medium and the binding conditions. Capacity must be determined empirically during media screening and method development.

Use the HiTrap HIC Selection Kit, comprising six prepacked HiTrap 1 ml columns containing media with different hydrophobic characteristics, to rapidly screen for the most suitable medium for a specific application (Figure 38). Select the medium that gives the best selectivity, resolution and loading capacity at the lowest salt concentration.



Sample: cytochrome C, ribonuclease A, lysozyme,  $\alpha$ -chymotrypsinogen, 6 mg protein/ml, (1:3:1:1) in start buffer  
Sample volume: 1 ml  
Sample load: 6 mg protein/ml medium  
Flow: 1.0 ml/min, (150 cm/h)  
Start buffer: 1.7 M  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 M  $\text{Na}_2\text{HPO}_4$ , pH 7.0  
Elution buffer: 0.1 M  $\text{Na}_2\text{HPO}_4$ , pH 7.0  
Gradient: 0%–100% elution buffer in 10 CV

**Fig 38.** Comparison of the different selectivity characteristics in a HiTrap HIC Selection Kit.

- Use prepacked HiTrap columns (1 ml or 5 ml) for method scouting, group separations, small-scale purification, sample concentration or clean-up. When increased capacity is required, use prepacked columns HiLoad 16/10 Phenyl Sepharose HP (20 ml) or HiLoad 26/10 Phenyl Sepharose HP (53 ml).

Phenyl Sepharose High Performance and Phenyl Sepharose 6 Fast Flow (low sub) usually exhibit similar hydrophobic properties. For capture or intermediate purification, Phenyl Sepharose 6 Fast Flow (low sub) may give similar resolution at higher flow rates.

- Butyl Sepharose High Performance is available only as a Custom Designed Product (see page 50). The butyl ligand offers an alternative selectivity to Phenyl Sepharose High Performance.

**Table 10.** Packing volumes and bed heights for Phenyl Sepharose High Performance and Phenyl Sepharose 6 Fast Flow media.

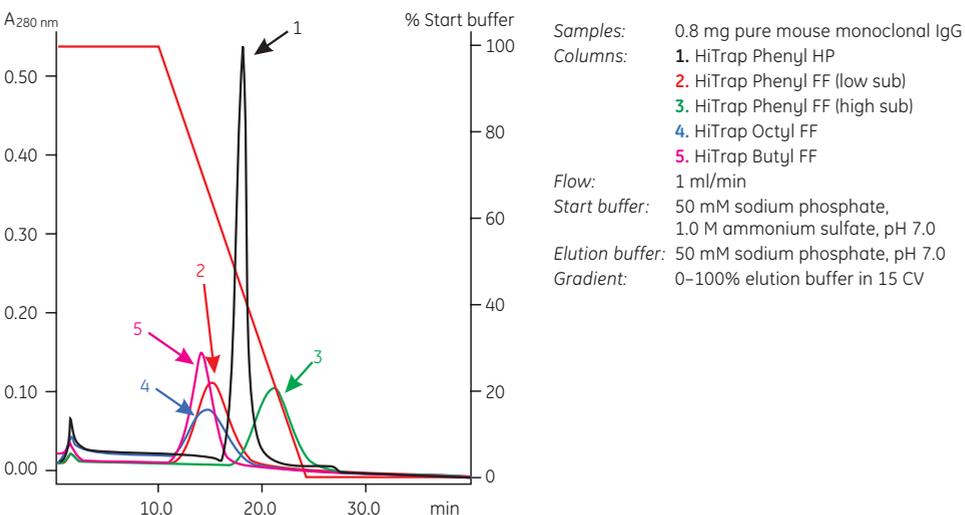
Empty glass column i.d./length (mm)	Volume	Bed height
Tricorn 5/100	up to 2 ml	up to 10 cm
Tricorn 5/150	up to 3 ml	up to 15 cm
Tricorn 5/200	up to 4 ml	up to 20 cm
Tricorn 10/100	up to 8 ml	up to 10 cm
Tricorn 10/150	up to 12 ml	up to 15 cm
Tricorn 10/200	up to 16 ml	up to 20 cm
XK 16/20	up to 30 ml	up to 15 cm
XK 26/20	up to 80 ml	up to 15 cm
XK 26/40	up to 196 ml	>15 cm

Select a production column such as FineLINE for larger volumes.

## Purification examples

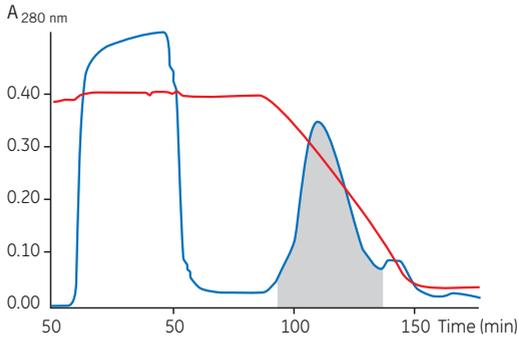
### Media screening: development of a monoclonal antibody purification

The most common contaminants of monoclonal antibody preparations are albumin and transferrin. However, since most monoclonal antibodies are more hydrophobic than these contaminants, HIC can be used to bind the antibody as the contaminants wash through the column. Figure 39 shows an example of screening using columns from the HiTrap HIC Selection Kit in order to select a HIC medium that could offer the best selectivity and resolution for purification of a monoclonal IgG. Phenyl Sepharose High Performance produced a well-resolved peak containing IgG (note that this peak is not necessarily the pure IgG, but may represent a series of components with similar hydrophobicity to the IgG).



**Fig 39.** Media screening for monoclonal antibody purification.

## Capture: monoclonal antibody purification



Purification protocol: CIPP	
Starting material:	Hybridoma culture
Capture:	Phenyl Sepharose High Performance
Polishing:	Superdex 200 prep grade

**Sample:** Hybridoma cell culture supernatant, mouse IgG<sub>1</sub>, anti-IgE. Ammonium sulfate added to 0.5 M

**Column:** HiLoad 16/10 Phenyl Sepharose HP

**Start buffer:** 20 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0

**Elution buffer:** 20 mM potassium phosphate, pH 7.0

**Gradient:** 0–100% elution buffer in 10 CV

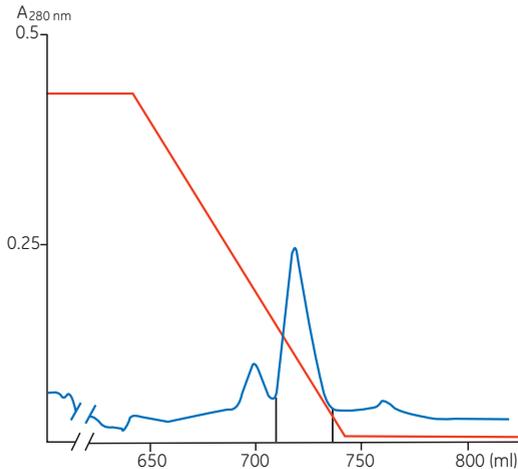
**Flow:** 100 cm/h

**Fig 40.** Concentration and purification of a monoclonal antibody from hybridoma cell culture.

Figure 40 shows an example of high selectivity used in a capture step. The goal of the purification was to produce a monoclonal antibody of sufficient purity for *in vitro* diagnostic use. The mouse IgG<sub>1</sub> anti-IgE, produced in a hybridoma cell culture, bound very strongly to Phenyl Sepharose High Performance, and most fetal calf serum proteins passed through the column. No intermediate step was required as the capture step gave a purity level >95%. The sample was concentrated into a small volume that could be transferred directly to a polishing step.

## Intermediate purification: recombinant HIV reverse transcriptase

In this example an *E. coli* lysate was subjected to ammonium sulfate precipitation (see Appendix 1) followed by a capture step using ion exchange chromatography. After addition of ammonium sulfate, HIC was used to concentrate and further purify the sample using a gradient elution prior to a final polishing step using another ion exchange medium.



Purification protocol: CIPP	
Starting material:	<i>E. coli</i> lysate
Capture:	Q Sepharose Fast Flow
Intermediate purification:	Phenyl Sepharose High Performance
Polishing:	Mono Q™

**Column:** HiLoad 16/10 Phenyl Sepharose HP

**Sample:** 400 ml (10 mg protein) diluted to 600 ml with 3 M ammonium sulfate

**Start buffer:** 10 mM Tris HCl, 1 M ammonium sulfate, 10% glycerol, 1 mM DTT, pH 8.0

**Elution buffer:** 10 mM Tris HCl, 10% glycerol, 1 mM DTT, pH 8.0

**Flow:** 3 ml/min, (90 cm/h)

**Fig 41.** Concentration and purification of HIV reverse transcriptase on HiLoad 16/10 Phenyl Sepharose HP.

## Performing a separation

Guidelines for the selection of media, buffer, salt and pH 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 and samples after all salts and additives have been included. Use high-quality water and chemicals. Filter solutions using 0.45 µm or 0.22 µm filters. To avoid formation of air bubbles in a packed column and to ensure reproducible results, the column and buffers should be at the same temperature when preparing for a run.
- To avoid problems with precipitation, check the salt stability window of the sample components. Avoid working at concentrations near to the stability limit of the target protein. For samples with unknown hydrophobic properties, try the following:

start buffer: 1.5 M ammonium sulfate, 50 mM phosphate buffer, pH 7.0

elution buffer: 50 mM phosphate buffer, pH 7.0

- Note that flow rates may need to be reduced due to the viscosity of the chosen start buffer, sample characteristics, loading requirements and the equipment used.

## First-time use or after long-term storage

- To remove ethanol, wash with 5 column volumes of distilled water or elution buffer.  
Flow: 1 ml/min, HiTrap 1 ml  
5 ml/min, HiTrap 5 ml  
0.8 ml/min, HiLoad 16/10, 20 ml  
2.2 ml/min, HiLoad 26/10, 53 ml  
25 cm/h for Sepharose High Performance packed in larger columns
- Wash with 5 column volumes of start buffer.  
Flow: 1 ml/min, HiTrap 1 ml  
5 ml/min, HiTrap 5 ml  
3 ml/min, HiLoad 16/10, 20 ml  
8 ml/min, HiLoad 26/10, 53 ml  
50 cm/h for Sepharose High Performance packed in larger columns
- Perform a blank elution to check conductivity.

## Separation by gradient elution

Flow: 1 ml/min, HiTrap 1 ml  
5 ml/min, HiTrap 5 ml  
3 ml/min, HiLoad 16/10, 20 ml  
8 ml/min, HiLoad 26/10, 53 ml  
50–100 cm/h for Sepharose High Performance packed in larger columns

Collect fractions throughout the separation.

- Equilibrate column with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable.
- Adjust the sample to the chosen salt concentration (and pH if necessary). Filter and apply to the column.

3. Wash with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable so that all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes, increasing the proportion of elution buffer until the salt concentration reaches a minimum, that is, salt-free buffer.
5. Wash with 2–5 column volumes of salt-free elution buffer to elute remaining hydrophobically bound material.
6. Re-equilibrate with 5–10 column volumes of start buffer or until conductivity reaches the required value.

## Separation by step elution

Flow: 1 ml/min, HiTrap 1 ml  
5 ml/min, HiTrap 5 ml  
3 ml/min, HiLoad 16/10, 20 ml  
8 ml/min, HiLoad 26/10, 53 ml  
50–100 cm/h for Sepharose High Performance packed in larger columns

Collect fractions throughout the separation.

1. Equilibrate the 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 salt concentration (and pH if necessary). Filter and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable so that all unbound material has washed through the column.
4. Elute with 5 column volumes of elution buffer + salt at chosen concentration.
5. Repeat step 4 at lower salt concentrations until the target protein(s) has been eluted.
6. Wash with 2–5 column volumes of salt-free elution buffer to elute remaining hydrophobically bound material.
7. Re-equilibrate with 5–10 column volumes of start buffer or until conductivity reaches the required value.

-  Save time by using higher flow rates during the salt-free wash and re-equilibration steps, but do not exceed the maximum recommended flow for the medium.
-  Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 2.
-  Never leave columns or equipment in high salt solutions.

## Cleaning

Correct preparation of samples and buffers and application of a salt-free buffer 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.

 Whenever possible, reverse the direction of flow during cleaning so that contaminants do not pass through the entire column length. 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 such as precipitated proteins.

Flow: 1 ml/min, HiTrap 1 ml  
5 ml/min, HiTrap 5 ml  
3 ml/min, HiLoad 16/10, 20 ml  
8 ml/min, HiLoad 26/10, 53 ml  
40 cm/h, with a contact time of 1–2 hours, for Sepharose High Performance packed in larger columns

Note that flow rates may need to be reduced due to the condition of the column and the viscosity of the sample, buffers or storage solutions.

1. Wash with up to 4 column volumes of 1 M NaOH.
2. Wash with at least 3 column volumes of water or until eluent pH is neutral.
- 3a. To start a new separation: re-equilibrate with at least 3 column volumes of start buffer or until the correct eluent pH is achieved.
- 3b. For storage: wash with at least 5 column volumes of storage solution. Allow UV baseline to stabilize before storing the column.

To remove lipids, lipoproteins and very hydrophobic proteins, see Appendix 1.

## Media characteristics

Composition: Sepharose High Performance is based on highly cross-linked, 6% agarose formed into spherical particles (mean size 34 µm) and substituted with hydrophobic ligands via uncharged, chemically stable O-ether linkages.

**Table 11.** Characteristics of Phenyl and Butyl Sepharose High Performance.

Product	Matrix	Ligand density per ml medium	pH stability*	Mean particle size
Phenyl Sepharose High Performance	6% cross-linked agarose, spherical particles	25 µmol	Long term: 3–13 Short term: 2–14	34 µm
Butyl Sepharose High Performance <sup>†</sup>	6% cross-linked agarose, spherical particles	not determined	Long term: 3–13 Short term: 2–14	34 µm

\*Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance.

Short-term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.

All ranges are estimates based on the experience and knowledge within GE Healthcare.

<sup>†</sup> Available as a Custom Designed Medium.

## Chemical stability

For daily use, Sepharose High Performance is stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), 70% ethanol, 1 M acetic acid, 30% isopropanol, 30% acetonitrile and up to 2% SDS.

## 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. Ensure that the column is sealed well to avoid drying out. Whenever possible, use a storage and shipping device, if supplied by the manufacturer. Store columns and unused media at 4°C to 30°C in 20% ethanol. Do not freeze.

Large-scale production columns are often stored in 0.01 M sodium hydroxide as an alternative to 20% ethanol.

 To avoid the formation of air bubbles, ensure that columns, buffers and equipment are at the same temperature before use.

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

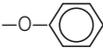
 Use Sepharose Fast Flow for capture or intermediate purification steps that require good resolution (recommended flows up to 300 cm/h). Use for intermediate purification or polishing if the required selectivity is not available in a medium of smaller particle size. Achieve optimum conditions from a choice of different selectivities.

 Run Sepharose Fast Flow columns on systems such as ÄKTAdesign, FPLC System and HPLC or systems using peristaltic pumps. Appendix 3 gives guidance on how to select the most suitable ÄKTAdesign system.

Sepharose Fast Flow media are based on a matrix of particles (mean size 90 µm) made from 4% or 6% agarose that is highly cross-linked for chemical and physical stability. The matrices are substituted with hydrophobic ligands (phenyl, butyl or octyl) coupled via a chemically stable O-ether or S-ether linkage, see Figure 42. Two different degrees of substitution of the phenyl ligand provide a further chance to optimize binding capacity and elution conditions. Particle size and bed volumes remain stable, despite changes in salt concentration, to ensure fast separations at high flow rates with good resolution. Methods can be easily scaled up from small HiTrap columns through to large-scale columns such as BPG or Chromaflow. The performance of Sepharose Fast Flow media is well documented, and there are many examples of the smooth transfer from the laboratory to pilot scale and on to production.

Reference lists highlighting the use of Sepharose Fast Flow media for hydrophobic interaction chromatography are available at [www.gehealthcare.com/protein-purification-labresearch](http://www.gehealthcare.com/protein-purification-labresearch)

### Ligand

Phenyl	
Butyl-S	$-\text{S}-(\text{CH}_2)_3-\text{CH}_3$
Butyl	$-\text{O}-(\text{CH}_2)_3-\text{CH}_3$
Octyl	$-\text{O}-(\text{CH}_2)_7-\text{CH}_3$

**Fig 42.** Ligands are coupled to Sepharose matrices via uncharged, chemically stable O-ether or S-ether linkages.

## Purification options



**Fig 43.** Most HIC media based on Sepharose 6 Fast Flow or Sepharose 4 Fast Flow are available prepacked in HiTrap or HiPrep columns and in media packs.

**Table 12.** HIC products based on Sepharose Fast Flow.

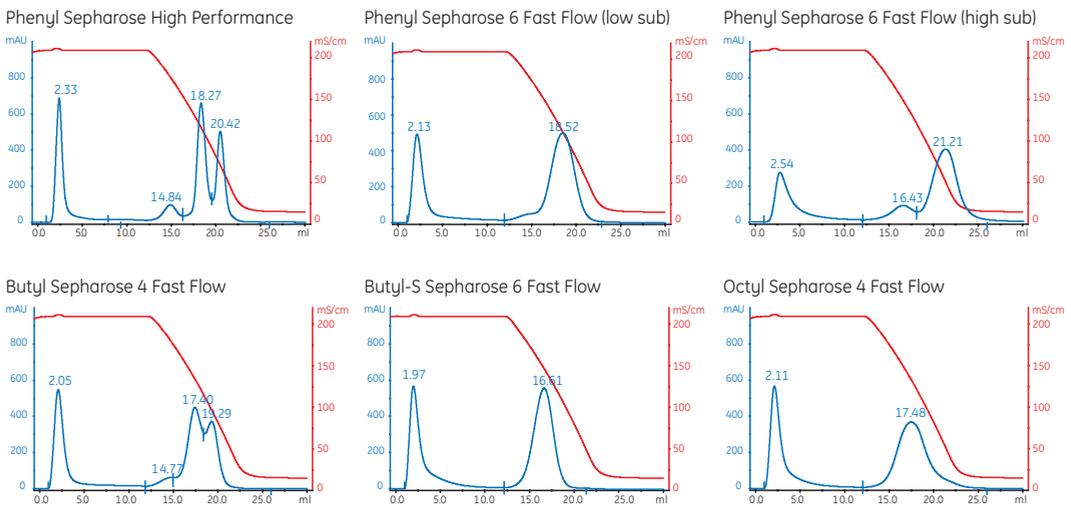
Product	Ligand density per ml medium <sup>‡</sup>	Recommended working flow*	Maximum flow*	Maximum operating back pressure <sup>†</sup> (MPa/psi) 1 MPa=10 bar
HiTrap Phenyl FF (high sub), 1 ml	40 µmol	up to 1 ml/min	4 ml/min	0.3/43
HiTrap Phenyl FF (high sub), 5 ml	40 µmol	up to 5 ml/min	20 ml/min	0.3/43
HiPrep 16/10 Phenyl FF (high sub), 20 ml	40 µmol	2–10 ml/min	10 ml/min	0.15/22
Phenyl Sepharose 6 Fast Flow (high sub)	40 µmol	50–400 cm/h	750 cm/h	0.3/43
HiTrap Phenyl FF (low sub), 1 ml	25 µmol	up to 1 ml/min	4 ml/min	0.3/43
HiTrap Phenyl FF (low sub), 5 ml	25 µmol	up to 5 ml/min	20 ml/min	0.3/43
HiPrep 16/10 Phenyl FF (low sub), 20 ml	25 µmol	2–10 ml/min	10 ml/min	0.15/22
Phenyl Sepharose 6 Fast Flow (low sub)	25 µmol	50–400 cm/h	750 cm/h	0.3/43
HiTrap Butyl FF, 1 ml	40 µmol	up to 1 ml/min	4 ml/min	0.3/43
HiTrap Butyl FF, 5 ml	40 µmol	up to 5 ml/min	20 ml/min	0.3/43
HiPrep 16/10 Butyl FF, 20 ml	40 µmol	2–10 ml/min	10 ml/min	0.15/22
HiTrap Butyl-S FF, 1 ml	10 µmol	up to 1 ml/min	4 ml/min	0.3/43
HiTrap Butyl-S FF, 5 ml	10 µmol	up to 5 ml/min	20 ml/min	0.3/43
Butyl Sepharose 4 Fast Flow	40 µmol	50–300 cm/h	400 cm/h	0.1/14
Butyl-S Sepharose 6 FF	10 µmol	50–400 cm/h	750 cm/h	0.3/43
HiTrap Octyl FF, 1 ml	5 µmol	up to 1 ml/min	4 ml/min	0.3/43
HiTrap Octyl FF, 5 ml	5 µmol	up to 5 ml/min	20 ml/min	0.3/43
HiPrep 16/10 Octyl FF, 20 ml	5 µmol	2–10 ml/min	10 ml/min	0.15/22
Octyl Sepharose 4 Fast Flow	5 µmol	50–300 cm/h	400 cm/h	0.1/14

\* Recommendations are for separations at room temperature in aqueous buffers. See Appendix 3 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa. Note that final working flow will depend also on factors such as column size and bed height, sample characteristics and loading conditions, the equipment used and the back pressure that the equipment can withstand.

<sup>†</sup> Maximum operating back pressure refers to the pressure above which the medium begins to compress.

<sup>‡</sup> Custom Designed Media, also available, see page 50.

- It is important to note that the binding capacity of a HIC medium is highly dependent on the properties of the target protein and contaminants, the selectivity of the medium and the binding conditions. Capacity must be determined empirically during media screening and method development.
- To transfer separations originally performed using Phenyl Sepharose CL-4B or Octyl Sepharose CL-4B (no longer commercially available), try Phenyl Sepharose 6 Fast Flow, Butyl Sepharose 4 Fast Flow or Octyl Sepharose 4 Fast Flow as a starting point.
- Use the HiTrap HIC Selection Kit, comprising six prepacked HiTrap columns containing media with different hydrophobic characteristics, to rapidly screen for the most suitable medium for a specific application (Figure 44). Select the medium that gives the best selectivity, resolution and loading capacity at the lowest salt concentration.



**Sample:** cytochrome C, ribonuclease A, lysozyme,  $\alpha$ -chymotrypsinogen  
6 mg protein/ml, (1:3:1:1) in start buffer

**Sample**

**volume:** 1 ml

**Sample load:** 6 mg protein/ml medium

**Flow:** 1.0 ml/min, (150 cm/h)

**Start buffer:** 1.7 M  $(\text{NH}_4)_2\text{SO}_4$ , 0.1 M  $\text{Na}_2\text{HPO}_4$ , pH 7.0

**Elution buffer:** 0.1 M  $\text{Na}_2\text{HPO}_4$ , pH 7.0

**Gradient:** 0%–100% elution buffer in 10 CV

**Fig 44.** Comparison of the different selectivity characteristics in a HiTrap HIC Selection Kit.

- Use prepacked HiTrap columns (1 ml or 5 ml) for method scouting, group separations, small-scale purification, sample concentration or clean-up. When increased capacity is required, use prepacked columns HiLoad 16/10 Phenyl Sepharose HP (20 ml) or HiLoad 26/10 Phenyl Sepharose HP (53 ml).

Use Butyl-S Sepharose 6 Fast Flow, the least hydrophobic medium in the Fast Flow series, for binding and elution of relatively strong hydrophobic proteins at comparatively low salt concentrations.

Phenyl Sepharose High Performance and Phenyl Sepharose 6 Fast Flow (low sub) usually exhibit similar hydrophobic properties. For capture or intermediate purification, Phenyl Sepharose 6 Fast Flow (low sub) may give similar resolution at higher flow rates.

**Table 13.** Packing volumes and bed heights for Sepharose Fast Flow media for HIC.

Empty glass column i.d./length (mm)	Volume	Bed height
Tricorn 5/100	up to 2 ml	up to 10 cm
Tricorn 5/150	up to 3 ml	up to 15 cm
Tricorn 5/200	up to 4 ml	up to 20 cm
Tricorn 10/100	up to 8 ml	up to 10 cm
Tricorn 10/150	up to 12 ml	up to 15 cm
Tricorn 10/200	up to 16 ml	up to 20 cm
XK 16/20	up to 30 ml	up to 15 cm
XK 26/20	up to 80 ml	up to 15 cm
XK 26/40	up to 196 ml	>15 cm
XK 50/20	up to 274 ml	up to 14 cm
XK 50/30	up to 559 ml	up to 28.5 cm

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

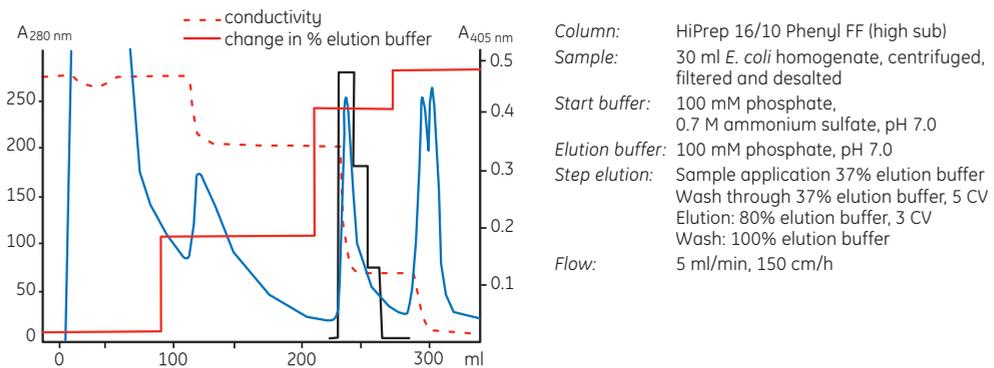
## Purification examples

### Media screening

See Figure 20, page 29 for an example of media screening.

### Capture: enzyme purification

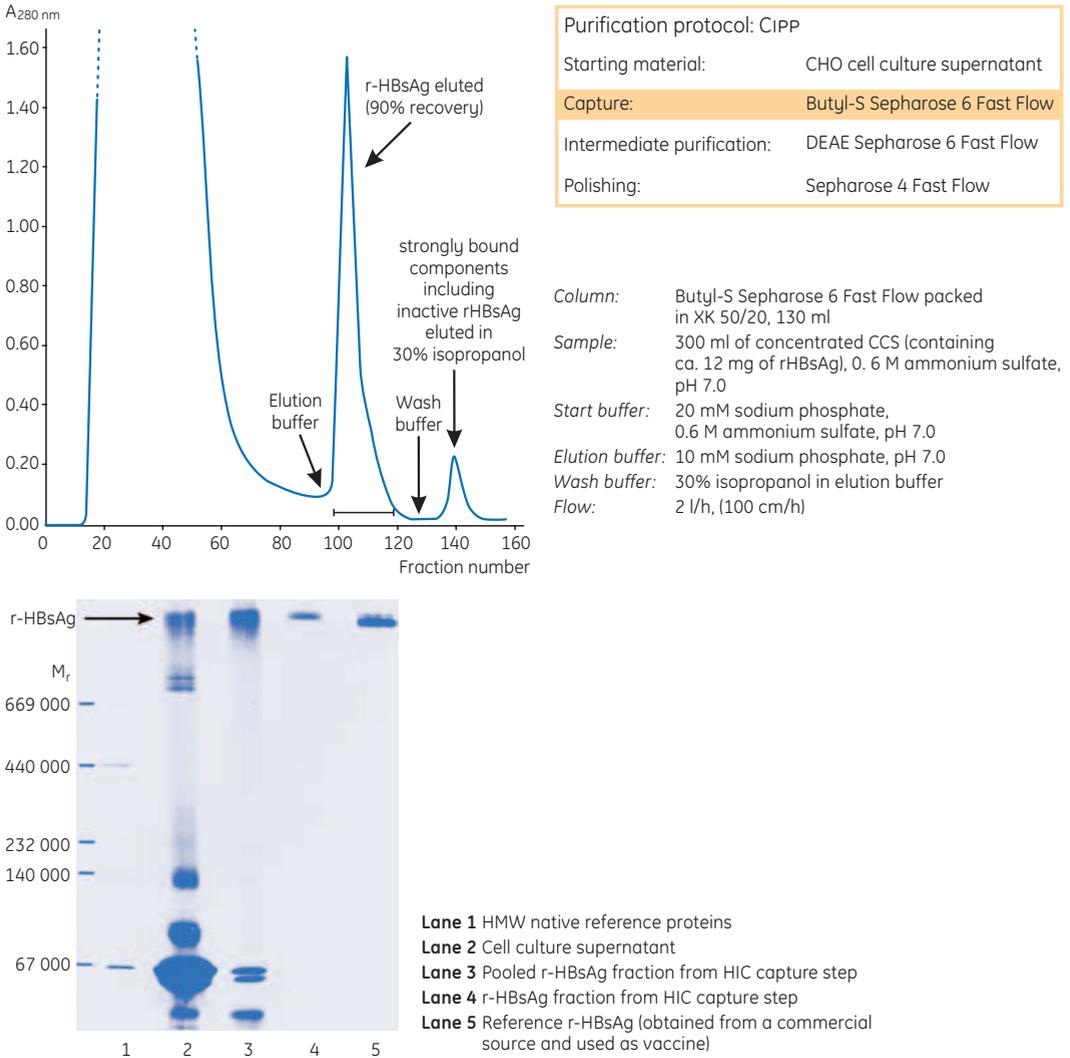
Figure 45 shows a capture step for partial purification of an enzyme, alkaline phosphatase, from *E. coli* homogenate. Conditions have been selected to ensure that the target protein binds to the column while most of the contaminants wash directly through. The enzyme is concentrated and a very effective purification step is achieved.



**Fig 45.** Purification of alkaline phosphatase on Phenyl Sepharose Fast Flow (high sub). The black line represents enzyme activity.

## Capture: recombinant Hepatitis B virus surface antigen (r-HBsAg) from CHO cells

Hepatitis B virus (HBV) is an infectious agent that causes acute and chronic hepatitis, cirrhosis, and primary hepatocellular carcinoma. It is estimated that approximately 5% of the world's population is infected by the virus. Effective vaccines for the non-infected population can be produced at large-scale using recombinant technology to generate the recombinant Hepatitis B surface Antigen (r-HBsAg). Figure 46 shows the large-scale purification of r-HBsAg from a CHO cell culture supernatant. Since this protein is extremely hydrophobic, it binds strongly to most HIC media. However, the mild hydrophobic properties of Butyl-S Sepharose Fast Flow enabled a successful purification in which over 90% of impurities were removed during the capture step.

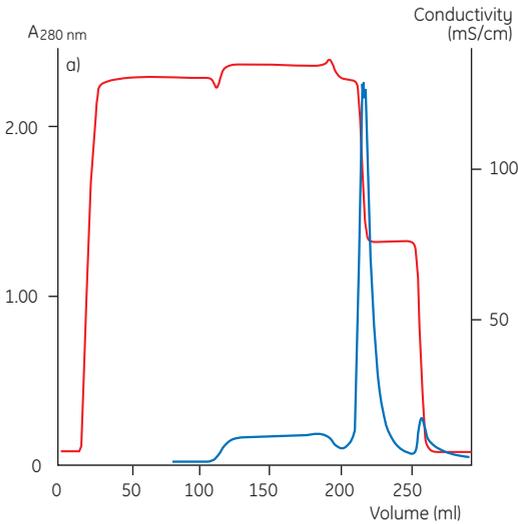


**Fig 46.** Large-scale purification of rHBsAg. Over 90% of impurities are removed by the HIC capture step (lane 3). Electrophoretic analysis (Gradient PAGE 4–30% under non-denaturing conditions) shows how purity increases after each chromatography step. r-HBsAg is a particle of 22 nm hence the band is seen at the top of the gel.

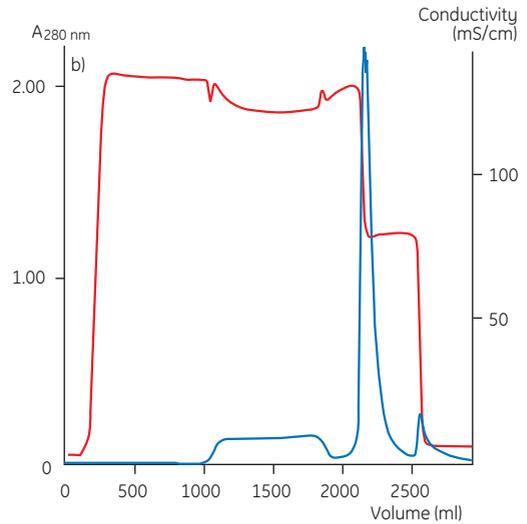
## Intermediate purification: Fab fragment

Figure 47 shows an optimized elution scheme for intermediate purification of a Fab fragment, developed from the media screening in Figure 20. Conditions were optimized on a 20 ml column until a step elution could be used to maximize throughput and make full use of the concentrating effect of HIC. The step could then be scaled up directly onto a 200 ml column (also shown in the same Figure).

Purification protocol: CIPP	
Starting material:	<i>E. coli</i> lysate
Capture:	STREAMLINE SP
Intermediate purification:	Phenyl Sepharose 6 Fast Flow (high sub)
Polishing:	SOURCE 15S



**Sample:** Fab fraction from STREAMLINE SP, 80 ml  
**Columns:** Phenyl Sepharose 6 Fast Flow (high sub) in XK 16/20 (10 cm bed height), 20 ml  
**Start buffer:** 1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 50 mM NaAc, pH 5.0  
**Elution buffer:** 50 mM NaAc, pH 5.0  
**Gradient:** Step gradient to 50% elution buffer  
**Flow:** 5 ml/min

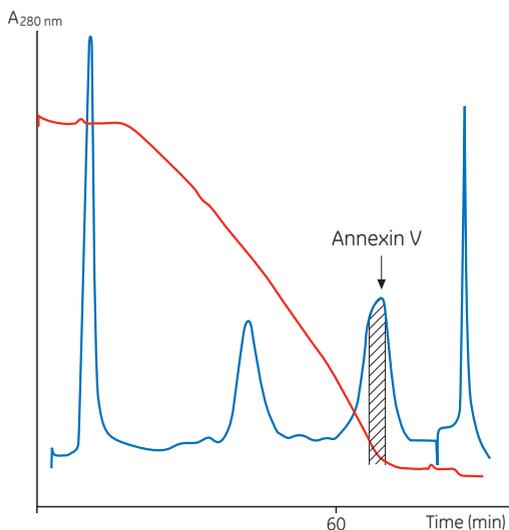


**Sample:** Fab fraction from STREAMLINE SP, 800 ml  
**Columns:** Phenyl Sepharose 6 Fast Flow (high sub) in XK 50/20, 200 ml  
**Start buffer:** 1 M (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 50 mM NaAc, pH 5.0  
**Elution buffer:** 50 mM NaAc, pH 5.0  
**Gradient:** Step gradient to 50% elution buffer  
**Flow:** Equilibration: 100 ml/min  
 Loading and elution: 50 ml/min

**Fig 47.** Intermediate purification and scale-up of a Fab fragment on Phenyl Sepharose 6 Fast Flow (high sub).

## Intermediate purification: recombinant protein Annexin V

Figure 48 shows Butyl Sepharose 4 Fast Flow used as an intermediate step during purification of a recombinant protein, annexin V, expressed in *E. coli*.



**Sample:** Partially purified Annexin V expressed in *E. coli*, 5 ml  
**Column:** Butyl Sepharose 4 Fast Flow in XK 16/20 column  
**Start buffer:** 20 mM sodium phosphate, 1 M ammonium sulfate, pH 7.0  
**Elution buffer:** 20 mM sodium phosphate, pH 7.0  
**Gradient:** 0 to 50% elution buffer, 20 CV  
**Flow:** 100 cm/h

Fig 48. Intermediate purification of a recombinant protein.

## Performing a separation

Guidelines for the selection of media, buffer, salt and pH 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 and samples after all salts and additives have been included. Use high-quality water and chemicals. Filter solutions using filters of 1 µm or less. To avoid formation of air bubbles in a packed column and to ensure reproducible results, the column and buffers should be at the same temperature when preparing for a run.
- To avoid problems with precipitation, check the salt stability window of the sample components. Avoid working at concentrations near to the stability limit of the target protein. For samples with unknown hydrophobic properties, try the following:

start buffer: 1.5 M ammonium sulfate, 50 mM phosphate buffer, pH 7.0

elution buffer: 50 mM phosphate buffer, pH 7.0

- Note that flow rates may need to be reduced due to the viscosity of the chosen start buffer, sample characteristics, loading requirements and the equipment used.

## ***First-time use or after long-term storage***

1. To remove ethanol, wash with 5 column volumes of distilled water or elution buffer.

Flow: 1 ml/min, HiTrap 1 ml  
5 ml/min, HiTrap 5 ml  
2 ml/min, HiPrep 16/10, 20 ml  
50 cm/h for Sepharose Fast Flow packed in larger columns

2. Wash with 5 column volumes of start buffer.

Flow: 1 ml/min, HiTrap 1 ml  
5 ml/min, HiTrap 5 ml  
5 ml/min, HiPrep 16/10, 20 ml  
up to 150 cm/h for Sepharose Fast Flow packed in larger columns

3. Perform a blank elution to check conductivity profile.

## ***Separation by gradient elution***

Flow: 1 ml/min, HiTrap 1 ml  
5 ml/min, HiTrap 5 ml and HiPrep 16/10, 20 ml  
up to 150 cm/h for Sepharose Fast Flow packed in larger columns

Collect fractions throughout the separation.

1. Equilibrate column with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable.
2. Adjust the sample to the chosen salt concentration (and pH if necessary). Filter and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable so that all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10–20 column volumes, increasing the proportion of elution buffer until the salt concentration reaches a minimum, that is, salt-free buffer.
5. Wash with 2–5 column volumes of salt-free elution buffer to elute remaining hydrophobically bound material.
6. Re-equilibrate with 5–10 column volumes of start buffer or until conductivity reaches the required value.

## ***Separation by step elution***

Flow: 1 ml/min, HiTrap 1 ml  
5 ml/min, HiTrap 5 ml and HiPrep 16/10, 20 ml  
up to 150 cm/h for Sepharose Fast Flow packed in larger columns

Collect fractions throughout the separation.

1. Equilibrate the 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 salt concentration (and pH if necessary). Filter and apply to the column.
3. Wash with 5–10 column volumes of start buffer or until the UV baseline and conductivity are stable so that all unbound material has washed through the column.
4. Elute with 5 column volumes of elution buffer + salt at chosen concentration.
5. Repeat step 4 at lower salt concentrations until the target protein(s) has been eluted.

6. Wash with 2–5 column volumes of salt-free elution buffer to elute remaining hydrophobically bound material.
7. Re-equilibrate with 5–10 column volumes of start buffer or until conductivity reaches the required value.

-  Save time by using higher flow rates during the salt-free wash and re-equilibration steps, but do not exceed the maximum recommended flow for the medium.
-  Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 2.
-  Never leave columns or equipment in high-salt solutions.

## Cleaning

Correct preparation of samples and buffers and application of a salt-free buffer 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.

-  Whenever possible, reverse the direction of flow during cleaning so that contaminants do not pass through the entire column length. 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 such as precipitated proteins.

Flow: 1 ml/min, HiTrap 1 ml  
5 ml/min, HiTrap 5 ml  
5 ml/min, HiPrep 16/10, 20 ml  
40 cm/h, with a contact time of 1–2 hours, for Sepharose Fast Flow packed in larger columns

Note that flow rates may need to be reduced due to the condition of the column and the viscosity of the sample, buffers or storage solutions.

1. Wash with up to 4 column volumes of 1 M NaOH.
2. Wash with at least 3 column volumes of water or until eluent pH is neutral.
- 3a. To start a new separation: re-equilibrate with at least 3 column volumes of start buffer or until the correct eluent pH is achieved.
- 3b. For storage: wash with at least 5 column volumes of storage solution. Allow UV baseline to stabilize before storing the column.

To remove lipids, lipoproteins and very hydrophobic proteins, see Appendix 1.

## Media characteristics

Composition: Sepharose 6 Fast Flow media are based on cross-linked, 6% agarose forming spherical particles (mean size 90 nm) coupled to phenyl or butyl ligands via uncharged, chemically stable, O-ether or S-ether linkages. Two levels of phenyl ligand substitution are available.

Sepharose 4 Fast Flow media are based on cross-linked 4% agarose forming spherical particles (mean size 90 nm) and coupled with butyl or octyl ligands via uncharged, chemically stable, O-ether linkages.

**Table 14.** Characteristics of Sepharose Fast Flow media for HIC.

Product	Matrix	Ligand density per ml medium	pH stability*	Mean particle size
Phenyl Sepharose 6 Fast Flow (high sub)	6% cross-linked agarose, spherical particles	40 µmol	Long term: 3–13 Short term: 2–14	90 µm
Phenyl Sepharose 6 Fast Flow (low sub)	6% cross-linked agarose, spherical particles	20 µmol	Long term: 3–13 Short term: 2–14	90 µm
Butyl-S Sepharose 6 Fast Flow	6% cross-linked agarose, spherical particles	10 µmol	Long term: 3–13 Short term: 2–14	90 µm
Butyl Sepharose 4 Fast Flow	4% cross-linked agarose, spherical particles	50 µmol	Long term: 3–13 Short term: 2–14	90 µm
Octyl Sepharose 4 Fast Flow	4% cross-linked agarose, spherical particles	5 µmol	Long term: 3–13 Short term: 2–14	90 µm

\*Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance.

Short-term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.

All ranges are estimates based on the experience and knowledge within GE Healthcare.

## Chemical stability

For daily use, HIC media based on Sepharose 6 Fast Flow or Sepharose 4 Fast Flow are stable in all common aqueous buffers, 1 M NaOH, 70% ethanol, 30% isopropanol, denaturing agents (6 M guanidine hydrochloride), 1 M acetic acid, 30% isopropanol, 30% acetonitrile and up to 2% SDS.

## 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. Ensure that the column is sealed well to avoid drying out. Whenever possible use a storage and shipping device, if supplied by the manufacturer. Store columns and unused media at 4°C to 30°C in 20% ethanol. Do not freeze.

Large-scale production columns are often stored in 0.01 M sodium hydroxide as an alternative to 20% ethanol.



To avoid the formation of air bubbles, ensure that columns, buffers and equipment are at the same temperature before use.

# Chapter 4

## HIC in a purification strategy (CIPP)

To ensure efficient, reproducible purification giving the required degree of purity, it is beneficial to develop a multi-step process using the purification strategy of Capture, Intermediate Purification and Polishing (CIPP), shown in Figure 49.

CIPP is used in both the pharmaceutical industry and in the research laboratory to ensure faster method development, a shorter time to pure product and good economy. This chapter gives a brief overview of this approach, which can be recommended for any multi-step protein purification. The *Protein Purification Handbook* from GE Healthcare is a guide for planning efficient and effective protein purification strategies. An important first step for any purification is correct sample preparation; this is covered in more detail in Appendix 1 and Chapter 2.

Hydrophobic interaction chromatography (HIC) offers selectivity based on the hydrophobic properties of the target protein and can be a useful complement to other chromatography techniques that separate according to specificity, charge or size. HIC can be used for capture, intermediate purification or polishing steps, according to the demands for the specific application. The need for samples to be in an elevated salt concentration to promote hydrophobic interaction makes HIC well-suited for capture steps after samples have been subjected to clean-up by ammonium sulfate precipitation or for intermediate steps directly after an ion exchange separation. Under both circumstances the partially purified sample is already in a high-salt solution and, other than the addition of more salt, no further preparation is required, saving time. Since samples can be concentrated and eluted in a reduced volume when using HIC, fractions can also be transferred directly to gel filtration. HIC can be used with step-wise elution for a rapid capture step or with gradient elution to achieve the highest resolution in a polishing step.

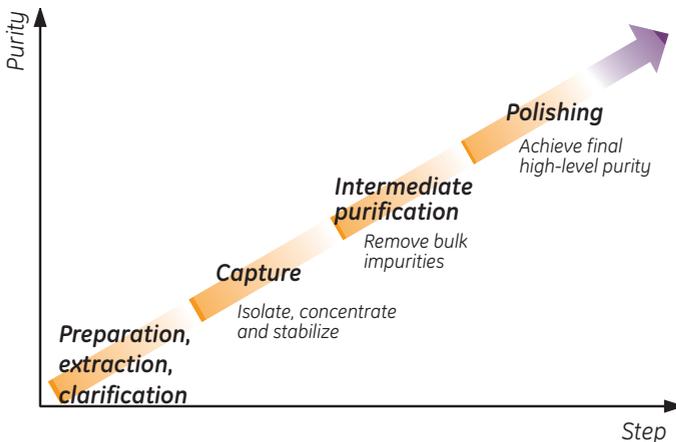


Fig 49. Strategy for protein purification.

## Applying CIPP

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



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

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

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

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

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



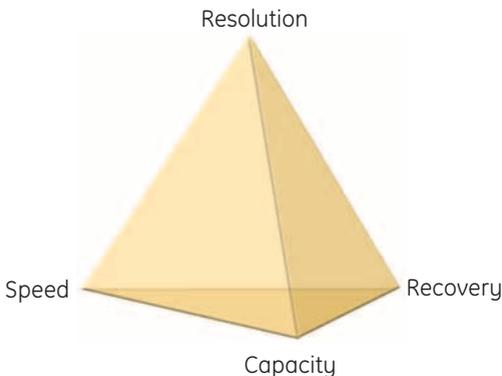
The optimal selection and combination of purification techniques for *Capture, Intermediate Purification and Polishing* is crucial for an efficient purification.

## Selection and combination of purification techniques

Proteins are purified using purification techniques that separate according to differences in specific properties, as shown in Table 15.

**Table 15.** Protein properties used during purification.

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



**Fig 50.** Every technique offers a balance between resolution, capacity, speed and recovery.

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

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

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

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

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

A guide to the suitability of each purification technique for the stages in CIPP is shown in Table 16.

**Table 16.** Suitability of purification techniques for CIPP.

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

-  Minimize sample handling between purification steps by combining techniques to avoid the need for sample conditioning. The product should be eluted from the first column in conditions suitable for the start conditions of the next column (see Table 16).
-  Ammonium sulfate, often used for sample clarification and concentration (see Appendix 1), leaves the sample in a high-salt environment. Consequently HIC, which requires high salt to enhance binding to the media, becomes the ideal choice as the capture step. The salt concentration and the total sample volume will be significantly reduced after elution from the HIC column. Dilution of the fractionated sample or rapid buffer exchange using a desalting column will prepare it for the next IEX or AC step.
-  Gel filtration is a non-binding technique unaffected by buffer conditions, but with limited volume capacity. GF is well suited for use after any of the concentrating techniques (IEX, HIC, AC) since the target protein will be eluted in a reduced volume and the components from the buffer will not affect the gel filtration process.

Selection of the final strategy will always depend upon specific sample properties and the required level of purification. Logical combinations of techniques are shown in Figure 51.

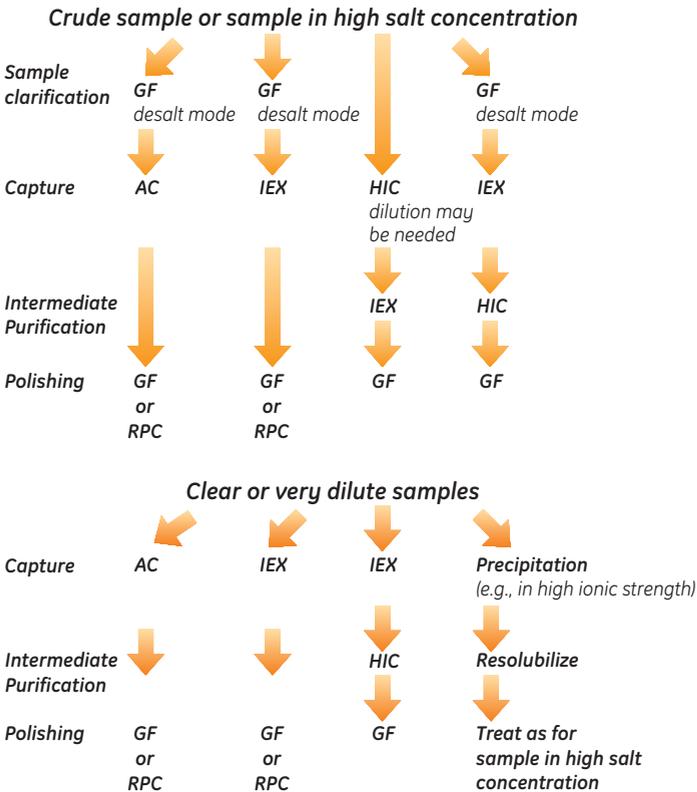


Fig 51. Logical combinations of chromatography techniques.

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

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

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

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

# HIC as a capture step

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

Focus is on capacity and speed in a capture step. It may be advisable to compromise on resolution in order to maximize the capacity and/or speed of the separation in this first step.

HIC media for *capture* steps should offer high speed and high capacity.

1. Sepharose Fast Flow (90 µm mean particle size) — good resolution at flows up to 300 cm/h.
2. Use Sepharose High Performance (34 µm particle size) HIC media for capture or scale-up when selectivity is satisfactory, high resolution is a priority and if lower flow rates (to compensate for a higher back pressure) are acceptable.
3. Use SOURCE (15 µm mean particle size) if the required selectivity is not available in a medium of larger particle size.

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

## Purification of recombinant human epidermal growth factor (h-EGF)

This purification represents a classical three-step purification process, using HIC-IEX-GF, for the purification of human epidermal growth factor (h-EGF) expressed as an extracellular product by *Saccharomyces cerevisiae*.

Purification protocol: CIPP	
Starting material:	Cell culture supernatant
Capture:	Phenyl Sepharose 6 Fast Flow (high sub)
Intermediate purification:	Q Sepharose High Performance
Polishing:	Superdex 75 prep grade

Fig 52. Classical HIC-IEX-GF purification strategy.

The strategy was developed as follows: initial media screening experiments for the capture step were performed on four different HIC media (Figure 53). Phenyl Sepharose 6 Fast Flow (high sub) was selected as giving the best selectivity for EGF, a high binding capacity with low back pressure.

### Media characteristics for EGF purification

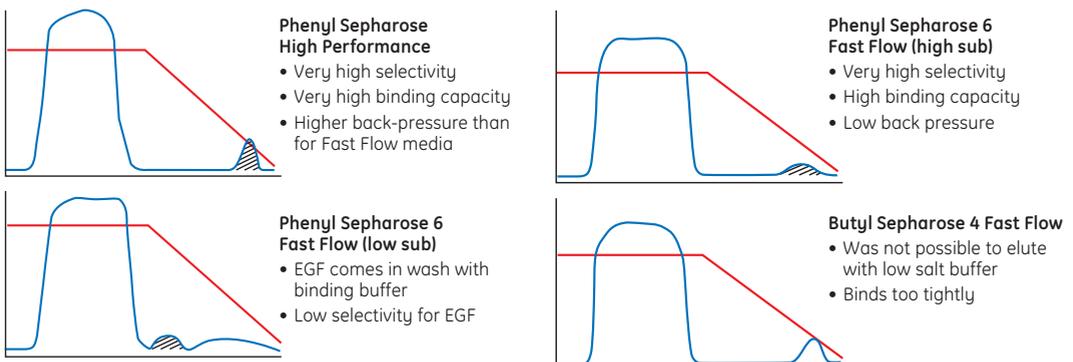
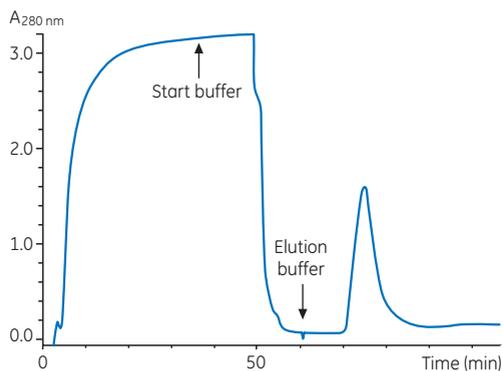
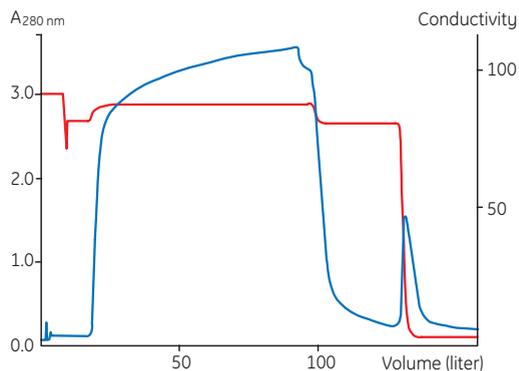


Fig 53. Media screening.



**Column:** Phenyl Sepharose 6 Fast Flow (high sub) in XK/16/20 column, 10 cm bed height, 20 CV  
**Sample:** Yeast supernatant, ammonium sulfate added to 0.5 M  
**Sample volume:** 450 ml  
**Sample load:** 0.41 mg h-EGF/ml medium  
**Flow:** 300 cm/h, 10 ml/min (loading)  
 60 cm/h, 2 ml/min (elution)  
**Start buffer:** 20 mM sodium phosphate, pH 7.0  
 0.5 M ammonium sulfate  
**Elution buffer:** 20 mM sodium phosphate, pH 7.0  
**Purification time:** 90 min



**Column:** Phenyl Sepharose 6 Fast Flow (high sub) in BPG 300/500, 10 cm bed height, 3.5 L CV  
**Sample:** Yeast supernatant, ammonium sulfate added to 0.5 M  
**Sample volume:** 80 l  
**Sample load:** 0.36 mg h-EGF/ml medium  
**Flow:** 300 cm/h, 212 l/h (loading)  
 60 cm/h, 42 l/h (elution)  
**Start buffer:** 20 mM sodium phosphate, pH 7.0  
 0.5 M ammonium sulfate  
**Elution buffer:** 20 mM sodium phosphate, pH 7.0  
**Purification time:** 90 min

**Fig 54.** Development and scale-up of the capture step.

As Figure 54 shows, development work was performed at a small scale using a step elution on XK columns packed with Phenyl Sepharose 6 Fast Flow (high sub) and then scaled up to production scale using a BPG 300/500 column.

A high-resolution, anion exchanger, Q Sepharose High Performance, was chosen for intermediate purification in order to reach a high degree of purity in the second step (> 96%).

Gel filtration on Superdex™ 75 prep grade was selected for final polishing in order to achieve a high final purity by separating polymers and unwanted buffer salts from the EGF product.

The start material was clarified cell culture supernatant supplied by Chiron-Cetus Corp., Emeryville, USA. Concentration of EGF in the start material was 0.018 mg/ml and the overall protein content was 63 mg/ml. This three-step procedure gave a product purity of 99% as determined by RPC, and an overall yield of 73%.

## HIC for intermediate purification

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



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

Media for intermediate purification should offer high capacity and high resolution. Select as follows:

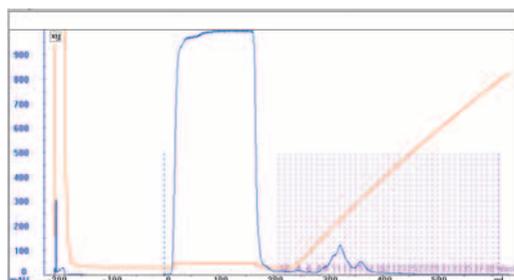
1. Sepharose High Performance (34 µm mean particle size) — for high resolution at flows up to 150 cm/h.
2. Sepharose Fast Flow (90 µm mean particle size) — for good resolution at flows up to 300 cm/h or when required selectivity is not available on a Sepharose High Performance matrix.

3. SOURCE 15 (15  $\mu\text{m}$  mean particle size) — if the required selectivity is not available in a medium of larger particle size. Samples should be free from particulate matter.

## Purification of Fab fragment

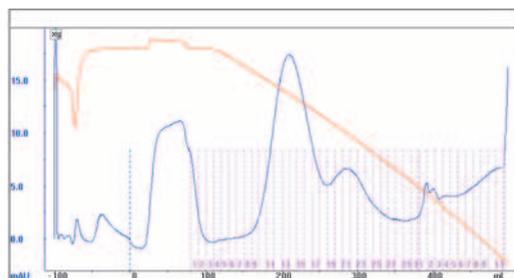
Figure 55 shows a classic protocol, IEX-HIC-GF, for purification of a Fab fragment against the gp 120 envelope of the HIV-1 virus. The fragment, which was expressed in the periplasmic space of *E. coli*, has a molecular mass of  $M_r$  50 000 and isoelectric point (pI)  $\sim$ 11. The high pI made cation exchange the most suitable ion exchange step for the initial capture step. The high capacity and good resolution of Phenyl Sepharose 6 Fast Flow (high sub) was well suited to intermediate purification, followed by a polishing step on gel filtration.

Purification protocol: CIPP	
Starting material:	<i>E. coli</i> periplasmic expressed target protein
Capture:	SP Sepharose Fast Flow
Intermediate purification:	Phenyl Sepharose 6 Fast Flow (high sub)
Polishing:	Superdex 75 prep grade



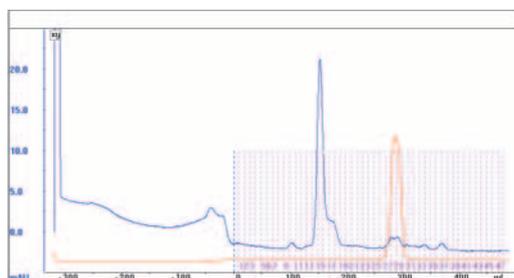
Active fractions pooled: 10–14

**Column:** SP Sepharose Fast Flow packed in XK 16/10, 20 ml  
**Sample volume:** 150 ml  
**Buffer:** BufferPrep CIEX, pH range 3–7.5, running pH 4.5  
**Gradient:** 0–1.0 M NaCl in 20 CV  
**Flow:** 150 cm/h, (5 ml/min)  
**System:** ÄKTAexplorer



Active fractions pooled: 11–16

**Column:** Phenyl Sepharose 6 Fast Flow (high sub) packed in XK 16/10, 20 ml  
**Sample volume:** 50 ml, pooled from SP Sepharose Fast Flow  
**Start buffer:** 1.0 M ammonium sulfate, 30 mM phosphate, pH 6.0  
**Elution buffer:** 30 mM phosphate, pH 6.0  
**Flow:** 150 cm/h, (5 ml/min)  
**Gradient:** 0–100% elution buffer in 20 CV  
**System:** ÄKTAexplorer



**Column:** HiLoad 26/60 Superdex 75 prep grade, 320 ml  
**Buffer:** 150 mM NaCl, 20 mM phosphate, pH 7.0  
**Sample volume:** 8 ml, pooled from Phenyl Sepharose 6 Fast Flow  
**Flow:** 50 cm/h, (4.4 ml/min).

Fig 55. Purification of a Fab fragment.

# HIC as a polishing step

At the polishing stage of a purification protocol most impurities have been removed except for trace amounts or closely related substances such as structural variants of the target protein, nucleic acids, viruses or endotoxins. The purpose of the separation is to reduce these variants and any other trace contaminants to acceptable levels for the application. In contrast to capture steps where a fast, high capacity, step elution is most commonly used, a polishing step will therefore focus on achieving the highest possible resolution.

Media for polishing steps should offer the highest possible resolution. Select as follows:

1. SOURCE 15 (15  $\mu\text{m}$  mean particle size) — polishing in laboratory or large-scale applications that require high resolution and high throughput (flows up to 1800 cm/h).
2. Sepharose High Performance (34  $\mu\text{m}$  mean particle size) if SOURCE media do not offer the required selectivity.
3. Sepharose Fast Flow (90  $\mu\text{m}$  mean particle size) if the required selectivity is not available in a medium of smaller particle size.

- Optimize the gradient elution to maximize selectivity. Use high-efficiency media with small bead sizes to improve resolution.
- Note that if HIC is used as a polishing step, it may be necessary to remove excess salt using a desalting/buffer exchange step.

## Purification of a recombinant *Pseudomonas aeruginosa* exotoxin A, PE553D

Figure 56 shows a four-step purification process, using expanded bed adsorption (EBA) followed by HIC-IEX-HIC, for the purification of a genetically modified recombinant *P. aeruginosa* exotoxin A ( $M_r$  66 000) expressed in the periplasm of *E. coli*. The strategy used here resulted in a highly purified exotoxin A that took less than half the time of a conventional approach.

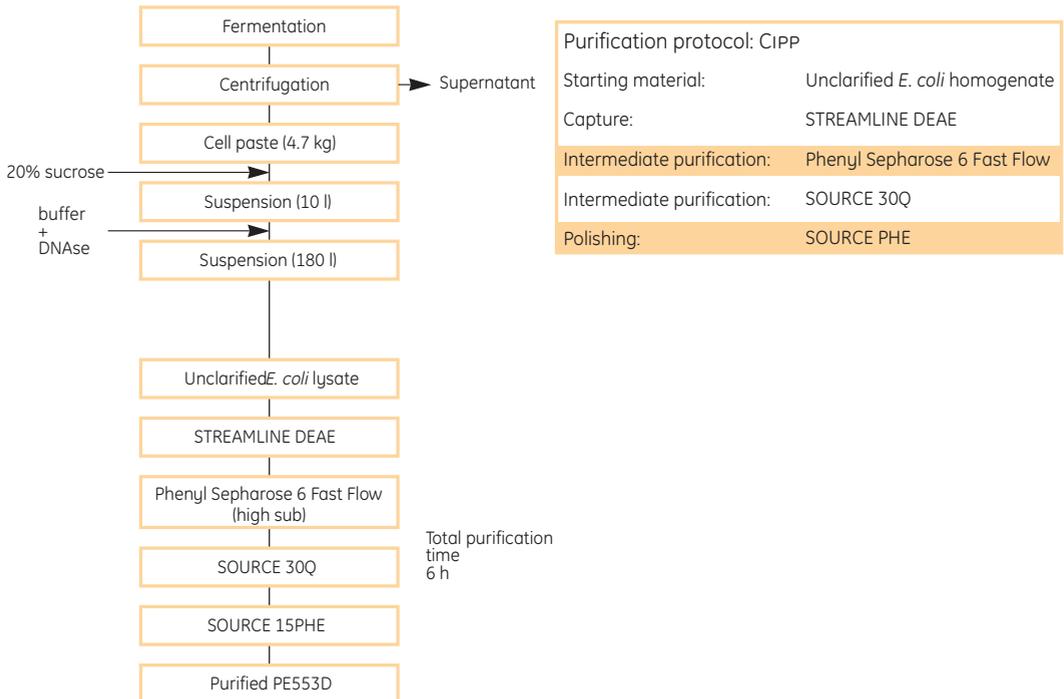
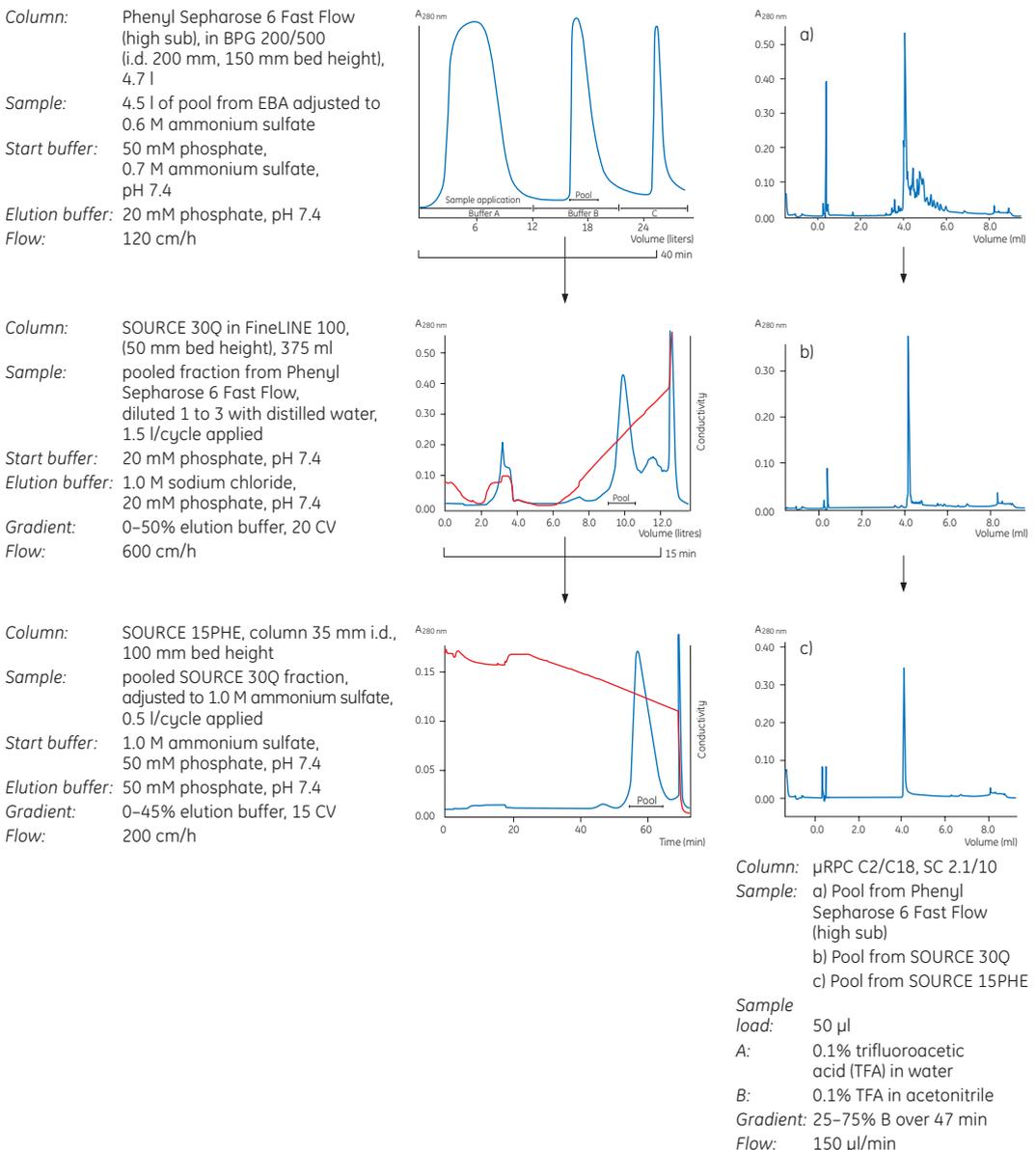


Fig 56. Four-step purification process.

Exotoxin A was captured directly from unclarified *E. coli* homogenate by expanded bed adsorption using STREAMLINE DEAE adsorbent in STREAMLINE 200 column. The collected fraction was transferred directly to an intermediate purification step using step elution on Phenyl Sepharose 6 Fast Flow (high sub) to remove a substantial part of the UV absorbing material that could interfere with the following steps (Figure 57a). Note that a Sepharose 6 Fast Flow matrix was used, rather than Sepharose High Performance, as this was a large-scale purification and a rapid step elution rather than a higher resolution gradient elution was required.

A second intermediate purification used an anion exchanger, SOURCE 30Q, to remove the majority of the remaining contaminants, based on differences in their net surface charge (Figure 57b).

HIC was used again for the polishing step, this time using a gradient elution to take full advantage of the high resolution offered by the smaller particle size of SOURCE 15PHE (Figure 57c). The process resulted in a pure protein, according to PAGE and RPC analysis, and the overall recovery was 51%.

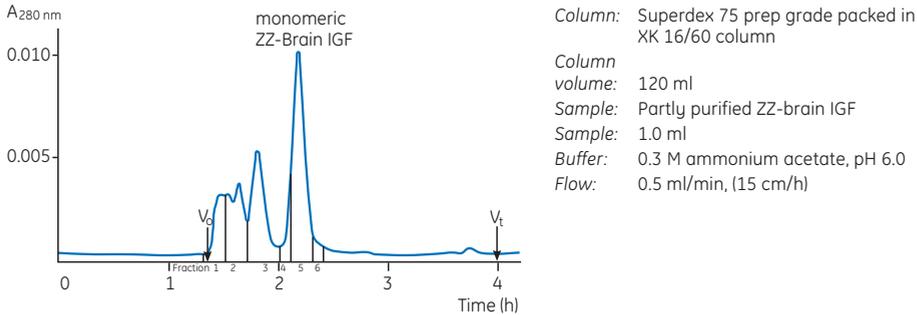


**Fig 57.** Purification of Exotoxin A.

## Alternative techniques for polishing steps

Most commonly, separations by charge, hydrophobicity or affinity will have been used in earlier stages of a purification strategy so that high-resolution gel filtration is ideal for the final polishing step. The product can be purified and transferred into the required buffer in one step, and dimers and aggregates can be removed, as shown in Figure 58.

Gel filtration is also the slowest of the chromatography techniques and the size of the column determines the volume of sample that can be applied. It is therefore most logical to use gel filtration after techniques that reduce sample volume so that smaller columns can be used. Media for polishing steps should offer the highest possible resolution. Superdex is the first choice of gel filtration at laboratory scale and Superdex prep grade for large-scale applications.



**Fig 58.** Final polishing step: separation of monomers, dimers and multimers on Superdex 75 prep grade.

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



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

# Chapter 5

## Reversed phase chromatography: principles and methods

### Introduction

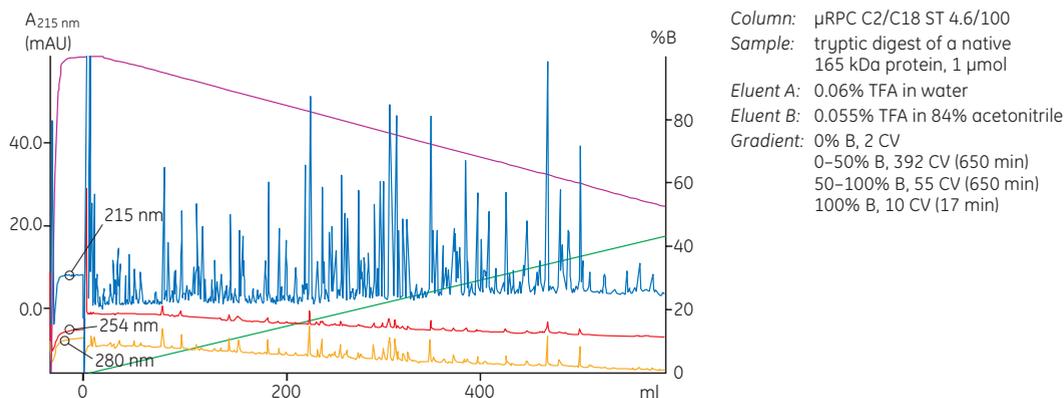
This chapter reviews the principles and methods of reversed phase chromatography (RPC) for purification and analysis of proteins, peptides and oligonucleotides.

RPC has become increasingly important for high-resolution separation and analysis of proteins, peptides and nucleic acids. The technique is ideal for applications such as peptide mapping or purity checking and is often used for final polishing of oligonucleotides and peptides.

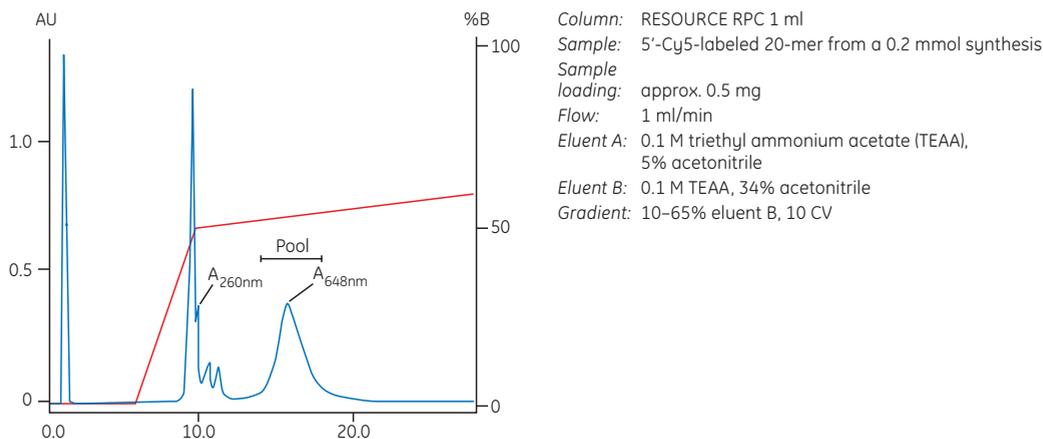
RPC separates molecules according to differences in their hydrophobicity. In theory, HIC and RPC are closely related techniques since both are based upon interactions between hydrophobic patches on the surface of biomolecules and the hydrophobic surfaces of a chromatography medium. However, in practice, the techniques are very different. The surface of an RPC medium is usually more hydrophobic than that of a HIC medium. This leads to stronger interactions that, for successful elution, must be reversed using non-polar, organic solvents such as acetonitrile or methanol. HIC media offer an alternative way of exploiting the hydrophobic properties of biomolecules by working in a more polar and less denaturing environment.

 Proteins are detected at 280 nm or 254 nm, oligonucleotides at 260 nm and peptides at 215 nm.

RPC offers great flexibility in separation conditions. *Extremely high-resolution* separations can be achieved, resolving components that have only minor differences in hydrophobicity. Separations can be performed using isocratic elution; however, most frequently, *gradient elution* is used in order to minimize the run time (Figure 59). RPC can also be used to separate molecules of significantly different hydrophobicity using a *step elution* as illustrated in Figure 60.



**Fig 59.** High-resolution separation of peptides from a 165 kDa protein digested with trypsin. The digest should contain about 150 protein fragments and more than 100 peaks were resolved, most of them base line separated. The separation is monitored at 215 nm to identify all peptides, and at 254 and 280 nm to identify fragments containing aromatic amino acid residues.



**Fig 60.** Oligonucleotides are often labeled, for example, with fluorescein, Dye667 and Dye550. RPC can be used to separate the more hydrophobic, labeled oligonucleotides from the free oligonucleotides. For purity checking, an RPC medium with different selectivity would be used under high-resolution separation conditions.

RPC is also used for *desalting and concentrating* the hydrophobic components of a sample. Ionic salts pass through the RPC column while hydrophobic components bind, effectively concentrating them on the column until removal by a simple *step elution*. RPC is therefore used for sample preparation as well as separations requiring high selectivity. The ability to desalt and use organic solvents makes RPC an ideal technique to couple with mass spectrometry.

RPC is often successfully combined with other chromatography techniques to achieve identification and characterization of previously unknown proteins and peptides. *Multidimensional liquid chromatography* (MDLC) followed by mass spectrometry is a fast and accurate solution for protein identification and characterization in proteomics, and RPC is one of the key techniques used in the process.

In the presence of non-polar solvents, complex enzymes and multi-component proteins are more likely to lose activity than peptides, oligonucleotides or highly stabilized, cross-linked proteins. The interaction of proteins or polypeptides with a hydrophobic surface in the presence of organic solvents generally leads to some loss of tertiary structure, often giving rise to different conformational states that may interact differently with an RPC medium. However, denaturation and consequent loss of activity can be minimized by returning the biomolecule to conditions that favor the native structure, as demonstrated by the widespread use of RPC for large-scale purification of recombinant and synthetic proteins and peptides, such as insulin and growth hormone. Unless precipitation occurs, denaturation is not a problem when using RPC to purify a protein or peptide for primary structure determination.

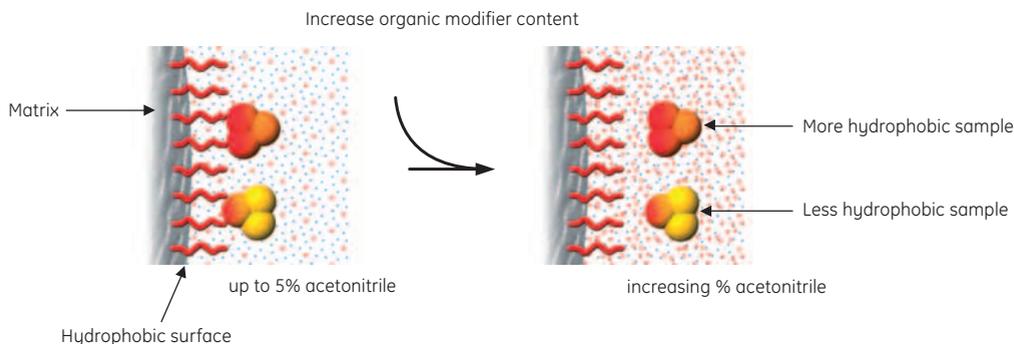
## Terminology

Certain terminology occasionally associated with RPC reflects the developmental history of the technique. The term “reversed phase” derives from “normal phase” chromatography, a technique utilizing a hydrophilic *stationary phase* together with *mobile phases* consisting of organic solvents such as hexane or methylene chloride. In RPC the stationary phase is hydrophobic so that a water/organic solvent mobile phase is used, that is, the stationary phase is more hydrophobic than the mobile phase. RPC media may be referred to as *adsorbents* while eluent solutions may be referred to as mobile phases.

## RPC in theory

The separation of biomolecules by RPC depends on a *reversible hydrophobic interaction* between sample molecules in the eluent and the medium. Initial conditions are primarily aqueous, favoring a high degree of organized water structure surrounding the sample molecule. Frequently, a small percentage of organic modifier, typically from 3–5% acetonitrile, is present in order to achieve a “wetted” surface. As sample binds to the medium, the hydrophobic area exposed to the eluent is minimized.

*Separation* relies on sample molecules existing in an equilibrium between the eluent and the surface of the medium. The distribution of the sample depends on the properties of the medium, the hydrophobicity of the sample and the composition of the eluent (mobile phase), as illustrated in Figure 61. Initially, conditions favor an extreme equilibrium state where essentially 100% of the sample is bound. Since proteins and peptides carry a mix of accessible hydrophilic and hydrophobic amino acids and are rather large, the interaction with the medium has the nature of a multi-point attachment.



**Fig 61.** Proteins and peptides bind to an RPC medium under aqueous conditions and elute as the eluent becomes more hydrophobic.

To bring about *elution* the amount of organic solvent is increased so that conditions become more hydrophobic. Binding and elution occur continuously as sample moves through the column. The process of moving through the column is slower for those samples that are more hydrophobic. Consequently, samples are eluted in order of *increasing hydrophobicity*.

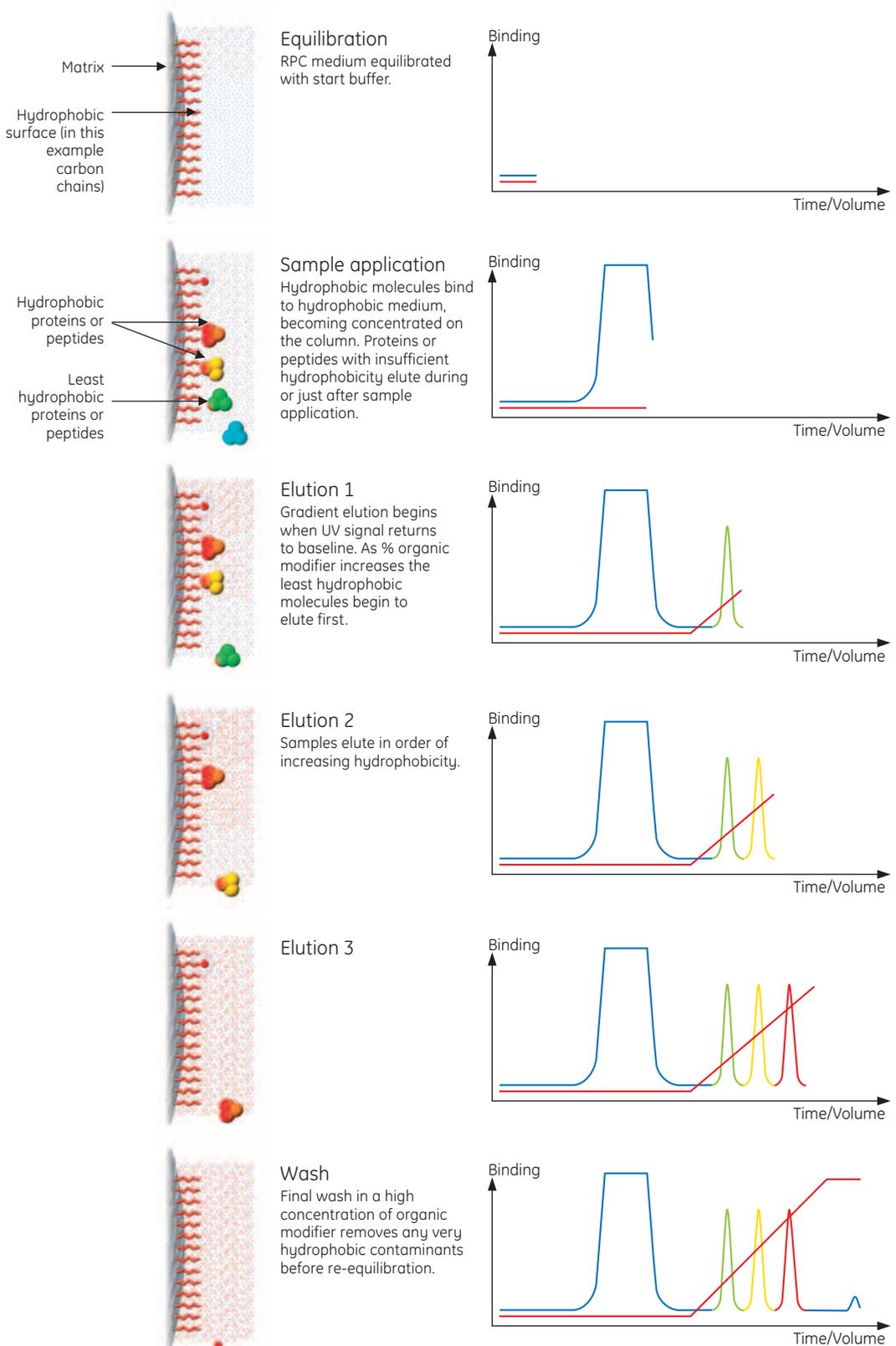
## Steps in an RPC separation

There are two main types of RPC media, one based on a hydrophilic matrix of *silica beads* covered with a bonded *hydrophobic* phase of carbon chains, typically n-alkyl or aromatic hydrocarbons, and one based on a naked, *hydrophobic polymer* matrix. Highly porous matrices provide a large internal surface area for high binding capacity. Matrices with uniform particle size can be used at higher flow rates.

As with other chromatography techniques, an RPC medium is packed into a column to form a *packed bed*. The bed is then equilibrated with eluent to fill the matrix pores and the space in between the particles. The performance achievable on RPC, especially the resolution, is strongly influenced by the efficiency of the column packing. The use of prepacked columns is therefore highly recommended, particularly when working with particles sizes below 15  $\mu\text{m}$ .

A typical biological sample contains a complex mixture of molecules with a correspondingly diverse range of hydrophobicity. Most biomolecules are sufficiently hydrophobic to bind strongly to RPC media under aqueous conditions, in the presence of a low concentration of organic modifier, and to elute within a very narrow window of organic modifier concentration. *Gradient elution* is, therefore, the most practical method for RPC separation of complex biological samples. Samples are concentrated during the binding process. The key stages in a separation are shown in Figure 62.

Sample is applied under conditions that favor binding, typically using an aqueous solution containing an *ion-pairing agent*, such as trifluoroacetic acid (TFA), to enhance the hydrophobic interaction (see page 99) and a low concentration of organic modifier such as 5% acetonitrile. After application, and when all non-bound molecules have passed through (i.e., the UV signal has returned to baseline), conditions are altered in order to elute the bound sample. Elution begins by increasing the concentration of *organic modifier*, such as acetonitrile. Molecules with the lowest hydrophobicity will elute first. By controlling the increase in organic modifier, molecules are eluted differentially. Those molecules with the highest degree of hydrophobicity will be most strongly retained and eluted last. A wash step removes most of the tightly bound molecules at the end of elution. The column is then re-equilibrated before the next run.



**Fig 62.** Steps in an RPC separation using gradient elution.

## Resolution

RPC is the highest resolution chromatography technique available. Resolution is a combination of the degree of separation between the peaks eluted from the column (*selectivity*), the ability of the column to produce narrow, symmetrical peaks (*efficiency*), the amount (mass) of sample applied and the *retention time* of the samples on the column. These factors are influenced by practical issues such as matrix properties, solvent conditions, column packing and flow rates, all of which are covered in detail in the practical section of this chapter.

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 63.

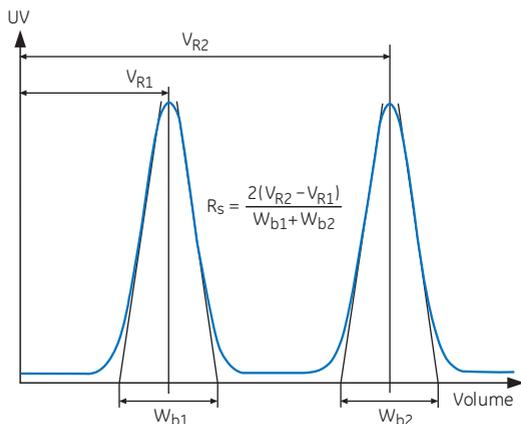


Fig 63. Determination of the resolution ( $R_s$ ) between two peaks.

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 64) 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  $R_s > 1.5$ . At this value, peak purity is 100%.

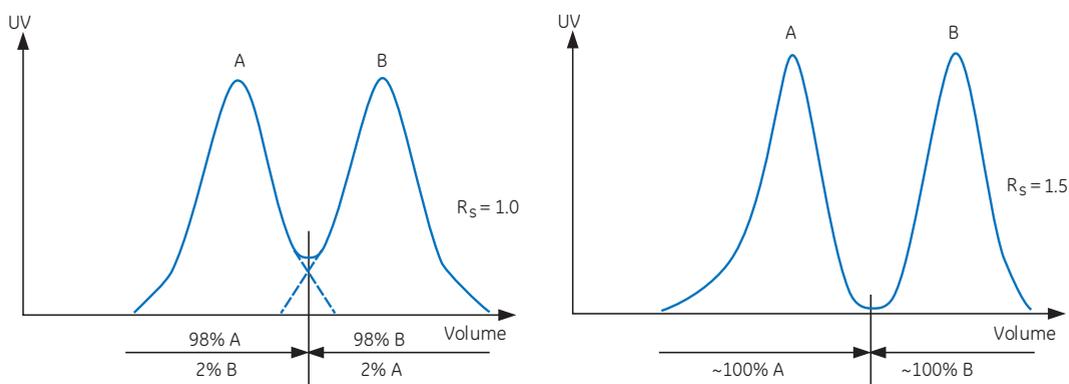


Fig 64. Separation results with different resolutions.

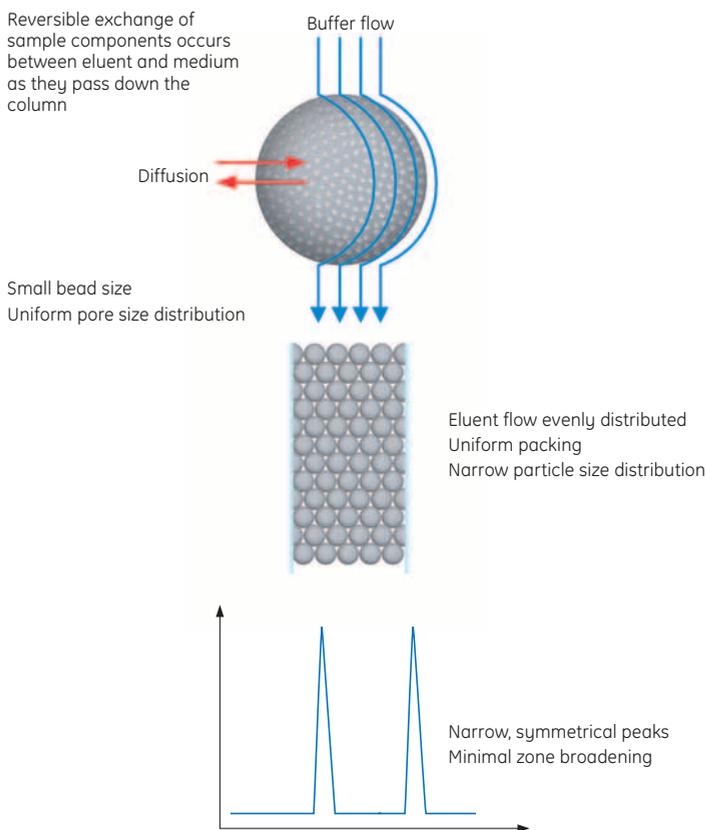


A single, well-resolved peak is not necessarily a pure substance, but may represent a series of components that could not be separated under the chosen elution conditions.

## Efficiency

Column efficiency (the ability to elute narrow, symmetrical peaks from a packed bed) relates to the *zone broadening* that occurs on the column and is described in terms of the number of theoretical plates (see Appendix 2 for determination of column efficiency).

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. Poorly packed columns lead to channeling (uneven passage of solutions through the column), zone broadening and hence loss of resolution. Figure 65 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 minimize diffusion effects and so produce the narrowest peaks under the correct elution conditions, in a well-packed column.



**Fig 65.** Factors that affect column efficiency.

Resolution in terms of efficiency can be improved by decreasing the particle size of the matrix. A decrease in particle size contributes to higher back pressure so that flow rates need to be reduced and run times lengthened. This may be compensated to some extent by using shorter columns.

Perhaps more than any other chromatography technique, resolution on RPC is strongly influenced by the efficiency of the column packing. The smaller the particle size, the more difficult it is to pack a column efficiently. Since most analytical RPC media are made from particles below 15  $\mu\text{m}$ , the use of commercially available, prepacked columns is highly recommended in order to achieve the best performance.

Temperature can significantly affect final resolution. Most often selectivity increases at lower temperatures while, with increasing temperature, eluent viscosity decreases leading to more efficient mass transfer (diffusion) of hydrophobic components between the eluent and medium thereby improving resolution. Temperature may therefore have to be optimized to balance selectivity against efficiency to achieve optimal resolution. There are examples of high and low temperature separations, although elevated temperatures are most often used when separating low molecular weight molecules.

### Column length

The resolution of high molecular weight biomolecules such as proteins and peptides is less sensitive to column length than the resolution of small organic molecules. Since the binding of high molecular weight molecules on a hydrophobic surface is very sensitive to small changes in eluent composition, these molecules elute over a very narrow range of organic modifier concentration. The use of gradient elution to ensure elution of all bound molecules, each with different hydrophobicity, reduces the significance of column length.

### Selectivity

Good selectivity (the degree of separation between peaks) is an important factor in determining resolution (Figure 66) and depends largely on the nature of the RPC medium, the nature and composition of the eluent and the gradient used for elution as well as other parameters such as temperature and the nature of the sample components.

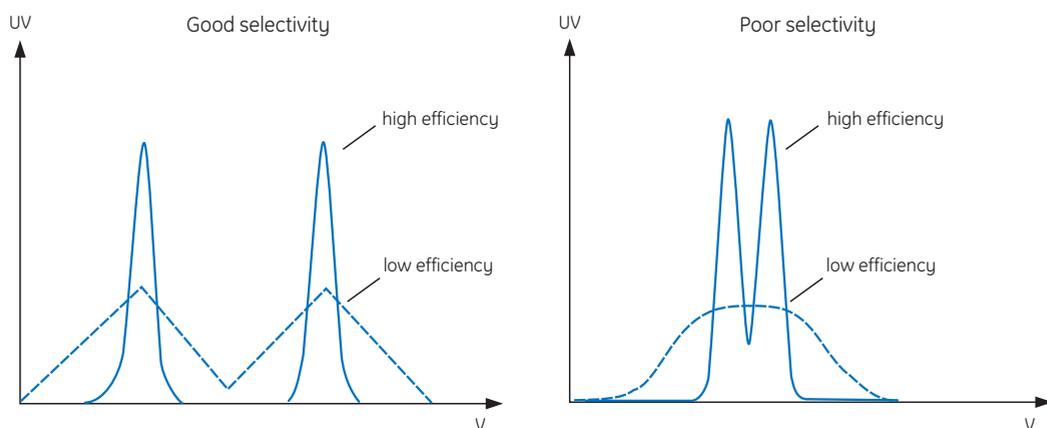


Fig 66. Effect of selectivity and efficiency on resolution.

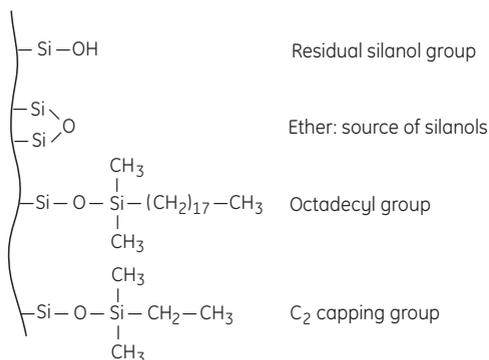
### Components of an RPC medium

Factors that may affect final resolution and selectivity include the chemical composition of the matrix, the particle size, the nature of the hydrophobic ligands, ligand surface density (if any), capping chemistry (if used) and pore size.

#### The silica matrix

Any matrix used for a binding/elution technique must be chemically and physically stable and preferably porous to ensure adequate binding capacity. Historically, silica was one of the earliest materials to be used. Typically, silica particles are coupled via silanol groups to hydrophobic ligands of various chain lengths and surface density, as illustrated in Figure 67.

These media were originally developed for purification of small organic molecules and have recently been used for purification of peptides. Silica is chemically stable at low to neutral pH and in the organic solvents typically used for RPC of small molecules.



**Fig 67.** Some typical structures on the surface of a silica-based RPC medium. The hydrophobic octadecyl group is one of the most common ligands.

### Ligands on silica matrices

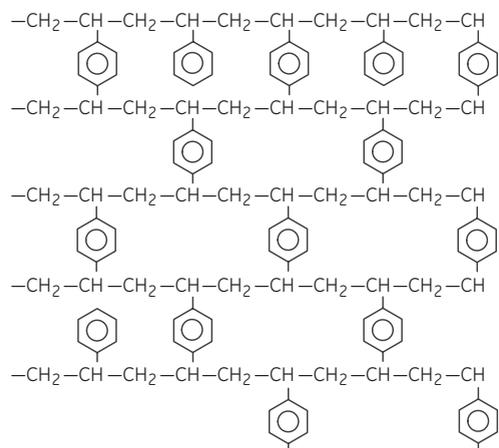
In general, more hydrophobic samples require less hydrophobic ligands for a successful separation. Conversely, more hydrophilic samples require strongly hydrophobic ligands in order to achieve sufficient binding for subsequent separation.

 Most commonly, synthetic peptides, shorter peptides and oligonucleotides are separated on C18 ligands.

A major disadvantage of silica is its chemical instability in aqueous solutions at high pH (above pH 7.5) and, in certain applications, its tendency to cause mixed-mode retention (page 100).

### The polymer matrix

Synthetic organic polymers, such as beaded polystyrene, provide excellent chemical stability, particularly under strongly acidic or basic conditions (from pH 1 to pH 12). These stable matrices offer key advantages when separating complex mixtures of protein or peptide: a broad working pH range offers greater control over selectivity (see pH and sample properties, page 106); greater chemical stability facilitates any cleaning that may be required after working with biological samples (Figure 68). In addition, high physical stability and uniform particles facilitate high flow rates, particularly during cleaning or re-equilibration steps, thereby improving throughput and productivity and minimizing back pressure.



**Fig 68.** Partial structure of a polystyrene-based RPC medium.

Matrices and ligands used in RPC media from GE Healthcare are shown in Table 17.

**Table 17.** Matrices used for RPC media available from GE Healthcare.

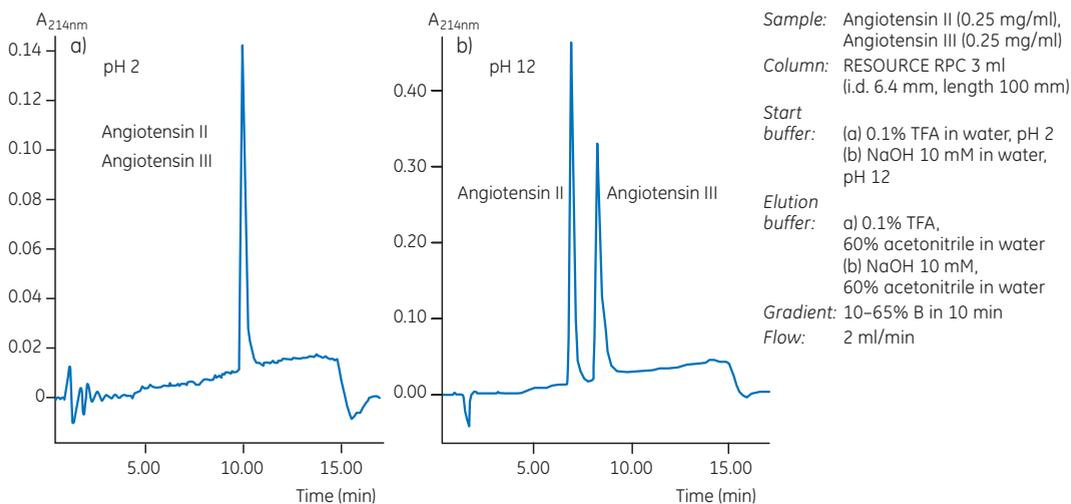
Product	Matrix	Ligand	Mean particle size	pH stability
μRPC C2/C18 (120 Å pore size)	Polar (hydrophilic) silica	Non-polar (hydrophobic) carbon chains : C2 /C18	3 μm	2–8
SOURCE 5RPC	Non-polar (hydrophobic) polystyrene/divinyl benzene, highly spherical, monodispersed	Matrix surface	5 μm	1–12
SOURCE 15RPC	As above	Matrix surface	15 μm	1–12
SOURCE 30RPC	As above	Matrix surface	30 μm	1–12

- Use columns packed with 3 μm (μRPC C2/C18) or 5 μm (SOURCE 5RPC) particles for micro-preparative and analytical separations.
- Use 5 μm or 15 μm media for intermediate purification or polishing of laboratory-scale separations. A 15 μm media will be better suited for use with crude samples.
- Use 15 μm or 30 μm media for large-scale preparative and process separation, for example, SOURCE 15RPC or SOURCE 30RPC. These media offer lower pressure requirements at high flow rates and have been optimized to ensure high throughput (amount of sample processed within a defined time) while retaining high performance. SOURCE 30RPC is ideal for the polishing stage of industrial processes.

## Eluents

### pH

Optimum pH is one of the most important parameters to establish. Figure 69 shows the significant difference in selectivity that could be achieved by increasing the pH used for the separation of two angiotensins under otherwise identical conditions.



**Fig 69.** Selectivity altered by a change in pH.

When using *silica-based media*, separations are often performed at pH 2–4 although pH values up to 7.5 can be used. Low pH conditions give good sample solubility while enhancing binding (see ion-pairing agents, page 99) and minimizing the risk of mixed-mode retention (see mixed-mode retention and ion suppression, page 100).

When using *polystyrene-based media* separations can be performed over a broad pH range from 1–12. Since the properties of proteins and peptides can alter significantly at different pH values, a change in pH can give a new selectivity for the sample molecules that contain R-groups with pKa values in the range 3.1–12.0. Altering pH can also improve control over selectivity and, in some cases, improve solubility and yield of biological activity. Basic peptides often tail during elution from RPC columns at low pH so better resolution can be achieved above pH 8. In addition, a wider working pH range facilitates method optimization.

Addition of an ion-pairing agent such as an acid may increase the hydrophobicity of the sample molecules thereby increasing retention and, in some cases, the final selectivity.

Examples of compounds used to maintain the required pH are given in Table 18.

**Table 18.** Examples of compounds used to maintain pH and/or act as ion-pairing agents.

Eluent	pH	Ion-pairing agent	Concentration	Comment
Hydrochloric acid	2.0	no	0.01 M	Volatile.
Sodium hydroxide	12	no	0.01 M	Non-volatile. Use only with polystyrene-based media.
Eluent components that also act as ion-pairing agents for positively charged molecules, e.g., proteins, peptides and hydrophilic peptides				
Trifluoroacetic acid (TFA)	2–3	Pairing ion: $\text{CF}_3\text{COO}^-$	(typically 0.1%)	Acidic amino acid side chains become undissociated. Ion pairs with amino groups. Low UV absorbance. Volatile. Note: stay below 0.3% to maintain a stable baseline.
Ammonium acetate	6–10 and 4.3–5.3	Pairing ion: $\text{CH}_3\text{COO}^-$	10–100 mM	Volatile.
Phosphoric acid	2–3	Pairing ion: $\text{H}_2\text{PO}_4^-$ , $\text{HPO}_4^{2-}$ , $\text{PO}_4^{3-}$	10–100 mM	Less hydrophobic than TFA. Weak ion-pairing properties. Non-volatile. Adjust to chosen pH with NaOH.
Eluent components that also act as ion-pairing agents for negatively charged molecules, e.g., oligonucleotides				
Triethylamine (TEA)	4–8	Pairing ion: $\text{NH}^+(\text{C}_2\text{H}_5)_3$	10–100 mM	Suppresses negative charges. Used mainly to mask negatively charged silanol groups. Also acts as an ion-pairing agent with negatively charged sample components.
Tetramethylammonium chloride	4–12	Pairing ion: $^+\text{N}(\text{CH}_3)_4$	5–100 mM	
Tetrabutylammonium chloride	4–12	Pairing ion: $^+\text{N}(\text{C}_4\text{H}_9)_4$	5–100 mM	

## Ion-pairing agents

A common way to increase the hydrophobicity of charged components, enhance binding to the medium, and so alter retention time, is to add ion-pairing agents to the eluent. These agents bind via ionic interactions with charged groups and thereby suppress their influence on overall hydrophobicity (Figure 70). Since most proteins and peptides are slightly basic, ion-pairing agents are often acids such as trifluoroacetic acid (TFA) whereas a base such as triethylamine is used for negatively charged molecules.

In some cases the addition of ion-pairing agents is an absolute requirement for binding to the RPC medium. For example, an ion-pairing agent such as trifluoroacetic acid is essential to ensure binding of hydrophilic peptides. Examples of compounds that act as ion-pairing agents are given in Table 18.

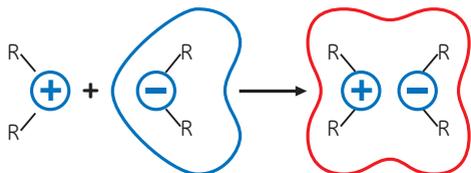
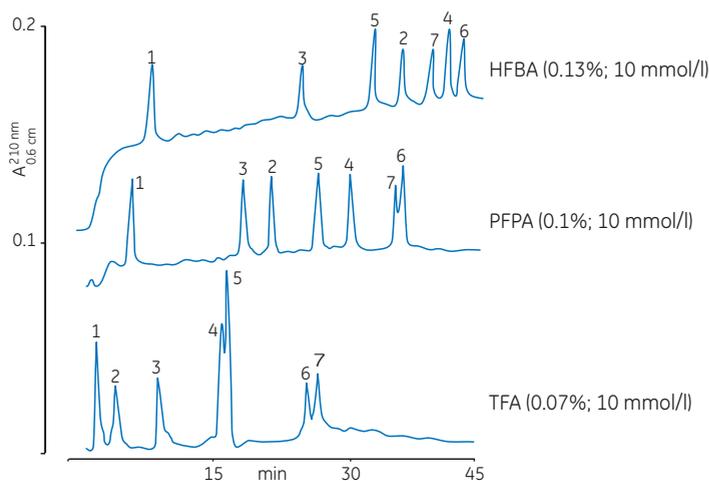


Fig 70. Ion-pairing agents alter net hydrophobic properties.

The type and concentration of an ion-pairing agent can also affect retention behavior and subsequent selectivity, as shown in Figures 71 and 72.

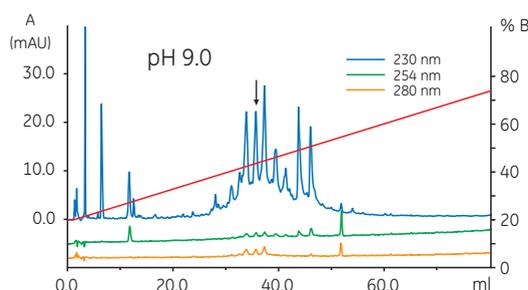


- 1: Met-enk.
- 2: ACTH<sub>1-24</sub>
- 3:  $\alpha$ -MSH
- 4: ACTH<sub>1-39</sub>
- 5: Somatostatin
- 6: Bovine insulin
- 7: Human calcitonin

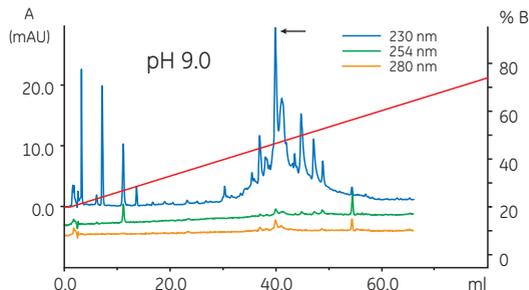
Figure taken from Bennett, H. P. J. Manipulation of pH and ion-pairing reagents to maximize the performance of reversed-phase columns, in *High-Performance Liquid Chromatography of Peptides and Proteins: Separation, Analysis, and Conformation* (Mant, C. T. and Hodges, R. S. eds). CRC Press, Boca Raton, pp 319-326 (1991). Used with permission. © 1991 CRC Press.

Fig 71. Ion-pairing agents significantly affect selectivity and even elution order. Note that pH is also involved in this example.

Eluent A: NH<sub>4</sub>OH/HCOOH  
Eluent B: CH<sub>3</sub>CN, 60%



Eluent A: NH<sub>4</sub>HCO<sub>3</sub>  
Eluent B: CH<sub>3</sub>CN, 60%

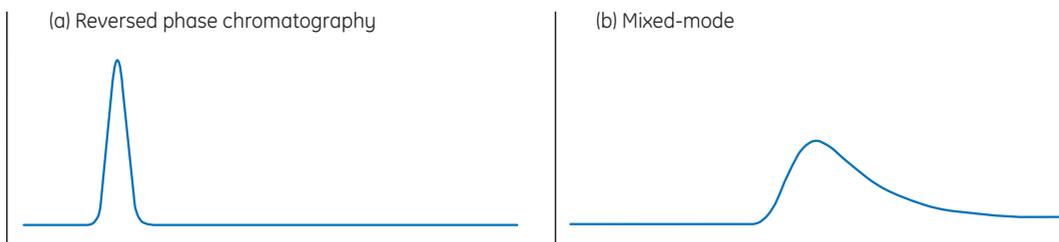


Sample: Amyloid- $\beta$  1-42  
Sample load: 622  $\mu$ g of crude material  
Column: SOURCE 5RPC ST 4.6/150  
Gradient: 20-80% eluent B over 87.5 ml  
Flow rate: 0.5 ml/min

Fig 72. Effect of ion-pairing agents on selectivity, pH constant.

## Mixed-mode retention and ion suppression

Mixed-mode retention is seen as an increase in retention times together with significant peak broadening as illustrated in Figure 73.



**Fig 73.** Typical effect of mixed-mode retention. Peaks are broader, skewed and retention time increases.

The phenomenon only needs to be considered when using silica-based media since polystyrene and other synthetic organic polymers used in RPC are homogenous and stable between pH 1–12. Peak broadening and increased retention times are caused by ionic interactions taking place between negatively charged silanol groups exposed on the surface of the silica medium and positively charged amino groups on the sample molecules.

During manufacture, silanol groups remain on the surface of the silica after ligand immobilization because steric hindrance from C8 or C18 chains prevents complete derivatization of all the available silanol groups. To reduce the risk of mixed-mode retention manufacturers react the silanol groups with smaller alkylsilane reagents such as chlorotrimethyl- and chlorotriethylsilanes. The process is referred to as “end-capping”. The extent of end-capping also affects selectivity, so reproducibility in the capping process is critical.

Ionizable silanol groups arise not only from inadequate end-capping, but also from column aging, the latter often accelerated by prolonged exposure to aqueous solutions.

Choosing commercially available silica-based media with high batch-to-batch reproducibility and stringent quality control methods can help to minimize the chance of mixed-mode retention.

Using an eluent below pH 3 will prevent interaction between silanols and positively charged sample molecules. The presence of charged silanols is often masked by the addition of TEA to the eluent.

## Elution

### Organic modifiers

To bring about elution, an organic modifier is added to the eluent in order to increase the elution strength. The organic modifier must be miscible in water and UV transparent to enable detection of the eluting molecules. The boiling point must be sufficiently low to enable evaporation of the modifier after elution. Table 19 reviews the commonly used modifiers in terms of their suitability for protein and peptide separations. Figure 74 shows how organic modifiers differ in their elution strength.

**Table 19.** Acetonitrile is the preferred organic modifier for protein and peptide separations.

Organic modifier	Suitability	Boiling point (°C)	UV cut-off (nm)	Viscosity (cP at 20°C)	Comments
Methanol	Organic small molecules	65	210	Medium- low: 0.60	May destabilize protein structure.
Ethanol	Organic small molecules and peptides	78	205	Medium- low: 1.20	May destabilize protein structure.
2-propanol	Proteins Peptides	82	210	High: 2.30	Least effect on protein structure.
Acetonitrile	Organic small molecules  Proteins Peptides	82	190	Low: 0.36	Most effect on protein structure. More powerful denaturant than alcohols. Toxic.

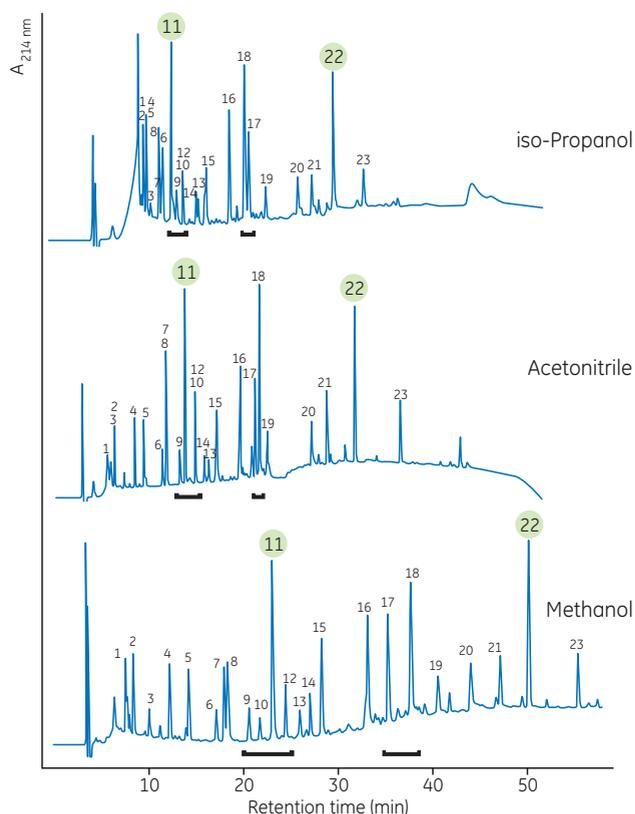


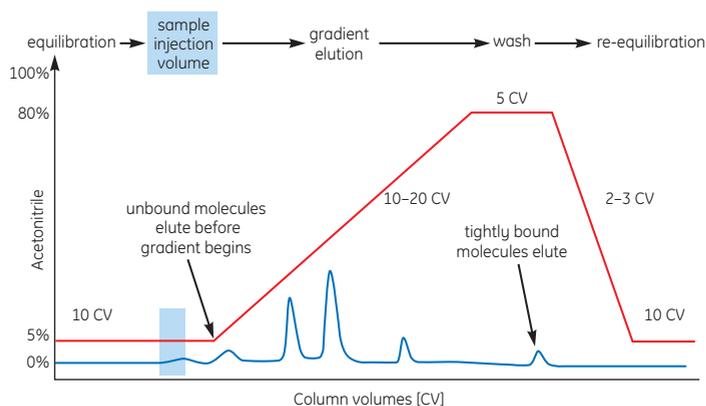
Figure taken from Aguilar, M.-I. and Hearn, M. T. W. High-resolution reversed-phase high-performance liquid chromatography of peptides and proteins. *Meth. Enzymol.* **270**, 3–26 (1996). Used with permission. © 1996 Academic Press, Inc.

**Fig 74.** Organic modifiers differ in their elution strength rather than in their influence on selectivity when separating proteins and peptides.

## Types of elution

*Gradient elution* is used most frequently for preparative and analytical, high-resolution separations of proteins and peptides, in order to minimize separation times. The UV absorbance and theoretical gradient traces shown in Figure 75 represent the elution of sample components and the increase in concentration of organic modifier in the eluent (%B) during gradient elution.

Although RPC separations are frequently described in terms of flow (ml/min or cm/h, see Appendix 3) and time (min), expressing eluent volumes as *column volumes* to describe a separation profile, for example, 5 CV=5 ml for a column with a 1 ml bed volume, greatly facilitates method development and the transfer of methods to columns of different dimensions when scaling up.



**Fig 75.** Typical high-resolution, RPC separation using gradient elution.

For *high-resolution analysis* a broad gradient is used in order to bind as many components as possible and then elute them differentially to obtain a comprehensive profile.

For *preparative* applications gradient elution conditions can be optimized in order to separate a target molecule from all contaminants.

*Step elution* is used most typically for desalting (buffer exchange). Here a low-resolution separation is used to separate hydrophobic components from hydrophilic contaminants and salts.

Under *isocratic elution*, separation is brought about using only one eluent. Isocratic elution is used mainly for high-resolution analysis of small organic molecules, but can occasionally be used to advantage as part of an optimized separation, for example, to maximize resolution in a region where contaminants elute very closely to a target molecule during a preparative application.

Isocratic elution is also used when desalting a sample. Desalting by RPC is used primarily for sample preparation before a chromatographic separation, for example, to remove salts before an IEX separation or before online or offline analysis by mass spectrometry. Large volumes of sample can be applied (limited only by the binding capacity of the medium). Hydrophobic molecules bind as hydrophilic molecules, including salts, pass through. The bound, concentrated molecules are then eluted using a small volume of a hydrophobic eluent, typically containing acetonitrile. This volatile solvent can then be removed by evaporation and the residue re-dissolved in a new buffer. Gel filtration chromatography is also used for desalting, separating low molecular weight contaminants and salts from higher molecular weight biomolecules (see Appendix 1). However, although gel filtration is a simple, gentle technique, it has the disadvantages that the volume of sample that can be applied to a column is limited and that samples are diluted. Figure 76 shows a theoretical comparison between gel filtration and RPC when used for desalting.

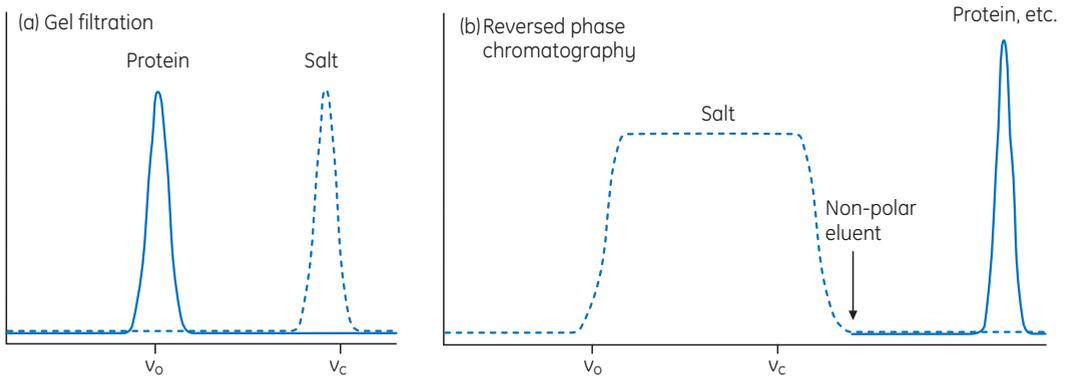


Fig 76. Desalting on RPC.

## Binding capacity

The *available binding capacity* of an RPC medium is a quantitative measurement of its ability to bind sample components under defined static conditions. If the defined conditions include the flow rate at which the medium was operated, the amount bound is referred to as the *dynamic binding capacity*. Porosity is a crucial factor in determining the binding capacity. The entire hydrophobic surface of macroporous media is available for binding of sample components, but high molecular weight components may be excluded from media of smaller pore size and only a small fraction of the hydrophobic surface will be used. High porosity with an open pore structure (e.g., SOURCE RPC media) is therefore an advantage when separating large molecules such as proteins and peptides. The pores must be large enough to allow all the molecules of interest to enter freely in order to achieve a high binding capacity. Capacity values are also influenced by other properties such as the nature of the sample components, the eluent used during binding, temperature, pH etc.

Binding capacities are supplied under “Purification options” with each medium in this chapter. However, these values can be used only as guides. Optimal selectivity in relation to the quantity of sample molecules applied to a column must be determined practically.

## RPC in practice

This section covers detailed practical aspects of an RPC separation, together with hints and tips to improve resolution and overall performance. Advice focuses primarily on separation of biomolecules such as proteins and peptides.

In practice the steps for a typical RPC separation can be summarized as in Figure 77.

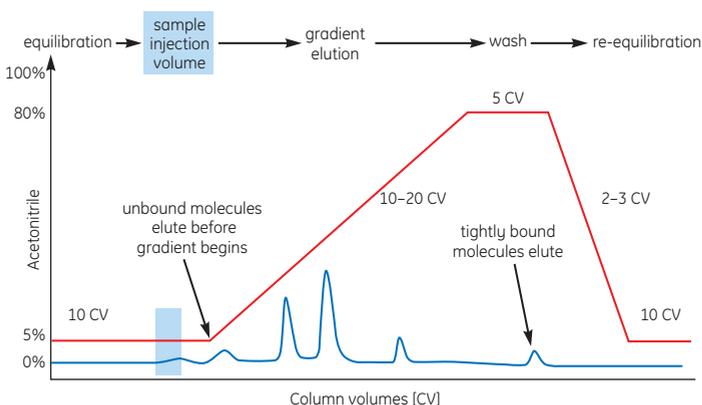


Fig 77. Typical RPC separation using gradient elution.

As discussed under the theoretical section, a successful RPC separation is influenced by many parameters and will always need to be optimized to meet the requirements for the application. Steps toward selecting and optimizing media and conditions for an RPC separation of biomolecules are given here in order of priority:

1. Select a medium that provides best resolution under the simplest starting conditions, for example, TFA and acetonitrile in the eluent (proteins and peptides show little difference in selectivity when using silica- or polymer-based media under these conditions).
2. Scout for the pH that provides best resolution. This may be the key factor that defines whether to use a silica- or polymer-based medium.
3. If necessary, scout for a suitable ion-pairing agent to improve selectivity.
4. Optimize the gradient elution to maximize selectivity (the gradient slope only influences the distance between peaks, it will not change their elution order). Select the steepest gradient (lowest gradient volume) that provides acceptable results.
5. Scout for the highest flow rate that gives an acceptable separation.

## **Media and column selection**

Choosing the correct RPC medium and column dimensions is critical for a successful separation and should be based on the goal of the application and the nature of the sample components.

### **Sample components: hydrophobicity**

The selection of an RPC medium in relation to the hydrophobicity of the sample components must be made empirically. Unlike other chromatographic techniques, it is almost impossible to predict the retention of biomolecules in RPC. Important parameters affecting retention of a peptide appear to be a combination of the amino acid sequence of the peptide together with any secondary structure, such as  $\alpha$ -helices and  $\beta$ -pleated sheets. The situation for proteins is further complicated by their tertiary structure.

-  Select less hydrophobic media when separating components that are known to be highly hydrophobic to facilitate elution. Samples that bind strongly to a medium will be more easily eluted from a less hydrophobic medium.

### **Goal of separation**

Applications involving fractionation of multi-component samples, such as peptide mapping, require extremely high resolution. Preparative reversed phase applications, such as the purification of synthetic peptides, are more concerned with throughput, and resolution may be traded off against speed and capacity. However, if used in the final polishing step, resolution will be crucial.

Resolution in reversed phase chromatography depends on the efficiency of the column and the selectivity. The parameters that can significantly affect selectivity are discussed earlier in this chapter (page 95).

-  Use commercially available prepacked columns whenever possible since maximum efficiency depends on the packing process and the particle size of the medium. The smallest 3  $\mu\text{m}$  particles give the highest efficiency followed by the larger 5  $\mu\text{m}$  media, such as SOURCE 5RPC and so on.

## Scale of separation

-  Use columns packed with 3  $\mu\text{m}$  ( $\mu\text{RPC C2/C18}$ ) or 5  $\mu\text{m}$  (SOURCE 5RPC) particles for micro-preparative and analytical separations.
-  Use 5  $\mu\text{m}$  or 15  $\mu\text{m}$  media for intermediate purification or polishing of laboratory-scale separations. A 15  $\mu\text{m}$  media will be better suited for use with crude samples.
-  Use 15  $\mu\text{m}$  or 30  $\mu\text{m}$  media for large-scale preparative and process separation, for example, SOURCE 15RPC or SOURCE 30RPC. These media offer lower pressure requirements at high flow rates and have been optimized to ensure high throughput (amount of sample processed within a defined time) while retaining high performance. SOURCE 30 RPC is ideal for the polishing stage of industrial processes.

Note that the nature of RPC separations may cause slight changes in selectivity when changing particle size.

## Eluent conditions

-  When initial selectivity or sample stability and solubility factors indicate the use of an eluent above pH 7.5, always use a polymer-based medium such as SOURCE RPC.
-  When working with crude protein or peptide samples, it is an advantage to use polymer-based media that can be easily cleaned with alkali (silica-based media dissolve above pH 7.5).

## Column length

-  Increasing column length may improve resolution when working with large sample volumes.
-  Longer column lengths may improve resolution of a complex peptide mixture, for example, the resolution of peptides from a peptide digest.  
Longer column lengths may improve resolution of closely-related peptides or proteins if a shallow gradient of organic modifier is used.

## Eluent selection and preparation

Use the highest purity, HPLC grade solvents, acids, bases, salts, ion-pairing agents and water whenever possible. Chemical purity is important since contaminants may produce unwanted extra peaks, ghost peaks or contaminate the final product.

All components must be transparent to UV below 220 nm and soluble under the low polarity conditions used during a separation. Although proteins absorb at 280 nm and synthetic oligonucleotides at 250–260 nm, detection below 220 nm (usually at 215 nm) is necessary when separating short peptides that lack aromatic amino acid residues such as Trp and Tyr. Components recommended in Tables 18 and 19 have been chosen on the basis of providing optimal separation in combination with low background absorbance.

-  When possible, use volatile components. These can be removed by evaporation from the eluted fractions, along with the organic modifier. Non-volatile salts or acids must be removed by an additional desalting step.

## pH and ion-pairing agents

Table 18 shows the most commonly used acids and bases for setting the pH of an eluent and shows their influence as ion-pairing agents. Since the net charge of proteins and peptides varies with pH, their net hydrophobicity also varies with pH. Eluent pH is therefore an important influence on elution order and final selectivity.

-  For samples with unknown properties, start with the most commonly used strong acid that also acts as an ion-pairing agent: 0.1% trifluoroacetic acid as eluent A (reduce to 0.065% if baseline stability needs to be improved).

Add ion-pairing agents at concentrations recommended in Table 18, to enhance binding of hydrophobic components to the medium. Note that other ion-pairing agents are not combined with TFA.

In the case of silica-based media, use ion-pairing agents to minimize mixed-mode retention (page 100), which can impair resolution.

-  For samples with known specific properties, refer to Table 18.



The presence of ion-pairing agents can affect UV absorbance, and changes may be seen as the concentration of organic modifier changes. This may result in ascending or descending baselines during gradient elution. Always run a blank gradient to determine the effect of any additives prior to performing a separation. Adjust the concentration if necessary (refer to eluent balancing, page 112). Note that changing the concentration can change the degree of ionization of sample components and alter their behavior during separation.

## Organic modifiers

-  For samples with unknown properties, start with the most commonly used organic modifier, acetonitrile.

With a cut-off below 210 nm, acetonitrile has a much lower background absorbance than other common solvents at these low wavelengths, providing better baseline stability as the content of organic modifier is varied during a separation and ensuring optimum detection sensitivity.

-  Ion-pairing agents may need to be added (see above).
-  Use 2-propanol when requiring stronger eluting properties or to maintain sample stability. Note that the higher viscosity results in lower column efficiency and increased back pressure.
-  If the elution profile is still unsatisfactory, refer to Table 19, for a review of other organic modifiers. Note that changing the organic modifier can affect retention time. Changes in the elution order of proteins are likely to be a result of denaturation that significantly alters their hydrophobicity.

## Typical eluent protocols for separation of proteins and peptides

This section presents some of the more commonly used eluent protocols. Most protocols contain 5% or less of organic modifier in eluent A and 80% or less of organic modifier in eluent B. Note that concentrations above 80% may affect PEEK tubing which is often present in high performance chromatography systems.

### Silica-based media

Eluents A and B should contain at least 0.1% of a strong acid to act as an ion-pairing agent, to maintain a low pH and to minimize mixed-mode retention (page 100). Eluent B should contain a significantly higher content of organic modifier. For example:

Eluent A: 0.1% TFA in water, 5% acetonitrile

Eluent B: 0.1% TFA in acetonitrile (maximum 80%)

## Polymer-based media

Since polymer-based media can be used over a wider pH range and without concerns over mixed-mode retention and the need to suppress the ionic interactions of silanol groups, a wider range of eluent protocols can be used.

### For samples with unknown properties or known to require acidic conditions

Eluent A: 0.065% TFA in 2% acetonitrile

Eluent B: 0.050% TFA in 80% acetonitrile

### For samples known to require basic conditions

Eluent A: 0.125% ammonium solution pH 10 in 2% acetonitrile

Eluent B: 80% acetonitrile in eluent A

### Other systems

Eluent A: pH 2.1 0.1% formic acid, 2% acetonitrile

pH 2.0 0.1% acetic acid, 2% acetonitrile

pH 2.0 0.1% TFA, 2% acetonitrile

pH 4.5 10 mM sodium acetate, 2% acetonitrile

pH 7.0 10 mM potassium phosphate, 2% acetonitrile

pH 9.0 10 mM Tris-HCl, 2% acetonitrile in buffer

pH 12 10 mM NaOH, 2% acetonitrile in buffer

Eluent B: 70% acetonitrile in eluent A

 Although most eluents contain strong acids and organic solvents that give little buffering capacity, adequate buffering capacity should be maintained when working closer to physiological conditions.

## Preparation

1. Filter eluents that have had solids added, using a 0.22  $\mu\text{m}$  filter. This prevents particles from clogging the column.
2. Measure volumes of organic solvent and aqueous solutions separately and then mix (this eliminates volume variations that occur when mixing organic and aqueous phases directly).
3. Degas the solutions in a sonication bath (<15 min), under vacuum with magnetic stirring (<5 min) or by purging by helium (<5 min). This prevents bubble formation during elution. Be careful to keep the degassing time as low as possible to prevent evaporation of the organic solvent.
4. Add volatile ion-pairing agents.

 Use freshly prepared eluents.

 Always use a flow restrictor (compatible with an appropriate pressure range) connected after the detector of a chromatography system to prevent the accumulation of air in the detector.

 If eluents have to be stored, the containers must be sealed to avoid changes in composition caused by evaporation and, preferably, kept at 4°C. Do not store aqueous solutions at neutral pH for more than 2–3 days due to the risk of microbial growth. To reduce the risk of bubble formation, allow cold solutions to reach running temperature and degas them before use.

 Follow health and safety regulations when using and disposing of the strong acids and organic solvents used in RPC.

## Column and media preparation

RPC columns should be "conditioned" for first-time use, after long-term storage or when eluent conditions are changed significantly. The eluents used for conditioning the column should be the same as those used in the subsequent separation. A general procedure for conditioning RPC columns is as follows:

1. Wash the column with approximately 3 column volumes of eluent B at a low to moderate flow rate appropriate for the particular column.
2. Run a 2–3 column volume linear gradient from 100% eluent B to 100% eluent A at the same flow rate as in step 1.
3. Equilibrate the column with 10 column volumes of eluent A. Continue equilibration until all monitor signals are stable.



If eluents are changed, perform a blank run to check for artifacts that may appear due to UV absorbing impurities. Return to 100% eluent A and equilibrate to a stable baseline prior to sample injection.

## Sample preparation

Sample preparation is critical especially for high-resolution separations when media with particle sizes as small as 3  $\mu\text{m}$  are used. Simple steps to clarify the sample will avoid the risk of blockage, reduce the need for stringent washing procedures and avoid deterioration in column performance and increases in back pressure. For efficient binding, dissolve the sample in the initial eluent or in a solution with a lower content of organic modifier.



Samples must be clear and free from particulate matter. Desalt very crude samples using gel filtration (Appendix 1) or an RPC column packed with larger particles to remove contaminants that may foul a high-resolution RPC column. Some RPC columns are also supplied with an in-line "guard column" to protect the main column.

1. Dissolve the sample in eluent A.
2. Centrifuge samples at 10,000 g for 10 min or filter through a 0.22 or 0.45  $\mu\text{m}$  sterile filter. Use a solvent-resistant filter if there is an organic modifier in eluent A. Apply to the column as soon as possible to avoid any side reactions such as oxidation.

## Sample solubility

It is important to maintain sample solubility throughout the loading process and during separation in order to avoid precipitation on the column.



If back pressure increases significantly this may be a sign that sample is precipitating on the column. Recheck sample solubility in eluent A. A low percentage of organic modifier in eluent A, for example, 5% acetonitrile, helps to overcome solubility problems without disrupting the separation.



If there are problems with solubility when the sample is dissolved directly in eluent A, add formic acid or acetic acid (0.1%) to increase solubility. Keep the sample volume small compared to the column volume to avoid any interference by these additives. If large sample volumes are applied these additives will be seen as additional peaks eluting in the void volume after injection.



Ensure that sample is at the same temperature as solutions, columns and chromatographic equipment.



Do not overload the column as this can also cause precipitation.

## Concentration and viscosity

Viscosity varies with temperature and will increase as the percentage of organic modifier increases. Differences in composition between the sample and eluent A will be seen as a disturbance in the UV baseline shortly after injection.

## Sample load

Sample load (mass) is of greater significance than sample volume since RPC is a binding technique. The amount of sample that can be applied to a column depends on the binding capacity of the medium and the degree of resolution required (see selectivity and binding capacity, page 95).

Sample load influences resolution since the width of the peaks is directly related to the amount of substance present. To achieve satisfactory resolution, the total amount of sample bound should be less than the total binding capacity of the packed column.

 Apply 20–25% of the total binding capacity of the column for optimal resolution with gradient elution. Sample loads can be increased if resolution is satisfactory.

Capacity may decrease with increasing flow rates so that a balance must be found between achieving the maximum dynamic binding capacity and a fast separation, particularly when applying large sample volumes.

## Sample volume

As a binding technique, RPC is independent of sample volume. Large volumes of dilute sample can be applied in order to concentrate and separate the sample.

## Temperature

Maintain sample, eluents, columns and chromatography equipment at the same, constant temperature throughout a separation to ensure consistent, reproducible results. Temperature will affect sample and eluent viscosity and may influence resolution (page 95). An increase or decrease in temperature can improve resolution. Increasing temperature is most effective in improving resolution when separating low molecular weight samples.

### Gradient, isocratic or step elution

For high-resolution RPC separations a stepwise or continuous gradient is used to elute components. The ideal gradient shape and volume must be empirically determined for each separation. Gradient slopes are described as changes in percent eluent B per unit time (%B/min) or per unit volume (%B/ml).

In any gradient elution, the concentration of organic modifier is lower in eluent A than in eluent B and, regardless of the absolute change in percent organic modifier, the gradient always proceeds from a relatively hydrophilic condition (high aqueous content, low concentration of organic modifier) to a hydrophobic condition (lower aqueous content, higher concentration of organic modifier).

Gradients can be measured in volume mode or time mode. Note that changes in flow (at constant gradient slope) have little effect on a separation but, at constant flow, gradient slope has a significant effect.

1. Begin with a broad linear gradient to determine whether the molecules(s) of interest will bind and elute or how well the components of a complex mixture will be separated.
2. Run a blank gradient before sample injection in order to detect any baseline disturbances coming from the column or impurities/components in the eluent. Run from 5% B to 80% B over 10–20 column volumes.
3. Adjust eluent conditions if the baseline drift is too great (see balancing the eluents, page 112). Check eluent components and cleanliness of column if there are signs of contamination (see ghost peaks, page 112).
4. When a satisfactory baseline has been achieved, repeat the run, this time injecting sample.

## Optimization

After the initial gradient run, separation conditions can be altered to improve selectivity and resolution. Key parameters that can be altered are listed in Table 20.

**Table 20.** Parameters to consider during optimization.

Effect	Parameter
Alter selectivity	Change organic modifier (elution power: iso-propanol>acetonitrile>ethanol>methanol)
Alter selectivity/retention time	Change pH
Alter selectivity	Change ion-pairing agent
Improve resolution	Use a shallower gradient (i.e. increase gradient volume or time or use a segmented gradient). Very effective for protein separations since retention times are very sensitive to small changes in eluent.
Improve resolution	Decrease flow rate
Improve resolution	Alter temperature

The final gradient shape can be a combination of linear gradients and isocratic steps. The choice of gradient slope will depend on how closely contaminants elute around a target molecule or how well a number of peaks are resolved.

 Shallow gradients with short columns are generally optimal for high molecular weight biomolecules.

Step gradients (i.e., a series of isocratic elutions at different % eluent B) are useful for applications such as desalting and when performing process-scale applications providing the desired resolution can be obtained.

### Flow rate

Flow rate is an important factor for resolution of small molecules, including small peptides and protein digests. Using an optimal flow rate is also important in isocratic experiments to keep zone broadening to a minimum. Flow rate is less important during a gradient elution as long as the most suitable particle size has been selected.

-  Choose the highest flow rate to achieve maximum resolution over the shortest time.
-  Apply samples directly to the column at a flow rate suitable to ensure optimal time for binding.
-  Use a chromatography system capable of producing accurate gradients. The choice of equipment depends largely on the sample volume, the size and type of column and the type of medium. When programming a chromatography system in time mode, remember that changes in flow rate will affect gradient slope and, therefore, resolution.
-  Reduce flow rate in order to reduce back pressure, but remember that this will lengthen the run time and the separation may need to be re-optimized.
-  For large-scale preparative RPC, the flow rate used during sample loading is significant since this will influence the dynamic binding capacity (page 103). The optimal flow rate for sample application must be determined empirically.

## ***Wash and re-equilibration***

1. Wash the column with at least 5 column volumes of 100% eluent B to remove any bound molecules.
2. Apply a decreasing gradient over 2–3 column volumes from 100% eluent B to 0% eluent A to avoid damaging the column by a sudden change in composition.
3. Re-equilibrate the column using at least 10 column volumes of eluent A.

 Occasionally the hydrophobic interaction is so strong that harsher organic solvents may be required to elute all bound material. Since the separation of proteins by RPC is a balance between elution and the risk of denaturation and loss of biological activity, HIC should be considered as a more suitable technique if this occurs. Peptides contain a low degree of tertiary structure and are therefore more stable in organic solvents.

# Troubleshooting

## Ghosting

Poor-quality eluent components can cause a phenomenon referred to as “ghosting”. Trace levels of organic impurities bind to the medium, concentrating during equilibration and sample application. When elution begins, these contaminants appear in the chromatogram as unknown, or “ghost” peaks. The size of a ghost peak will usually depend on the equilibration time and the level of organic impurities in the eluent.

Ghosting may also be caused by incomplete elution of molecules in a previous run. Run a blank gradient, with no sample, as a check, especially if subsequent runs are to be performed with high-sensitivity detection.

## Baseline drift: balancing eluents

During a typical run the baseline can progressively increase or decrease in an approximately linear fashion as the proportion of eluent B increases. This phenomenon may originate from an ion-pairing agent (or strong acid component) or an organic modifier that absorbs significantly at the detection wavelength. The background absorbance caused by eluent components is corrected for during column equilibration. As the proportion of organic component increases so the absorbance properties change.

 Compensate for a drifting baseline by using different concentrations of UV-absorbing ion-pairing agents (or buffer acids) in eluent A and B and thereby balancing the “concentrations” with respect to UV-absorption properties to give an approximately straight baseline. Because of batch-to-batch variations in the absorption properties of eluent components and other differences between the conditions in different runs, it is not practical to give specific recommendations. The following example can assist to illustrate the principle: gradients from TFA in water to TFA in acetonitrile will usually require that the concentration of TFA in acetonitrile is 10–30% lower than in water. The balanced concentrations of UV-absorbing components should then be determined empirically. The difference in concentration of ion-pairing agent between the two eluents is generally not large enough to adversely affect the separation. A typical example would be to use 0.065% TFA in eluent A and 0.05% TFA in eluent B.

Situation	Cause	Remedy
Reduced or no flow through the column.	Outlet closed or pumps not working.	Open outlet. Check pumps for signs of leakage.
	Blocked filter, end-piece, adaptor, tubing or pre-column.	Remove and clean or replace if possible. Always filter samples and eluents before use.
Reduced flow through the column. Back pressure increases during a run or during successive runs.	Precipitation in the column.	Follow cleaning procedures. Adjust eluents to maintain sample solubility.
	Bed is compressed.	Replace column.
Back pressure increases during a run or during successive runs.	Turbid sample applied.	Adjust eluents to improve sample solubility e.g., increase organic modifier or adjust pH.
	pH caused precipitation.	Adjust pH to avoid precipitation.
Sample does not elute during gradient elution.	Final concentration of organic modifier too low.	Increase concentration of organic modifier in gradient or in eluent B.
	Eluting power of organic modifier too weak.	Change to a less hydrophobic RPC medium or change organic modifier.

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Situation	Cause	Remedy
Sample elutes before gradient elution begins.	Sample components not sufficiently hydrophobic.	Add or increase concentration of ion pairing agent or use an organic modifier with less eluting power or change to a more hydrophobic RPC medium.
	pH unsuitable.	Adjust pH to enhance binding.
	Impurities bound to the column.	Clean and re-equilibrate column.
	Concentration of organic modifier in initial eluent too high.	Decrease organic modifier concentration.
Leading or very rounded peaks in chromatogram.	Column overloaded.	Decrease sample load and repeat.
	Column contaminated.	Clean using recommended procedures.
Peaks are tailing.	Column poorly packed.	Repack or use a prepacked column.
	Sample has precipitated on column.	Clean column, replace top filter or precolumn if possible.
Peaks are tailing or have a leading edge.	Column packing compressed.	Check column efficiency (see Appendix 2). Repack using a lower flow rate. Use prepacked columns.
Peaks too small.	Sample absorbs light.	If appropriate, check absorbance range on monitor. If satisfactory, use a different wavelength. Check UV cut offs of eluent components.
	Different assay conditions have been used before and after the chromatographic step.	Use same assay conditions for all assays.
	Excessive peak broadening.	Check column efficiency. Repack if necessary. Adjust eluent to suppress ionization of silanols or replace pre-column. Replace column if necessary.
Resolution less than expected.	Large mixing spaces at top of or after column.	Top up surface of medium if possible. Reduce all post-column volumes.
	Sub-optimal elution conditions e.g., gradient too steep, flow rate too high.	Alter elution conditions: use shallower or plateau gradient, reduce flow rate.
	Column poorly packed.	Check column efficiency (see Appendix 2). Repack if possible. Use prepacked columns.
	Column overloaded.	Clean and re-equilibrate column. Decrease sample load.
	Lipoproteins or protein aggregates have precipitated.	Clean and re-equilibrate column. Adjust eluent to maintain solubility.
	Column aging.	Adjust eluent to improve ion suppression or replace pre-column. Replace column if necessary.
	Mixed-mode retention due to surface silanols.	Lower pH to suppress silanols or replace column.
	Sample not filtered properly.	Clean the column, filter the sample and repeat.
	Poor selectivity.	Add or adjust ion-pairing agent. Change to another medium.

*continues on following page*

Situation	Cause	Remedy
Samples do not bind or elute as expected.	Sample has not been filtered.	Prepare fresh samples.
	Column equilibration incomplete.	Repeat or prolong the equilibration step until baseline is stable.
	Lipoproteins or protein aggregates have precipitated.	Clean and re-equilibrate column.
Medium/beads appears in eluent.	Incorrect eluent conditions compared to previous runs (possibly due to evaporation).	Check conditions required. Prepare fresh solutions.
	Column operated at too high pressure.	Do not exceed recommended operating pressure for medium or column.
Low recovery of activity, but normal recovery of mass.	Sample may be denatured or inactive in the eluent.	Determine the pH and organic solvent stability of the sample. Reduce separation time to limit exposure to organic conditions or change to a less powerful organic modifier or use a less hydrophobic RPC medium.
Sample yield lower than expected.	Enzyme separated from co-factor or similar.	Test by pooling aliquots from the fractions and repeating the assay.
	Sample may have been degraded by proteases or nucleases.	Add inhibitors or minimize separation time.
	Absorption to filter during sample preparation.	Use another type of filter.
Sample yield lower than expected.	Sample precipitated.	Decrease sample load or change eluent conditions.
	Basic sample components bound to medium by ionic interaction.	Increase pH or add/adjust ion-pairing agent concentration.
More activity 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.
Air bubbles in the bed.	Removal of inhibitors during separation.	
	Eluents not properly degassed.	Degas eluents thoroughly.
Air bubbles in the bed.	Column packed or stored at cool temperature and then warmed up.	Remove small bubbles by passing degassed eluent through the column. Take special care if eluents 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.
Negative peaks.	Refractive index effects.	Ensure sample is dissolved in initial eluent.
Unexpected peaks or spikes in chromatogram. Peaks appear on blank gradients.	Impurities in the eluent.	Use high-quality HPLC grade reagents.
Peaks appear on blank gradients.	Incomplete elution of previous sample.	Wash the column according to recommended methods.
Spikes in chromatogram.	Air bubble trapped in UV low cell.	Always use degassed eluents. Ensure the flow restrictor has an appropriate pressure range. Rinse the chromatography system with 100% methanol.
UV baseline rises with gradient.	Eluents A and B absorb differently at the same wavelength.	Balance eluent components or use a different wavelength.
	Impurities in the eluent.	Use high-quality HPLC grade reagents.

*continues on following page*

<b>Situation</b>	<b>Cause</b>	<b>Remedy</b>
Retention time of a component increases over time. Peak width increases over time.	Mixed-mode retention due to surface silanols.	Lower pH to suppress silanols or replace column.
Excessive baseline noise.	UV absorption by components in eluent.	Monitor at different wavelength or reduce concentration of UV absorbing component (usually the ion-pairing agent) or change organic modifier if this is absorbing.

# μRPC C2/C18: for high-resolution separation of complex samples

- Use μRPC C2/C18 for high-resolution, analytical separations as required for peptide mapping and LC-MS techniques.
- Run μRPC C2/C18 columns on ÄKTAdesign or other high-performance chromatography systems. Appendix 3 gives guidance on how to select the most suitable chromatography system.

μRPC C2/C18 is a porous, microparticulate silica-based medium (Figure 78) with a particle size of 3 μm and covalently bound C2/C18 alkyl ligands. The small particle size ensures high column efficiency to facilitate high resolution in analytical applications such as peptide mapping and LC-MS analyses. SOURCE 5RPC provides an alternative selectivity and the ability to work above pH 7.5 if required.

## Purification options



Fig 78. μRPC C2/C18 is available in prepacked columns.

Table 21. RPC medium based on μRPC.

Product	Dynamic binding capacity per column *	Recommended loading for optimal resolution	Efficiency (Nm-1)	Recommended working flow†	Maximum flow†	Maximum operating back pressure‡ (MPa/psi) 1 MPa= 10 bar
μRPC C2/C18 SC 2.1/10¶, 0.35 ml	1–2 mg peptide	~0.5 mg	>100 000	0.01–0.25 ml/min	0.25 ml/min	25/3625
μRPC C2/C18 ST 4.6/100, 1.6 ml	4–9 mg peptide	~2 mg	>100 000	0.1–1.2 ml/min	1.2 ml/min	35/5000
μRPC C2/C18 ST 1.0/150, 120 μl	0.3–0.6 mg peptide	~150 μg	–no information available	25–50 μl/min	62 μl/min	31/4500
μRPC C2/C18 ST 300μm/150, 11 μl	30–60 μg peptide	~15 μg	–no information available	5 μl/min	5 μl/min	31/4500

\* The dynamic binding capacity of an RPC medium is dependent on several parameters including the properties of the target molecule, the selectivity and pore size of the medium, eluent conditions and flow rate.

† Flow rate used will depend also on the pressure specification of the chromatography system, the solvent used and the column bed height.

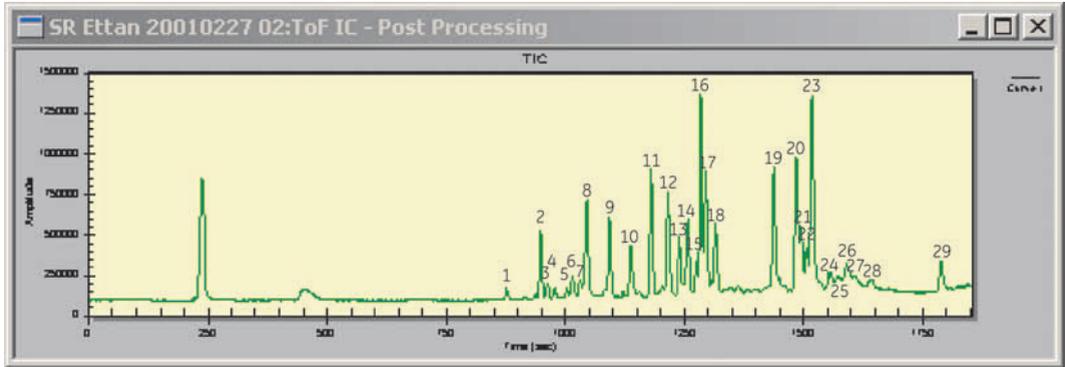
‡ Maximum operating back pressure refers to the pressure above which the medium begins to compress.

¶ Requires Column Holder 10 cm (17-1550-01) for attachment to ÄKTAdesign and other high-performance chromatography systems.

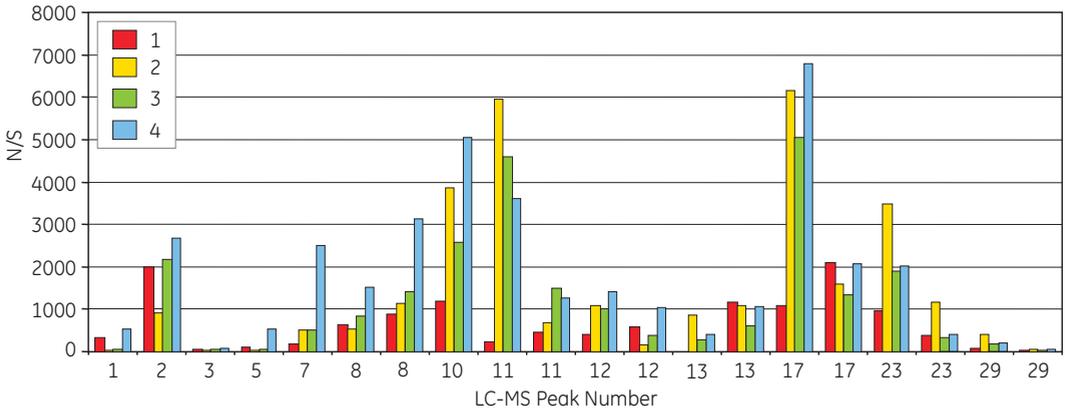
- For optimum resolution, match sample amount to column size.
- Use the longest column length to produce highest resolution.

## Separation examples

Figure 79 shows the use of a high-resolution RPC column for peptide mass fingerprinting. This example also illustrates how eluent conditions can significantly affect the sensitivity achieved during analysis by mass spectrometry.



MS S/N for Ions identified in Various LC-MS Peaks



Sample: Tryptic digest of  $\beta$ -lactoglobulin  
 Column:  $\mu$ RPC C2/C18 ST 1.0/150  
 Flow rate: 25  $\mu$ l/min  
 Gradient: 0–60% Eluent B over 30 min

### Eluent systems

- Eluent A: 0.065% TFA in water  
 Eluent B: 0.050% TFA in acetonitrile
- Eluent A: 1% formic acid in water  
 Eluent B: 1% formic acid in acetonitrile
- Eluent A: 0.1% formic acid in water  
 Eluent B: 0.1% formic acid in acetonitrile
- Eluent A: 1% acetic acid in water  
 Eluent B: 1% acetic acid in acetonitrile

**Fig 79.** Peptide mass fingerprinting: an ion chromatogram of a tryptic digest of  $\beta$ -lactoglobulin is shown.

## Performing a separation

Use these instructions as a basis from which to optimize a separation.

Eluent B should contain the same concentration of ion-pairing agent with a significantly higher content of organic modifier. For example:

### For samples with unknown properties or known to require acidic conditions

Eluent A: 0.1% TFA in 2% acetonitrile

Eluent B: 0.1% TFA in 80% acetonitrile

### For samples known to require basic conditions

Eluent A: 0.125% ammonium solution pH 10 in 2% acetonitrile

Eluent B: 80% acetonitrile in eluent A

## First-time use or after long-term storage

1. Flush out the storage solution with at least 5 column volumes of eluent A
2. Wash the column using a gradient of 5 column volumes from 0–100% eluent B (or continue at 100% eluent B until UV signal is stable).
3. Wash the column with a gradient of 2 column volumes from 100–0% eluent B.

## Separation by gradient elution

Flow: 0.5 ml/min, ( $\mu$ RPC C2/C18 ST 4.6/100)  
0.5 ml/min, ( $\mu$ RPC C2/C18 SC 2.1/10)  
50  $\mu$ l/min, ( $\mu$ RPC C2/C18 ST 1.0/150)  
5  $\mu$ l/min, ( $\mu$ RPC C2/C18 ST 300 $\mu$ m/150)  
Collect fractions throughout the separation.

1. Equilibrate the column with at least 10 column volumes of eluent A until the UV signal is stable.
2. Dissolve the sample in a small volume of eluent A. Filter or centrifuge to remove particulate matter if necessary. Apply to the column.
3. When the UV signal is stable, so that all unbound material has washed through the column, elute using a gradient of 10–20 column volumes from 0–100% eluent B.
4. Wash the column with at least 5 column volumes of 100% eluent B (or until UV signal is stable) to elute any remaining material.
5. Wash with a gradient of 2–3 column volumes from 100%–0% eluent B.
6. Re-equilibrate with 10 column volumes of eluent A or until UV signal is stable.



Do not exceed the maximum recommended flow for the medium.



Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 2.

## Cleaning

Correct preparation of samples and eluents, including filtration, the removal of any particulate matter and a final isocratic elution step in 100% eluent B, should keep most columns in good condition. However, reduced performance, reduced flow, loss of resolution, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove tightly bound, precipitated or denatured substances.

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

Contact time, organic solvent and pH are significant parameters for successful cleaning, and different protocols may have to be developed and used in combination according to the nature of the contaminants. Examples of cleaning protocols are as follows:

Eluent A: 0.1% TFA

Eluent B: 0.1% TFA in 80% acetonitrile

Flow: 0.25 ml/min, ( $\mu$ RPC C2/C18 ST 4.6/100)  
0.25 ml/min, ( $\mu$ RPC C2/C18 SC 2.1/10)  
25  $\mu$ l/min, ( $\mu$ RPC C2/C18 ST 1.0/150)  
5  $\mu$ l/min, ( $\mu$ RPC C2/C18 ST 300 $\mu$ m/150)

1. Equilibrate the column with at least 10 column volumes of eluent A until the UV signal is stable.
2. Wash using a gradient of 20–30 column volumes from 0–100% eluent B.
3. Wash the column with at least 10 column volumes of 100% eluent B.
4. Wash using a gradient of 20–30 column volumes from 100–0% eluent B.
5. Wash the column with at least 10 column volumes of eluent A.
6. Equilibrate the column in at least 10 column volumes in the eluent A that will be used for the separation if different the eluent used in step 5. Transfer between the two eluents should be performed using a 2–3 column volume gradient if the two eluents are significantly different.



Change to 0.1%TFA in 2-propanol for eluent B if column performance is not restored. Note that 2-propanol will increase back-pressure, and flow rates may need to be reduced.



For more rigorous cleaning:

1. Wash the column with 10 column volumes of 90% acetic acid.
2. Re-equilibrate immediately with 10 column volumes of eluent A. Do not leave the column in acetic acid!



Do not open the columns.

## Media characteristics

Composition: porous, microparticulate silica with covalently bonded C2/C18 alkyl chains.

Table 22. Characteristics of  $\mu$ RPC C2/C18 column.

Medium	Ligand	Pore size	Temperature stability for regular use	pH stability*	Mean particle size
$\mu$ RPC C2/C18	C2/C18 alkyl chains	120 Å	4°C to 70°C	Long term: 2–8 Short term: 2–8	3 $\mu$ m

\*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 GE Healthcare.

## Chemical stability

For daily use,  $\mu$ RPC is stable in:

- aqueous solutions pH 2–8 including trifluoroacetic acid (up to 0.3%), pentafluoropropionic acid (up to 0.3%), heptafluorobutyric acid (up to 0.3%), perchloric acid (up to 0.3%), formic acid (up to 60%), acetic acid (up to 90%), ammonium acetate (10–50 mM), phosphoric acid (10–50 mM), ionic and non-ionic detergents, guanidine hydrochloride.
- water-miscible organic solvents including methanol, ethanol, acetonitrile, 1-propanol, 2-propanol.
- ion-pairing agents such as trifluoroacetic acid, tributylphosphate, triethylammonium phosphate, tetrabutylammonium salts, hexylsulfate.



Note that ethanol, 1-propanol and 2-propanol will generate higher back-pressures, and flow rates may need to be reduced.



Avoid oxidizing agents and solutions <pH 2 and >pH 8.

## Storage

For column storage, wash with at least 5 column volumes of 70% methanol or 70–80% acetonitrile. Solutions should be free from any other additives such as TFA. Store at 4°C to 30°C. Ensure that the column is sealed well to avoid drying out.



Do not store in aqueous solutions.

## SOURCE: rapid separation with high resolution and easy scale-up

- Use SOURCE RPC media for purification and analysis of proteins, peptides and oligonucleotides.
- Use SOURCE RPC as an alternative to silica-based matrices when separations must be performed above pH 8 or when requiring different selectivity or higher capacity.
- Use SOURCE 30RPC for polishing stages of industrial processes requiring high flow rate and low back pressure.
- Use SOURCE 15RPC for polishing steps in laboratory or large-scale applications that require highest resolution and fast separation (flows up to 1800 cm/h).
- Use SOURCE 5RPC for highest-resolution, analytical separations as required for peptide mapping and LC-MS techniques.
- Run SOURCE RPC columns on systems such as ÄKTAdesign, FPLC System and HPLC. Appendix 3 gives guidance on how to select the most suitable ÄKTAdesign system.

SOURCE media are based on a matrix made from monodispersed, rigid, polystyrene/divinyl benzene (Figure 80). The media demonstrate extreme chemical and physical stability and, unlike silica-based media, can be used at extremes of pH. A range of particle sizes (30  $\mu\text{m}$ , 15  $\mu\text{m}$  or 5  $\mu\text{m}$ ) enables SOURCE RPC to be used from large-scale purification through to high-resolution analysis. The uniformity and stability of SOURCE particles ensures high flow rates at low back pressure. Such high flow rates are useful for speeding up cleaning and re-equilibration steps. Flow rates are more likely to be limited by the equipment available and the eluents used rather than the physical properties of the media.

Separation methods can be easily scaled up from prepacked columns such as RESOURCE and SOURCE 15RPC ST 4.6/100 through to large-scale columns such as FineLINE.



**Fig 80.** Scanning electron micrograph of SOURCE 15RPC shows the uniform size distribution.

## Purification options

**Table 23.** RPC media based on SOURCE matrices are available in prepacked columns and as media packs.

Product	Dynamic binding capacity per column or per ml medium*	Recommended working flow <sup>†</sup>	Maximum flow <sup>†</sup>	Maximum operating back pressure <sup>‡</sup> (MPa/psi) 1 MPa=10 bar
SOURCE 5RPC ST 4.6/150, 2.5 ml	~ 80 mg bacitracin	1 ml/min	n.d.	40/5800
SOURCE 15RPC ST 4.6/100, 1.7 ml	~ 17 mg BSA ~ 85 mg insulin	0.5–2.5 ml/min	5 ml/min	4/580
RESOURCE RPC, 1 ml	~ 10 mg BSA ~ 30 mg bacitracin ~ 50 mg insulin	1–5 ml/min	10 ml/min	4/580
RESOURCE RPC, 3 ml	~ 10 mg BSA ~ 30 mg bacitracin ~ 50 mg insulin	1–5 ml/min	10 ml/min	4/580
SOURCE 15RPC	~ 10 mg BSA ~ 30 mg bacitracin ~ 50 mg insulin	150–900 cm/h	1800 cm/h	4/580
SOURCE 30RPC	~ 14 mg BSA ~ 23 mg bacitracin ~ 72 mg insulin	100–1000 cm/h	2000 cm/h	1.5/220

\*Determined at 10% breakthrough by frontal analysis. The dynamic binding capacity of an RPC medium is dependent on several parameters including the properties of the target molecule, the selectivity and pore size of the medium, eluent conditions and flow rate. Capacities given here were determined using 0.1% TFA at a flow rate of 300 cm/h.

<sup>†</sup> See Appendix 3 to convert linear flow (cm/hour) to volumetric flow rates (ml/min) and vice versa. Flow rate used will depend also on the pressure specification of the chromatography system, the eluents used and the column bed height.

<sup>‡</sup> Maximum operating back pressure refers to the pressure above which the medium begins to compress.

-  In RPC many parameters, such as properties of the protein, flow rates and selectivity of the medium play a significant role in the determination of binding capacity. Final capacity must be determined by experimentation.
-  Use RESOURCE RPC 1 ml for rapid screening and method development. Transfer RESOURCE RPC 3 ml column for higher resolution and method development on a 10 cm bed height.
-  Use SOURCE 5RPC columns for highest resolution as required, for example, for peptide mapping.

**Table 24.** Packing volumes and bed heights for SOURCE media for RPC.

	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

Select a production column such as FineLINE for larger volumes.

## Purification examples

### Capture and purification of a synthetic peptide

Figure 81 shows the purification and mass spectrometric analysis of a crude mixture of synthetic amyloid- $\beta$  1–42. This peptide is generated from a large transmembrane precursor protein by proteolytic cleavage and may polymerize to form rigid, linear, non-branching fibrils building up to the senile plaques that represent one of the characteristics of Alzheimer's disease. Amyloid- $\beta$  1–42 was synthesized for use in studies of the mechanisms underlying fibril formation. However, while readily soluble under alkaline conditions, the peptide is virtually insoluble under conditions so that traditional purification by RPC at low pH cannot be used. The wide pH stability of SOURCE media is therefore well suited for such a purification challenge.

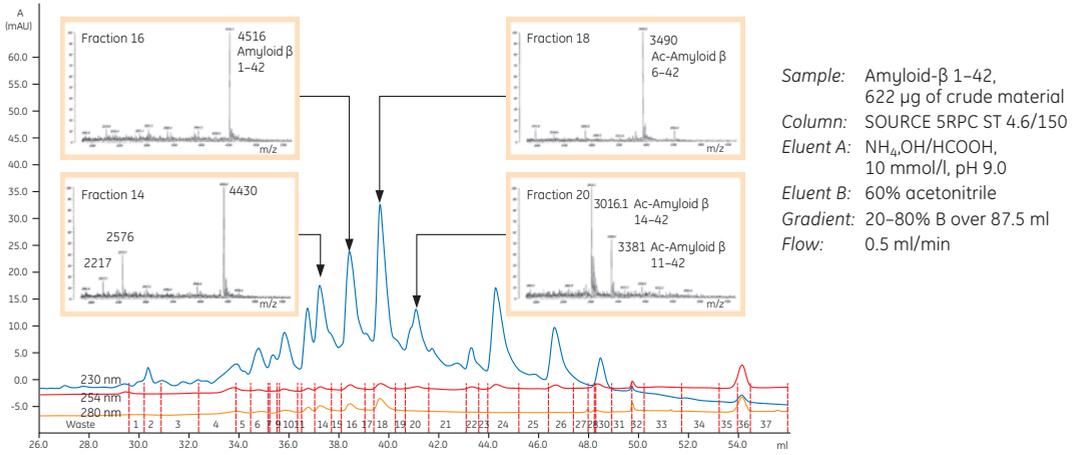


Fig 81. Purification and MS analysis of a synthetic peptide.

### Purification at high pH

Figure 82 shows the successful purification of beta-lipotropin fragment 1–10 (MW 950, Sigma) achieved by using high pH conditions (pH 12), possible only on polymer-based media. At pH 2 the contaminants are eluted in the beta-lipotropin peak.

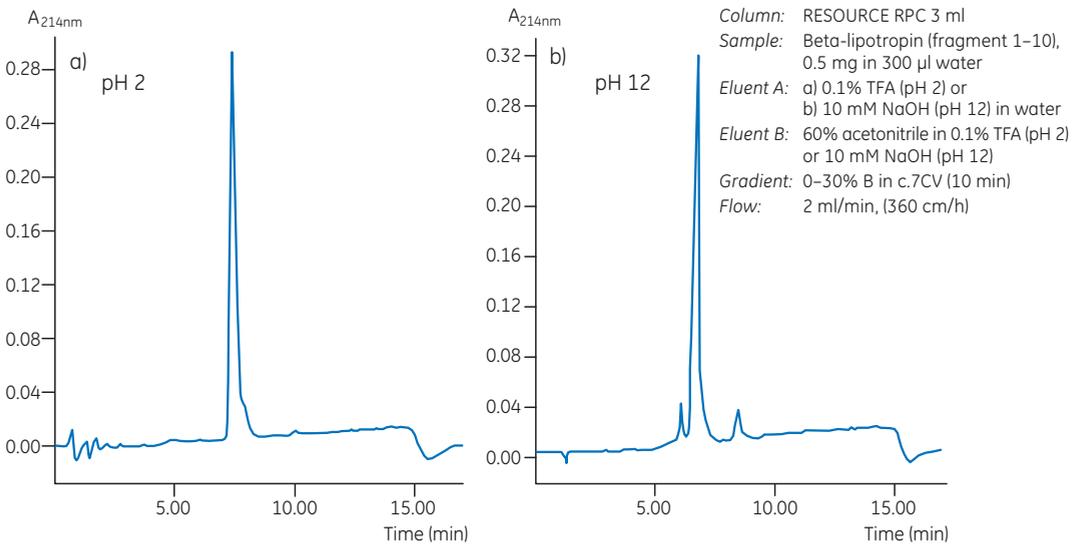
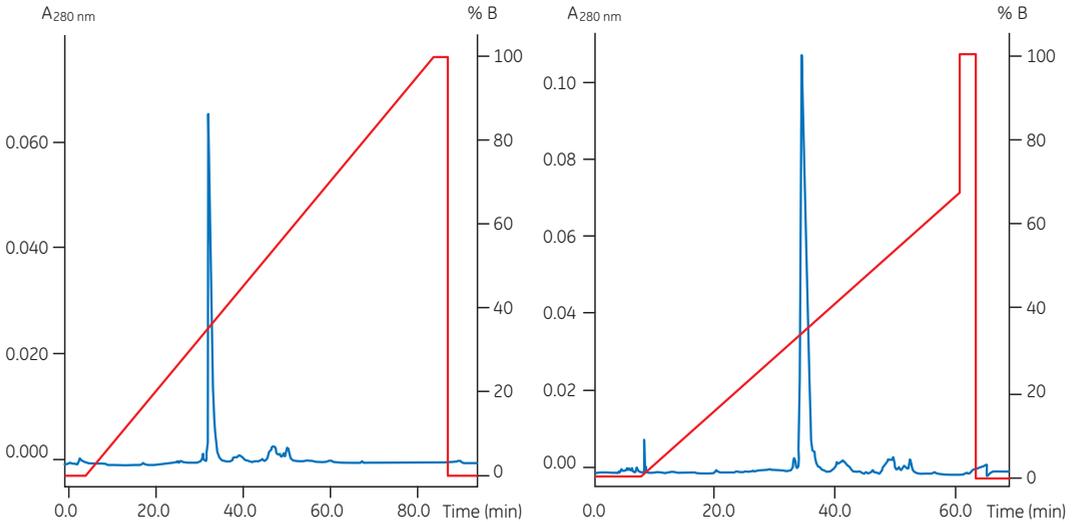


Fig 82. Purification at high pH.

## Polishing step and scale-up

Figure 83 shows a high-resolution preparative separation of recombinant human epidermal growth factor (EGF) expressed in yeast. Most impurities have been removed by an initial hydrophobic interaction chromatography step on Phenyl Sepharose 6 Fast Flow (high sub) followed by ion exchange on Q Sepharose High Performance. The final polishing step on SOURCE 15RPC was optimized on a RESOURCE 3 ml column before scale-up to a pilot-scale column.



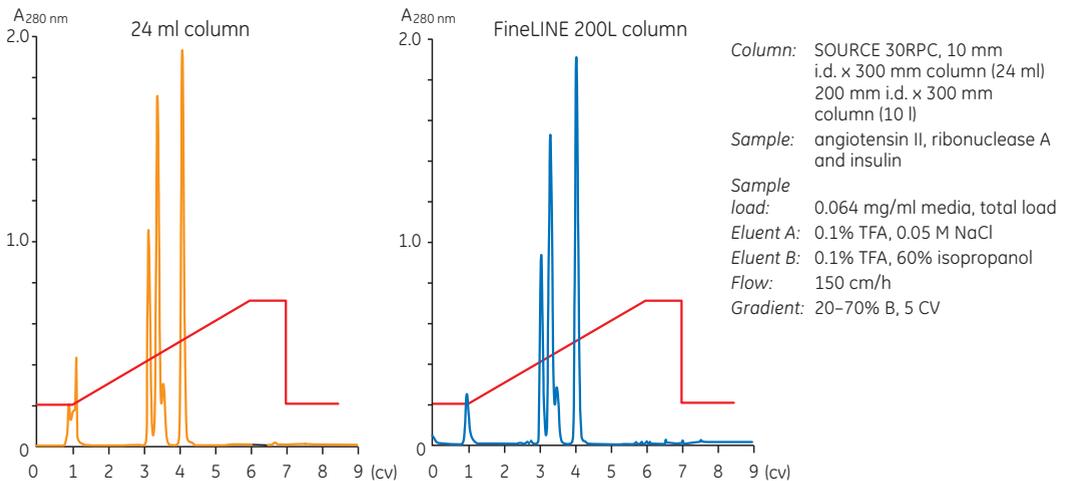
*Column:* RESOURCE RPC 3 ml  
*Sample:* 2.14 ml EGF pooled from separation on Q Sepharose High Performance  
*Eluent A:* 0.05% TFA, 5% acetonitrile  
*Eluent B:* 0.05% TFA, 80% acetonitrile  
*Gradient:* 0–100% B in 40 CV  
*Flow:* 1.6 ml/min, (300 cm/h)

*Column:* SOURCE 15RPC, 35 x 100 mm  
*Sample:* 62.5 ml EGF pool after Q Sepharose High Performance  
*Load:* 0.1 mg/ml media, 10 mg total load  
*Eluent A:* 0.05% TFA, 5% acetonitrile  
*Eluent B:* 0.05% TFA, 80% acetonitrile  
*Gradient:* 0–100% B in 40 CV  
*Flow:* 50 ml/min, (300 cm/h)

**Fig 83.** Preparative separation of a recombinant protein.

## Scaling up

Figure 84 shows the excellent scalability of SOURCE 30RPC. The medium is easily packed and maintains its performance during scale-up. In this example, the separation of a model protein mixture was scaled up by a factor of 400 from a 24 ml column to a 10 liter FineLINE 200L column.



*Column:* SOURCE 30RPC, 10 mm i.d. x 300 mm column (24 ml) 200 mm i.d. x 300 mm column (10 l)  
*Sample:* angiotensin II, ribonuclease A and insulin  
*Sample load:* 0.064 mg/ml media, total load  
*Eluent A:* 0.1% TFA, 0.05 M NaCl  
*Eluent B:* 0.1% TFA, 60% isopropanol  
*Flow:* 150 cm/h  
*Gradient:* 20–70% B, 5 CV

**Fig 84.** Scalability of SOURCE 30RPC.

## Performing a separation

Use the instructions given here as a basis from which to optimize a separation.

### For samples with unknown properties or known to require acidic conditions

Eluent A: 0.1% TFA in 2% acetonitrile

Eluent B: 0.1% TFA in 80% acetonitrile

### For samples known to require basic conditions

Eluent A: 0.125% ammonium solution pH 10 in 2% acetonitrile

Eluent B: 80% acetonitrile in eluent A

## First-time use or after long-term storage

1. Flush out the storage solution with at least 5 column volumes of eluent A
2. Wash the column using a gradient of 5 column volumes from 0–100% eluent B (or continue at 100% eluent B until UV signal is stable).
3. Wash the column with a gradient of 2 column volumes from 100–0% eluent B.

## Separation by gradient elution

Flow: 0.2 ml/min (SOURCE 5RPC ST 2.1/150)

1 ml/min, (SOURCE 5RPC ST 4.6/150)

2 ml/min, (SOURCE 15RPC ST 4.6/100)

1–5 ml/min, (RESOURCE 1 ml)

1–5 ml/min, (RESOURCE 3 ml) or, for larger columns, 200 cm/h (SOURCE 15RPC) and

100–1000 cm/h, (SOURCE 30RPC)

Collect fractions throughout the separation.

1. Equilibrate the column with at least 10 column volumes of eluent A until the UV signal is stable.
2. Dissolve the sample in a small volume of eluent A. Filter or centrifuge to remove particulate matter if necessary. Apply to the column.
3. When the UV signal is stable, that is, when all unbound material has washed through the column, elute using a gradient of 10–20 column volumes from 0–100% eluent B.
4. Wash the column with at least 5 column volumes of 100% eluent B (or until UV signal is stable) to elute any remaining material.
5. Wash with a gradient of 2–3 column volumes from 100%–0% eluent B.
6. Re-equilibrate with 10 column volumes of eluent A or until UV signal is stable.



Do not exceed the maximum recommended flow for the medium.



Check column performance regularly by determining column efficiency and peak symmetry. See Appendix 2.

## Cleaning

Correct preparation of samples and eluents, including filtration, the removal of any particulate matter and a final wash step in 100% eluent B, should keep most columns in good condition. However, reduced performance, reduced flow, increasing back pressure or complete blockage are all indications that the medium needs to be cleaned using more stringent procedures in order to remove tightly bound, precipitated or denatured substances.

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

Contact time, organic solvent and pH are significant parameters for successful cleaning, and different protocols may have to be developed and used in combination according to the nature of the contaminants. Examples of cleaning protocols are as follows:

Eluent A: 0.1% TFA

Eluent B: 0.1% TFA in 80% acetonitrile

Note: acetonitrile cannot be used for bioprocess applications, 2-propanol is an accepted alternative.

Flow: 0.5 ml/min, (SOURCE 5RPC ST 4.6/150)

0.5 ml/min, (SOURCE 15RPC ST 4.6/100)

1.0 ml/min, (RESOURCE 1 ml)

1.0 ml/min, (RESOURCE 3 ml) or, for larger columns, 100 cm/h (SOURCE 15RPC) and 100 cm/h, (SOURCE 30RPC)

1. Equilibrate the column with at least 10 column volumes of eluent A until the UV signal is stable.
2. Wash using a gradient of 20–30 column volumes from 0–100% eluent B.
3. Wash the column with at least 10 column volumes of 100% eluent B.
4. Wash using a gradient of 20–30 column volumes from 100–0% eluent B.
5. Wash the column with at least 10 column volumes of eluent A.
6. Equilibrate the column in at least 10 column volumes in the eluent A that will be used for the separation if different the eluent used in step 5. Transfer between the two eluents should be performed using a 2–3 column volume gradient if the two eluents are significantly different.

- Change to 0.1%TFA in 2-propanol for eluent B if column performance is not restored. Note that 2-propanol will increase back-pressure, and flow rates may need to be reduced.

- For removal of contaminants known to be acid- or alkali-soluble the following eluents can be used, following the same procedure as outlined above:

### Removal of acid-soluble contaminants

Eluent A: 90% acetic acid

Eluent B: 80% acetonitrile or 50% 2-propanol

### Removal of alkali-soluble contaminants

Eluent A: 0.5 M NaOH

Eluent B: 50% acetonitrile or 50% 2-propanol

-  If neither of the protocols for acid- or alkali-soluble contaminants is successful, wash the column in 5–10 column volumes of 6 M guanidine hydrochloride.
-  SOURCE RPC media can be cleaned using aggressive chemical agents since the polystyrene-based matrix is extremely stable. Sodium hydroxide is a very effective cleaning agent and SOURCE RPC can be equilibrated with several column volumes of 0.5–1 M NaOH for cleaning. The ability to use such a strong cleaning agent is a major advantage of using SOURCE RPC for large-scale separations. At production scale, other cleaning protocols may be applied in order to fulfill regulatory requirements.

## Media characteristics

Composition: rigid, monodisperse, polystyrene/divinyl benzene particles (5 µm, 15 µm or 30 µm) with an optimized pore size distribution.

**Table 25.** Media characteristics for SOURCE RPC media.

Product	Temperature stability for regular use	pH stability*	Mean particle size
SOURCE 5RPC	4°C to 60°C	Long term: 1–12 Short term: 1–14	5 µm
SOURCE 15RPC	4°C to 40°C	Long term: 1–12 Short term: 1–14	15 µm
SOURCE 30RPC	4° C to 40°C	Long term: 1–12 Short term: 1–14	30 µm

\*Long-term pH stability refers to the pH interval where the medium is stable over a long period of time without adverse side effects on the chromatography performance.

Short-term pH stability refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.

All ranges are estimates based on the experience and knowledge within GE Healthcare.

## Chemical stability

For daily use, SOURCE RPC media are stable in:

- all commonly used aqueous buffers: 1 M HCl, 1 M NaOH, 1 M HCl/90% methanol, 90% acetic acid, 0.45 M NaOH/40% 2-propanol, 6 M guanidine hydrochloride, 1-propanol, 20% ethanol, acetone.
- aqueous solutions pH 1–12 including trifluoroacetic acid (up to 0.3%), pentafluoropropionic acid (up to 0.3%), heptafluorobutyric acid (up to 0.3%), perchloric acid (up to 0.3%), formic acid (up to 0.3%), acetic acid (up to 60%), ammonium acetate (10–50 mM), phosphoric acid (10–50 mM).
- water-miscible organic solvents including methanol, ethanol, acetonitrile, 1-propanol, 2-propanol.
- ion-pairing agents such as trifluoroacetic acid, tributylphosphate, triethylammonium phosphate, tetrabutylammonium salts, hexylsulfate.



Note that ethanol, 1-propanol and 2-propanol will increase back-pressure, and flow rates may need to be reduced.



Avoid detergents, oxidizing agents and solutions <pH 1 and >pH 12.

## Storage

SOURCE 5RPC columns: wash with 70% acetonitrile. SOURCE 15RPC and SOURCE 30RPC columns: wash with at least 10 column volumes distilled water, equilibrate with at least 10 column volumes 20% ethanol or 70% acetonitrile. Note that columns used for bioprocess applications can only be stored in 20% ethanol.

Store at 4°C to 30°C. Ensure that the column is sealed well to avoid drying out. Store unused media at 4°C to 8°C in 20% ethanol. Do not freeze.

# Reversed Phase Chromatography and CIPP

RPC can give extremely high resolution of complex mixtures for analytical purposes and, when full recovery of activity and tertiary structure are not essential, RPC has the potential for high-resolution purification as well as being an effective option for low-resolution desalting steps. In a purification strategy, RPC is best suited as a polishing step when high resolution of similar components is required and the majority of contaminants have been removed. Refer to Chapter 2 for details on CIPP, a strategic approach to purification.

## ***RPC as a capture step***

RPC is a suitable method for the capture of synthetic peptides and synthetic oligonucleotides, but less suitable for capture of peptides and proteins from biological sources when lipids and other highly hydrophobic components that bind strongly, reduce the dynamic capacity for the molecule of interest, and can be difficult to remove from the column. IEX and HIC on larger particle sizes above 90  $\mu\text{m}$  are more appropriate.

## ***RPC for intermediate purification***

RPC can be suitable for intermediate purification when the ability to resolve similar components is of increased importance and the focus is on resolution and recovery in order to separate target molecule from most of the bulk impurities such as other proteins, peptides, nucleic acids, endotoxins and viruses.

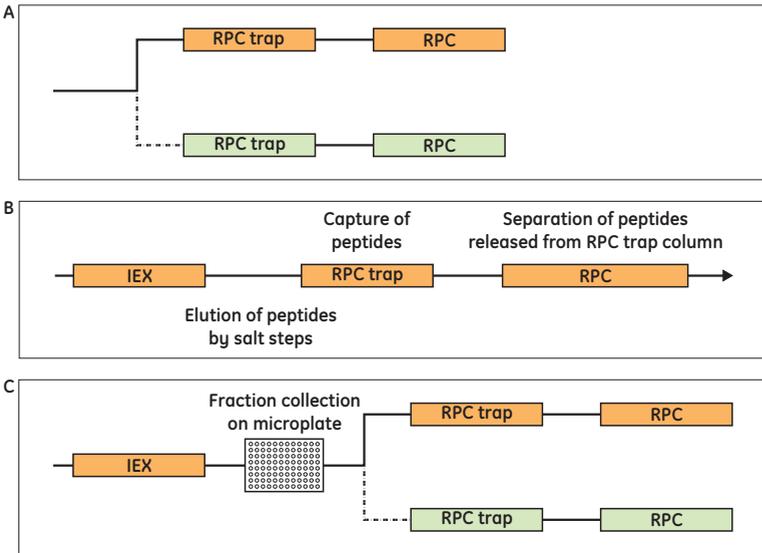
## ***RPC as a polishing step***

The polishing step is used to remove trace contaminants and impurities, leaving the purified biomolecule in a form suitable for its intended use. The goal is to achieve 100% purity in less than two steps and with high recovery. The excellent resolving power of RPC makes it the method of choice when dealing with slight structural variants (dimers, oligomers, aggregates, oxidized amino acids, protease-clipped molecules, deaminated amino acid residues) and other micro-heterogeneity. An example of RPC used as a polishing step is shown in Figure 83.

## **RPC in identification and characterization — multidimensional liquid chromatography (MDLC)**

The CIPP strategy is well suited for purification of a target protein or peptide. However, chromatography techniques are also successfully combined to achieve identification and characterization of previously unknown proteins and peptides often present in complex mixtures. Multidimensional liquid chromatography (MDLC) followed by mass spectrometry is a fast and accurate solution for protein identification and characterization in proteomics, and RPC is one of the key techniques used in the process. As peptide mixtures become more complex, more effort is needed to achieve optimal separation of the individual peptides. Eventually, a point is reached where all the peptides cannot be resolved with RPC alone. A single chromatographic peak may contain more than one peptide. Mass spectrometry can detect many peptides in each scan, even though they have not been physically separated during chromatography. However, in some cases, for very complex samples such as plasma digests and tissue lysates, RPC is severely limited in the extent to which it can separate the peptides. There may be so many peptides in the chromatographic peak that the mass spectrometer cannot detect them all. Potentially valuable information about the sample may be lost.

Multidimensional liquid chromatography overcomes the limitations of using one-dimensional chromatographic separations for complex samples by employing two or more chromatographic separation techniques sequentially.



**Fig 85.** Schematic representation of the different methods: (A) high throughput, (B) online salt step injection, (C) offline fractionation.

Figure 85 illustrates examples of how techniques are combined in order to achieve greater separation of complex peptide mixtures. IEX and RPC columns can be used in a single automated method or fractions can be collected offline after the first dimension IEX separation before continuing with RPC.

With online and offline MDLC the principle is the same: a sample is loaded onto a strong cation exchange column and divided into a number of fractions by elution with salt steps of increasing molarity or a linear salt gradient. These salt fractions are individually captured and desalted on trap columns, then eluted on RPC columns with an acetonitrile gradient and detected by mass spectrometry. The inherent complexity of a multi-protein sample is reduced by fractionation of the sample before it reaches the mass spectrometer. As a result, fewer peptides are presented to the mass spectrometer at the same time than would be the case for one-dimensional chromatography. This leads to improved probability of identifying the peptides.



# Appendix 1

## Sample preparation

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

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

### **Sample stability**

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

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



It is advisable to perform stability tests before beginning to develop a purification protocol.

The list below may be used as a basis for such testing:

- Test pH stability in steps of one pH unit between pH 2 and pH 9.
- Test salt stability with 0–2 M NaCl and 0–2 M  $(\text{NH}_4)_2\text{SO}_4$  in steps of 0.5 M.
- Test the stability toward acetonitrile and methanol in 10% steps between 0% and 50%.
- Test the temperature stability in 10°C steps from 4°C to 40°C.
- Test the stability and occurrence of proteolytic activity by leaving an aliquot of the sample at room temperature overnight. Centrifuge each sample and measure activity and UV absorbance at 280 nm in the supernatant.

## Sample clarification

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

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

### Centrifugation

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

- For small sample volumes or proteins that adsorb to filters, centrifuge at 10 000 ×g for 15 min.
- For cell lysates, centrifuge at 40 000–50 000 ×g for 30 min.
- Serum samples can be filtered through glass wool after centrifugation to remove any remaining lipids.

### Filtration

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

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

**Table 26.** Filter pore size selection based on bead size.

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



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

### Desalting

Desalting columns are suitable for any sample volume and will rapidly remove low molecular weight contaminants in a single step at the same time as transferring the sample into the correct buffer conditions. Centrifugation and/or filtration of the sample before desalting is still recommended. Detailed procedures for buffer exchange and desalting are given on page 136.

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

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

 Remove salts from proteins with molecular weight  $M_r > 5000$ .

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

# Specific sample preparation steps

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

## Fractional precipitation

Fractional precipitation is frequently used at laboratory scale to remove gross impurities from small sample volumes, and occasionally used in small-scale commercial production. Precipitation techniques separate fractions by the principle of differential solubility. Because protein species differ in their degree of hydrophobicity, increased salt concentrations can enhance hydrophobic interactions between the proteins and cause precipitation. Fractional precipitation can be applied to remove gross impurities in three different ways, as shown in Figure 86.

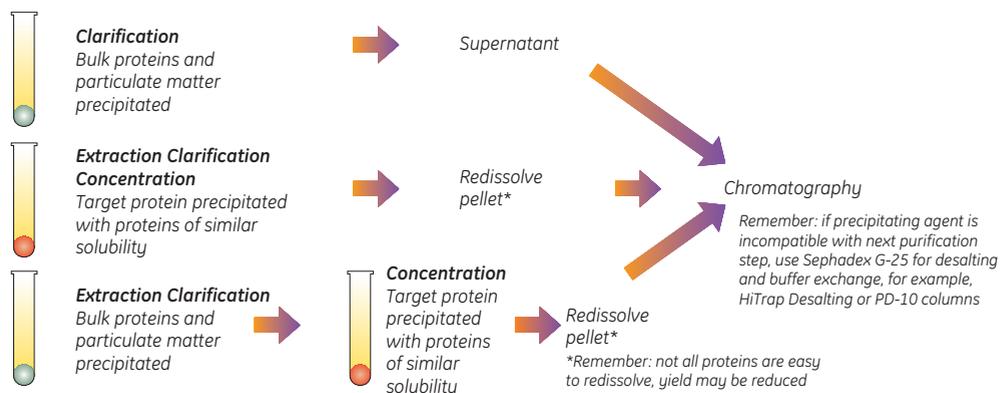


Fig 86. Three ways to use precipitation.

Examples of precipitation agents are reviewed in Table 27. The most common precipitation method using ammonium sulfate is described in more detail.

Table 27. Examples of precipitation techniques.

Precipitation agent	Typical conditions for use	Sample type	Comment
Ammonium sulfate	As described below.	> 1 mg/ml proteins especially immunoglobulins.	Stabilizes proteins, no denaturation; supernatant can go directly to HIC. Helps to reduce lipid content.
Dextran sulfate	Add 0.04 ml 10% dextran sulfate and 1 ml 1 M CaCl <sub>2</sub> per ml sample, mix 15 min, centrifuge 10 000 ×g, discard pellet.	Samples with high levels of lipoprotein e.g., ascites.	Precipitates lipoprotein.
Polyvinylpyrrolidone	Add 3% (w/v), stir 4 h, centrifuge 17 000 ×g, discard pellet.	Samples with high levels of lipoprotein e.g., ascites.	Alternative to dextran sulfate.
Polyethylene glycol (PEG, M <sub>r</sub> > 4000)	Up to 20% w/vol	Plasma proteins.	No denaturation, supernatant goes directly to IEX or AC, complete removal may be difficult. Stabilizes proteins.

continues on following page

**Table 27.** Examples of precipitation techniques (continued).

Precipitation agent	Typical conditions for use	Sample type	Comment
Acetone (cold)	Up to 80% vol/vol at 0°C. Collect pellet after centrifugation at full speed in an Eppendorf™ centrifuge.		May denature protein irreversibly. Useful for peptide precipitation or concentration of sample for electrophoresis.
Polyethyleneimine	0.1% w/v		Precipitates aggregated nucleoproteins.
Protamine sulfate	1% w/v		Precipitates aggregated nucleoproteins.
Streptomycin sulfate	1% w/v		Precipitation of nucleic acids.
Caprylic acid	(X/15) g where X = volume of sample.	Antibody concentration should be > 1 mg/ml.	Precipitates bulk of proteins from sera or ascites, leaving immunoglobulins in solution.

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

### Ammonium sulfate precipitation



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



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



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

Solutions needed for precipitation:

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

1 M Tris-HCl, pH 8.0.

Buffer for first purification step.

1. Filter (0.45 µm) or centrifuge the sample (10 000 ×g at 4°C).
2. Add 1 part 1 M Tris-HCl, pH 8.0 to 10 parts sample volume to maintain pH.
3. Stir gently. Add ammonium sulfate solution, drop by drop. Add up to 50% saturation\*. Stir for 1 hour.
4. Centrifuge 20 min at 10 000 ×g.
5. Remove supernatant. Wash the pellet twice by resuspension in an equal volume of ammonium sulfate solution of the same concentration (i.e., a solution that will not redissolve the precipitated protein or cause further precipitation). Centrifuge again.
6. Dissolve pellet in a small volume of the buffer to be used for the next step.
7. Ammonium sulfate is removed during clarification/buffer exchange steps with Sephadex™ G-25, using desalting columns (see page 136).

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

The quantity of ammonium sulfate required to reach a given degree of saturation varies according to temperature. Table 28 shows the quantities required at 20°C.

**Table 28.** Quantities of ammonium sulfate required to reach given degrees of saturation at 20°C.

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

### Resolubilization of protein precipitates

Many proteins are easily resolubilized in a small amount of the buffer to be used in the next chromatographic step. However, a denaturing agent may be required for less soluble proteins. Specific conditions will depend upon the specific protein. These agents must always be removed to allow complete refolding of the protein and to maximize recovery of mass and activity. A chromatographic step often removes a denaturant during purification. Table 29 gives examples of common denaturing agents.

**Table 29.**

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

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

## Buffer exchange and desalting

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

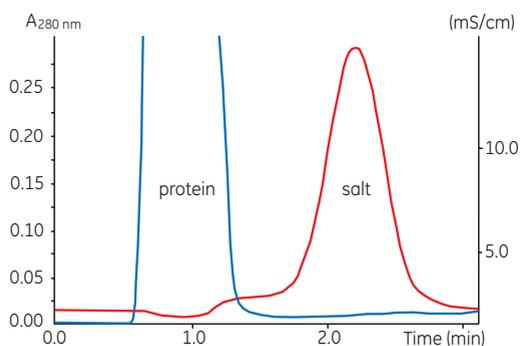
In a fast, single step, the sample is desalted, transferred into a new buffer and low molecular weight materials are removed.

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

Sample volumes up to 30% of the total volume of the desalting column can be processed. Sample concentration does not influence the separation as long as the concentration of proteins does not exceed 70 mg/ml when using normal aqueous buffers. The sample should be fully dissolved. Centrifuge or filter to remove particulate material.

- For small sample volumes it may be possible to dilute the sample with the start buffer that is to be used for chromatographic purification, but cell debris and particulate matter must still be removed.
- To prevent possible ionic interactions the presence of a low salt concentration (25 mM NaCl) is recommended during desalting and in the final sample buffer.
- Volatile buffers such as 100 mM ammonium acetate or 100 mM ammonium hydrogen carbonate can be used if it is necessary to avoid the presence of NaCl.

Figure 87 shows a typical buffer exchange and desalting separation. The process can be monitored by following changes in UV absorption and conductivity.



**Fig 87.** Buffer exchange of mouse plasma (10 ml) on HiPrep 26/10 Desalting.

For laboratory-scale operations, Table 30 shows a selection guide for prepacked, ready to use desalting and buffer exchange columns.

**Table 30.** Selection guide for desalting and buffer exchange.

Column	Sample volume	Sample elution volume
MicroSpin™ G-25	0.1–0.15 ml	0.1–0.15 ml
PD-10 (gravity column)	1.5–2.5 ml	2.5–3.5 ml
HiTrap Desalting 5 ml	0.25–1.5 ml	1.0–2.0 ml
HiPrep 26/10 Desalting	2.5–15 ml	7.5–20 ml

To desalt larger sample volumes:

- connect up to 5 HiTrap Desalting 5 ml columns in series to increase the sample volume capacity, for example, 2 columns: sample volume 3 ml, 5 columns: sample volume 7.5 ml.
- connect up to 4 HiPrep 26/10 Desalting columns in series to increase the sample volume capacity, for example, 2 columns: sample volume 30 ml, 4 columns: sample volume 60 ml. Even with 4 columns in series, the sample can be processed in 20 to 30 min, at room temperature, in aqueous buffers.

Instructions are supplied with each column. Desalting and buffer exchange can take less than 5 min per sample with greater than 95% recovery for most proteins.

#### Alternative 1: Manual desalting with HiTrap Desalting 5 ml using a syringe

1. Fill the syringe with buffer. Remove the stop plug. To avoid introducing air into the column, connect the column "drop to drop" to the syringe (via the adapter provided).
2. Remove the snap-off end at the column outlet.
3. Wash the column with 25 ml buffer at 5 ml/min to remove completely the 20% ethanol (supplied as storage buffer). If air is trapped in the column, wash with degassed buffer until the air disappears. Air bubbles introduced onto the column by accident during sample application do not influence the separation.
4. Apply the sample using a 2–5 ml syringe at a flow rate between 1–10 ml/min. Discard the liquid eluted from the column.
5. If the sample volume is less than 1.5 ml, change to buffer and proceed with the injection until a total of 1.5 ml has been eluted. Discard the eluted liquid.
6. Elute the protein with the appropriate volume selected from Table 31.

Collect the desalted protein in the volume indicated.

*Note: 5 ml/min corresponds to approximately 120 drops/min when using a HiTrap 5 ml column. A simple peristaltic pump can also be used to apply sample and buffers.*

 The maximum recommended sample volume is 1.5 ml. See Table 31 for the effect of reducing the sample volume applied to the column.

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

**Table 31.** Recommended sample and elution volumes using a syringe or Multipipette™.

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

## Alternative 2: Simple desalting with ÄKTApri<sup>™</sup> Plus

ÄKTApri<sup>™</sup> Plus contains pre-programmed templates for individual HiTrap Desalting 5 ml and HiPrep 26/10 Desalting columns.

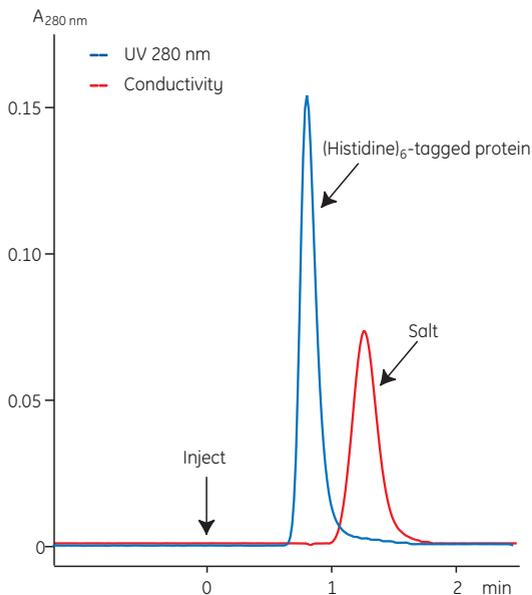


### Buffer Preparation

Prepare at least 500 ml of the required buffer.

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

Figure 88 shows a typical result obtained from ÄKTApri Plus. The UV (protein) and conductivity (salt) traces enable pooling of the desalted fractions.



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

**Fig 88.** Desalting of a (Histidine)<sub>6</sub>-tagged fusion protein on ÄKTApri Plus.

## Removal of lipoproteins

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

-  Centrifuge samples to avoid the risk of non-specific binding of the target molecule to a filter.
-  Samples such as serum can be filtered through glass wool to remove remaining lipids.

## Removal of phenol red

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

-  Use a desalting column to simultaneously remove phenol red (a low molecular weight molecule) and transfer sample to the correct buffer conditions for further purification, as described under Buffer exchange and desalting.

## Removal of low molecular weight contaminants

-  If samples contain a high level of low molecular weight contaminants, use a desalting column before the first chromatographic purification step, as described under Buffer exchange and desalting.



## Appendix 2

# Column packing and preparation

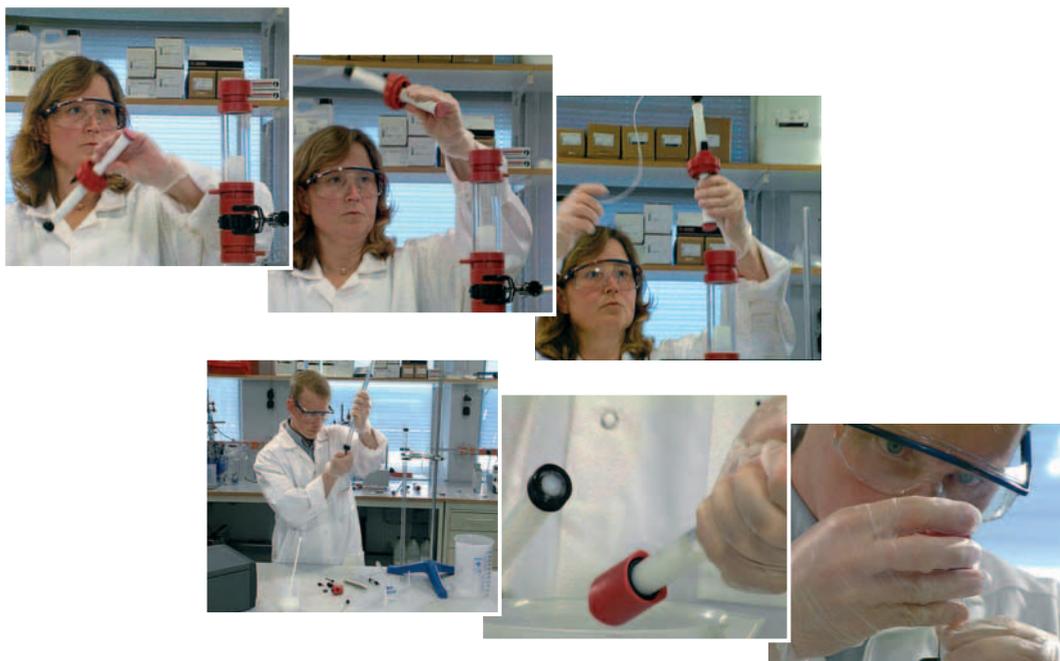
Prepacked columns from GE Healthcare will ensure reproducible results and the highest performance.

- Use small prepacked columns for media screening and method optimization to increase efficiency in method development, for example, HiTrap HIC Selection Kit, RESOURCE HIC Test Kit and RESOURCE RPC columns.

Efficient column packing is essential for a good separation, especially when using gradient elution. A poorly packed column gives rise to poor and uneven flow, peak broadening, and loss of resolution. If column packing is required, the following guidelines will apply at all scales of operation:

- When using a binding technique, use short, wide columns (typically 5–15 cm bed height) for rapid purification, even with low linear flow.
- The amount of medium required will depend on the binding capacity of the medium and the amount of sample. The binding capacity of a medium is always significantly influenced by the hydrophobic nature of the sample as well as the medium itself and must be determined empirically. Estimate the amount of medium required to bind the sample of interest and use five times this amount to pack the column. The amount of medium required can be reduced if resolution is satisfactory.
- Once separation parameters have been determined, scale up a purification by increasing the diameter of the column to increase column volume. Avoid increasing the length of the column as this will alter separation conditions.

HIC media can be packed in either Tricorn or XK columns available from GE Healthcare. SOURCE RPC can be packed in Tricorn or HR columns also available from GE Healthcare. A step-by-step demonstration of column packing can be seen in “Column Packing – The Movie”, available in CD format (see Ordering Information).



1. Equilibrate all materials to the temperature at which the separation will be performed.
2. Eliminate air by flushing column end pieces with the recommended buffer. Ensure no air is trapped under the column net. Close column outlet leaving 1–2 cm of buffer in the column.
3. Gently resuspend the medium.

Note that HIC media from GE Healthcare are supplied ready to use. Decanting of fines that could clog the column is unnecessary.



Avoid using magnetic stirrers since they may damage the matrix.

4. Estimate the amount of slurry (resuspended medium) required on the basis of the recommendations supplied.
5. Pour the required volume of slurry into the column. Pouring down a glass rod held against the wall of the column will minimize the introduction of air bubbles.
6. Immediately fill the column with buffer.
7. Mount the column top piece and connect to a pump.
8. Open the column outlet and set the pump to the desired flow rate.



When slurry volume is greater than the total volume of the column, connect a second glass column to act as a reservoir (see Ordering information for details). This ensures that the slurry has a constant diameter during packing, minimizing turbulence and improving column packing conditions.



If the recommended flow rate cannot be obtained, use the maximum flow rate the pump can deliver.



Do not exceed the maximum operating pressure of the medium or column.

9. Maintain the packing flow rate for at least 3 column volumes after a constant bed height is obtained. Mark the bed height on the column.



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

10. Stop the pump and close the column outlet. Remove the top piece and carefully fill the rest of the column with buffer to form an upward meniscus at the top.
11. Insert the adaptor into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adaptor slowly down the column (the outlet of the adaptor should be open) until the mark is reached. Lock the adaptor in position.
13. Connect the column to the pump and begin equilibration. Re-position the adaptor if necessary.



The medium must be thoroughly washed to remove the storage solution, usually 20% ethanol. Residual ethanol may interfere with subsequent procedures.



Many media equilibrated with sterile phosphate-buffered saline containing an antimicrobial agent may be stored at 4°C for up to 1 mo, but always follow the specific storage instructions supplied with the product.

## Column selection

Tricorn and XK columns are fully compatible with the high flow rates achievable with modern media and a broad range of column dimensions are available. Columns most suitable for packing HIC media are listed under the column packing section for each HIC medium (Chapter 3). In most cases the binding capacity of the medium and the amount of sample to be purified will determine the column size required. For a complete listing, refer to the GE Healthcare life sciences catalog.

## Column packing and efficiency

Column efficiency is expressed as the number of theoretical plates per meter chromatography bed (N) or as H (height equivalent to a theoretical plate, HETP), which is the bed length (L) divided by the plate number. Since column efficiency is related to the peak broadening that can occur on a column, it can be calculated from the expression:

$$N = 5.54 \times \left( \frac{V_R}{w_h} \right)^2$$

$V_R$  = volume eluted from the start of sample application to the peak maximum

$w_h$  = peak width measured as the width of the recorded peak at half of the peak height

H is calculated from the expression:

$$H = \frac{L}{N}$$

L = height of packed bed



Measurements of  $V_R$  and  $w_h$  can be made in distance (mm) or volume (ml) but both parameters must be expressed in the same unit.

Column performance should be checked at regular intervals by injecting acetone to determine column efficiency (N) and peak symmetry (asymmetry factor,  $A_s$ ). Since the observed value for N depends on experimental factors such as flow rate and sample loading, comparisons must be made under identical conditions. In HIC, efficiency is measured under isocratic conditions by injecting acetone (which does not interact with the medium) and measuring the eluted peak as shown in Figure 89.

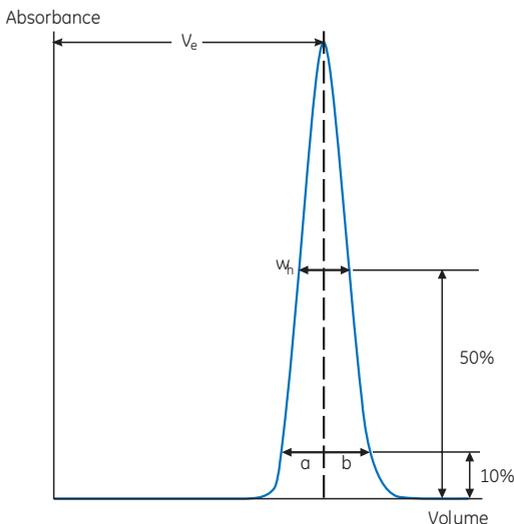


Fig 89. Measurement of efficiency.

As a general rule, a good H value is about two to three times the average particle diameter of the medium being packed. For a 90 µm particle, this means an H value of 0.018–0.027 cm.

The symmetry factor ( $A_s$ ) is expressed as:

$$A_s = \frac{b}{a}$$

where

a = 1st half peak width at 10% of peak height

b = 2nd half peak width at 10% of peak height

$A_s$  should be as close as possible to 1. A reasonable  $A_s$  value for a short column when used for HIC or RPC is 0.80–1.80.



An extensive leading edge is usually a sign that the medium is packed too tightly and extensive tailing is usually a sign that the medium is packed too loosely.



Run at least two column volumes of buffer through a newly packed column to ensure that the medium is equilibrated with start buffer. Use pH monitoring to check the pH of the eluent.

# Appendix 3

## Selection of purification equipment

A chromatography system is required in order to achieve a high-resolution separation using accurately controlled linear gradient elution, to take advantage of the high flow rates of modern media, or when the same column is to be used for many runs. The simplest HIC separations, such as elution by a step-gradient, can be performed using a syringe or peristaltic pump with prepacked HiTrap columns. ÄKTAdesign systems can be chosen to suit separation needs ranging from a simple step-gradient elution in the laboratory through to separations that must meet cGMP requirements. Ettan™ MDLC is a multidimensional liquid chromatography system for protein identification and characterization in proteomic research, ideally used in combination with an electrospray tandem mass spectrometer (ESI-MS/MS).

Way of working	Standard ÄKTAdesign configurations							
	process	primeplus	FPLC	purifier	explorer	pilot	xpress	crossflow
Manufacturing and production	•						•	
UNICORN™	•		•	•	•	•	•	•
One-step simple purification		•						
Reproducible performance for routine purification	•	•	•	•	•	•	•	•
System control and data handling for regulatory requirements	•		•	•	•	•	•	•
Automatic method development and optimization				•	•	•		•
Automatic buffer preparation				•	•			
Automatic pH scouting				•	•			
Automatic media or column scouting					•	•		
Automatic multistep purification					•		•	
Method development and scale-up					•	•		•
Sanitary design cGMP	•					•		•
Scale-up, process development, and transfer to production						•		•



Ettan MDLC



ÄKTApilot™



ÄKTExpress™



ÄKTExplorer



ÄKTAFPLC™



ÄKTAPurifier™



ÄKTAPrime Plus

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

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

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

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

where

Y = linear flow in cm/h

d = column inner diameter in cm

*Example:*

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

Y = linear flow = 150 cm/h

d = inner diameter of the column = 1.6 cm

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

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

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

where

Z = volumetric flow rate in ml/min

d = column inner diameter in cm

*Example:*

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

Z = Volumetric flow rate = 1 ml/min

d = column inner diameter = 0.5 cm

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

### From ml/min to using a syringe

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

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

## Appendix 4

# Conversion data: proteins, column pressures

Mass (g/mol)	1 $\mu\text{g}$	1 nmol
10 000	100 pmol; $6 \times 10^{13}$ molecules	10 $\mu\text{g}$
50 000	20 pmol; $1.2 \times 10^{13}$ molecules	50 $\mu\text{g}$
100 000	10 pmol; $6.0 \times 10^{12}$ molecules	100 $\mu\text{g}$
150 000	6.7 pmol; $4.0 \times 10^{12}$ molecules	150 $\mu\text{g}$

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

1 kb of DNA = 333 amino acids of coding capacity  
= 37 000 g/mol

270 bp DNA = 10 000 g/mol

1.35 kb DNA = 50 000 g/mol

2.70 kb DNA = 100 000 g/mol

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

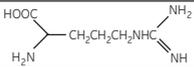
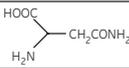
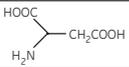
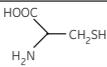
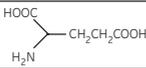
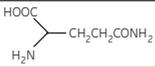
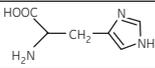
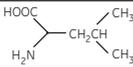
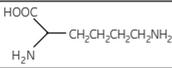
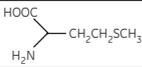
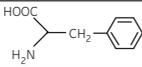
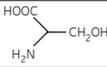
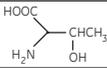
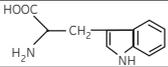
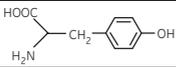
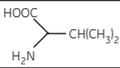
### **Column pressures**

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

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

# Appendix 5

## Table of amino acids

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

Formula	M <sub>r</sub>	Middle unit residue (-H <sub>2</sub> O)		Charge at pH 6.0-7.0	Hydrophobic (non-polar)	Uncharged (polar)	Hydrophilic (polar)
		Formula	M <sub>r</sub>				
C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub>	89.1	C <sub>3</sub> H <sub>5</sub> NO	71.1	Neutral	■		
C <sub>6</sub> H <sub>14</sub> N <sub>4</sub> O <sub>2</sub>	174.2	C <sub>6</sub> H <sub>12</sub> N <sub>4</sub> O	156.2	Basic (+ve)			■
C <sub>4</sub> H <sub>8</sub> N <sub>2</sub> O <sub>3</sub>	132.1	C <sub>4</sub> H <sub>6</sub> N <sub>2</sub> O <sub>2</sub>	114.1	Neutral		■	
C <sub>4</sub> H <sub>7</sub> NO <sub>4</sub>	133.1	C <sub>4</sub> H <sub>5</sub> NO <sub>3</sub>	115.1	Acidic(-ve)			■
C <sub>3</sub> H <sub>7</sub> NO <sub>2</sub> S	121.2	C <sub>3</sub> H <sub>5</sub> NOS	103.2	Neutral		■	
C <sub>5</sub> H <sub>9</sub> NO <sub>4</sub>	147.1	C <sub>5</sub> H <sub>7</sub> NO <sub>3</sub>	129.1	Acidic (-ve)			■
C <sub>5</sub> H <sub>10</sub> N <sub>2</sub> O <sub>3</sub>	146.1	C <sub>5</sub> H <sub>8</sub> N <sub>2</sub> O <sub>2</sub>	128.1	Neutral		■	
C <sub>2</sub> H <sub>5</sub> NO <sub>2</sub>	75.1	C <sub>2</sub> H <sub>3</sub> NO	57.1	Neutral		■	
C <sub>6</sub> H <sub>9</sub> N <sub>3</sub> O <sub>2</sub>	155.2	C <sub>6</sub> H <sub>7</sub> N <sub>3</sub> O	137.2	Basic (+ve)			■
C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub>	131.2	C <sub>6</sub> H <sub>11</sub> NO	113.2	Neutral	■		
C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub>	131.2	C <sub>6</sub> H <sub>11</sub> NO	113.2	Neutral	■		
C <sub>6</sub> H <sub>14</sub> N <sub>2</sub> O <sub>2</sub>	146.2	C <sub>6</sub> H <sub>12</sub> N <sub>2</sub> O	128.2	Basic(+ve)			■
C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub> S	149.2	C <sub>5</sub> H <sub>9</sub> NOS	131.2	Neutral	■		
C <sub>9</sub> H <sub>11</sub> NO <sub>2</sub>	165.2	C <sub>9</sub> H <sub>9</sub> NO	147.2	Neutral	■		
C <sub>5</sub> H <sub>9</sub> NO <sub>2</sub>	115.1	C <sub>5</sub> H <sub>7</sub> NO	97.1	Neutral	■		
C <sub>3</sub> H <sub>7</sub> NO <sub>3</sub>	105.1	C <sub>3</sub> H <sub>5</sub> NO <sub>2</sub>	87.1	Neutral		■	
C <sub>4</sub> H <sub>9</sub> NO <sub>3</sub>	119.1	C <sub>4</sub> H <sub>7</sub> NO <sub>2</sub>	101.1	Neutral		■	
C <sub>11</sub> H <sub>12</sub> N <sub>2</sub> O <sub>2</sub>	204.2	C <sub>11</sub> H <sub>10</sub> N <sub>2</sub> O	186.2	Neutral	■		
C <sub>9</sub> H <sub>11</sub> NO <sub>3</sub>	181.2	C <sub>9</sub> H <sub>9</sub> NO <sub>2</sub>	163.2	Neutral		■	
C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>	117.1	C <sub>5</sub> H <sub>9</sub> NO	99.1	Neutral	■		



# Appendix 6

## Analytical assays during purification

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

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

### Total protein determination

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

### Purity determination

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

### SDS-PAGE Analysis

#### Reagents Required

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

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

 The percentage of acrylamide in the SDS-gel should be selected according to the expected molecular weight of the protein of interest (see Table 32).

**Table 32.** Separation ranges of polyacrylamide gels.

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

\* The larger proteins fail to move significantly into the gel.

 For information and advice on electrophoresis techniques, please refer to the section Additional reading and reference material.

## Functional assays

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

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

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

Electrophoresis and protein transfer may be accomplished using a variety of equipment and reagents. For further details, refer to the *Protein Electrophoresis Technical Manual and Hybond ECL instruction manual*, both from GE Healthcare and available on [www.gehealthcare.com/lifesciences](http://www.gehealthcare.com/lifesciences)

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

## Detection and assay of tagged proteins

SDS-PAGE, Western blotting and ELISAs can also be applied to the detection and assay of genetically engineered molecules to which a specific tag has been attached. In some cases, an assay based on the properties associated with the tag itself can be developed, for example, the GST Detection Module for enzymatic detection and quantification of GST-tagged proteins. Further details on the detection and quantification of GST- and (histidine)<sub>6</sub>- tagged proteins are available in *The Recombinant Protein Handbook: Protein Amplification and Simple Purification* and the *GST Gene Fusion System Handbook* from GE Healthcare.

# Appendix 7

## Storage of biological samples



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

### General recommendations

- Add stabilizing agents, if essential. Stabilizing agents are often required for storage of purified proteins.
- Serum, culture supernatants and ascitic fluid should be kept frozen at  $-20^{\circ}\text{C}$  or  $-70^{\circ}\text{C}$ , in small aliquots.
- Avoid repeated freeze/thawing or freeze drying/re-dissolving that may reduce biological activity.
- Avoid conditions close to stability limits, for example, pH or salt concentrations, reducing or chelating agents.
- Keep refrigerated at  $4^{\circ}\text{C}$  in a closed vessel to minimize bacterial growth and protease activity. Above 24 hours at  $4^{\circ}\text{C}$ , add a preserving agent if possible (e.g., merthiolate 0.01%).



Sodium azide can interfere with many coupling methods and some biological assays and can be a health hazard. It can be removed by using a desalting column (see page 136).

### General recommendations for purified proteins

- Store as a precipitate in high concentration of ammonium sulfate, for example, 4.0 M.
- Freeze in 50% glycerol, especially suitable for enzymes.
- Avoid the use of preserving agents if the product is to be used for a biological assay. Preserving agents should not be added if *in vivo* experiments are to be performed. Instead store samples in small aliquots and keep frozen.
- Sterile filter to prolong storage time.
- Add stabilizing agents, for example, glycerol (5–20%), serum albumin (10 mg/ml), ligand (concentration is selected based on concentration of active protein) to help to maintain biological activity. Remember that any additive will reduce the purity of the protein and may need to be removed at a later stage.
- Avoid repeated freeze/thawing or freeze drying/re-dissolving that may reduce biological activity.



Sodium azide can interfere with many coupling methods and some biological assays. It can be removed by using a desalting column (see page 136).



Cryoproteins are a group of proteins, including some mouse antibodies of the IgG<sub>3</sub> subclass, that should not be stored at  $4^{\circ}\text{C}$  as they precipitate at this temperature. Keep at room temperature in the presence of a preserving agent.



# Appendix 8

## Column cleaning for HIC media

Correct preparation of samples and buffers and application of a salt-free buffer or distilled water wash 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.

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

### **Removal of common contaminants**

The following procedure should be satisfactory to remove common contaminants such as precipitated proteins. Note that flow rates may need to be reduced due to the condition of the column and the viscosity of the sample, buffers or storage solutions.

1. Wash with up to 4 column volumes of 1 M NaOH at flow rate recommended in Table 33.
2. Wash with at least 3 column volumes of water or until eluent pH is neutral.
- 3a. To start a new separation: re-equilibrate with at least 3 column volumes of start buffer or until the correct eluent pH is achieved at the flow rate recommended in Table 33.
- 3b. For storage: wash with at least 5 column volumes of storage solution. Allow UV baseline to stabilize before storing the column.

### **To remove lipids, lipoproteins and very hydrophobic proteins**

-  Organic solvents or detergents may be required to completely remove contaminants of this type. Always check for solvent compatibility in the instructions supplied with the medium or column.
-  Before using organic solvents, wash the medium with at least 4 column volumes of distilled water to avoid any salts precipitating on the column. When applying organic solvents or solutions it may be necessary to reduce the flow rate to avoid over-pressuring the column.

### **Alternative 1**

1. Wash with at least 2 column volumes of water at flow rate recommended in Table 33.
2. Wash with at least 4 column volumes of 70% ethanol or 30% isopropanol at the flow rate recommended in Table 33.
3. Wash with at least 4 column volumes of distilled water, same flow as step 1.
- 4a. To start a new separation: re-equilibrate with at least 3 column volumes of start buffer, or until the correct eluent pH is achieved at the flow rate recommended in Table 33.
- 4b. For storage: wash with at least 5 column volumes of storage solution. Allow UV baseline to stabilize before storing the column.

## Alternative 2

1. Wash with up to 2 column volumes of 0.05% non-ionic detergent (e.g., Triton™ X-100) in 1 M acetic acid at flow rate recommended in Table 33.
2. Wash with at least 5 column volumes of 70% ethanol (to remove detergent) at flow rate recommended in Table 33.
3. Wash with at least 4 column volumes of distilled water (same flow as step 1).
- 4a. To start a new separation: re-equilibrate with at least 3 column volumes of start buffer, or until the correct eluent pH is achieved, at the flow rate recommended in Table 33.
- 4b. For storage: wash with at least 5 column volumes of storage solution. Allow UV baseline to stabilize before storing the column.



Use detergents with care as they may bind to the medium thereby reducing binding capacity in subsequent runs.

**Table 33.** Recommended flow according to medium, column dimensions and eluent.

Column (volume) or medium	Water, start buffer, 1 M acetic acid, 2 M NaCl, 1 M NaOH*	6 M guanidine hydrochloride	70% ethanol or 30% isopropanol
SOURCE 15 4.6/100 PE	0.2 ml/min	0.1 ml/min	0.1 ml/min
RESOURCE 1 ml	1 ml/min	0.5 ml/min	0.5 ml/min
SOURCE in larger columns <sup>†</sup>	40 cm/h	20 cm/h	20 cm/h
HiTrap (1 ml)	1 ml/min	0.5 ml/min	0.5 ml/min
HiTrap (5 ml)	5 ml/min	2.5 ml/min	2.5 ml/min
HiPrep (20 ml)	5 ml/min	2.5 ml/min	2.5 ml/min
HiLoad 16/10 (20 ml)	3 ml/min	2.5 ml/min	2.5 ml/min
HiLoad 26/10 (53 ml)	8 ml/min	5 ml/min	5 ml/min
Sepharose High Performance in larger columns <sup>†</sup>	40 cm/h	20 cm/h	20 cm/h
Sepharose Fast Flow in larger columns <sup>†</sup>	40 cm/h	20 cm/h	40 cm/h

\* If contamination is thought to be significant, use a lower flow rate to increase the contact time when using 1 M NaOH.

<sup>†</sup> When cleaning larger columns such as FineLine, allow a contact time of 1–2 hours for any solution that is used as an initial cleaning step.

# Product index

<b>RESOURCE</b>	27, 28, 38, 42, 53–55, 59, 60, 90, 97, 121–126, 141, 156, 160, 161
RESOURCE HIC Test Kit	27, 28, 54, 141, 160
RESOURCE PHE	42, 54, 160
RESOURCE ETH	54, 160
RESOURCE ISO	38, 54, 160
RESOURCE RPC	90, 97, 122–124, 141, 161
<b>SOURCE</b>	18, 24, 27, 28, 43, 44, 53–62, 74, 83, 85–87, 97, 99, 103–105, 116, 121–127, 141, 156, 160, 161
SOURCE 15PHE	27, 54–56, 58–61, 86, 87, 160
SOURCE 15ETH	54, 55, 61, 160
SOURCE 15ISO	54, 55, 57, 61, 160
SOURCE 5RPC	97, 99, 104, 105, 116, 121–123, 125–127, 161
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SOURCE 30RPC	97, 105, 121, 122, 124–127, 161
<b>HiLoad</b>	44, 62–68, 71, 85, 156, 159, 160
HiLoad 16/10 Phenyl Sepharose HP	44, 63–65, 71, 160
HiLoad 26/10 Phenyl Sepharose HP	44, 63, 64, 71, 160
<b>HiPrep</b>	19, 44, 70, 72, 76, 77, 136–138, 156, 159, 160–162
HiPrep 16/10 Butyl FF	19, 70, 161
HiPrep 16/10 Octyl FF	19, 70, 161
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HiTrap Phenyl FF (low sub)	29, 64, 160
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Phenyl Sepharose 6 Fast Flow (high sub)	19, 27, 29, 63, 71, 74, 83–87, 124, 160
Phenyl Sepharose 6 Fast Flow (low sub)	19, 63, 64, 71, 83, 160
Butyl Sepharose 4 Fast Flow	50, 63, 71, 75, 83, 160, 161
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Octyl Sepharose 4 Fast Flow	63, 71, 160, 161
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μRPC C2/C18 SC	58, 87, 118, 119, 161
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Tricorn column	9, 51, 55, 64, 72, 122, 141, 143, 146, 162, 163
XK column	64, 72–75, 84, 85, 88, 141, 143, 146, 162, 163
HR column	141, 162

# Additional reading

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## Code No.

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### Purification

Antibody Purification Handbook	18-1037-46
Protein Purification Handbook	18-1132-29
Recombinant Protein Handbook: Protein Amplification and Simple Purification	18-1142-75
GST Gene Fusion System Handbook	18-1157-58
Affinity Chromatography Handbook: Principles and Methods	18-1022-29
Gel filtration Handbook: Principles and Methods	18-1022-18
Hydrophobic Interaction and Reversed Phase Chromatography: Principles and Methods	11-0012-69
Fast Desalting and Buffer Exchange of Proteins and Peptides	18-1128-62
Gel Filtration Columns and Media Selection Guide	18-1124-19
Ion Exchange Columns and Media Selection Guide	18-1127-31
Affinity Columns and Media Product Profile	18-1121-86
Convenient Protein Purification, HiTrap Column Guide	18-1129-81
Prepacked column chromatography columns with ÄKTAdesign systems	18-1173-49
ÄKTAprime Plus-Don't mess around with protein preparation	11-0034-88
Protein Purification Application that meet your needs	11-0027-81
ÄKTAdesign: Purification platform preferred by 100 000 scientists worldwide	28-4026-97
The Protein Purifier	18-1155-49
Protein Purification: Principles, High Resolution Methods and Applications, J-C. Jansson and L.Rydén	18-1128-68
Column Packing CD The Movie	18-1165-33

### Analysis

2-D Electrophoresis Handbook	80-6429-60
Protein Electrophoresis Technical Manual	80-6013-88

Many of these items can be downloaded from [www.gehealthcare.com/protein-purification-labresearch](http://www.gehealthcare.com/protein-purification-labresearch)

# References

Reference lists are available from [www.gehealthcare.com/protein-purification-labresearch](http://www.gehealthcare.com/protein-purification-labresearch)

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	<b>Code No.</b>
Reference list HiLoad Phenyl Sepharose HP and HiLoad Phenyl Sepharose FF	18-1156-97
Reference list HiTrap Butyl FF	18-1156-79
Reference list HiTrap Octyl FF	18-1156-80
Reference list HiPrep 16/10 Phenyl FF	18-1156-93
Reference list HiTrap HIC Selection Kit	18-1156-78
Reference list HiTrap Phenyl HP and HiTrap Phenyl FF	18-1156-81

# Ordering information

## Hydrophobic interaction media

SOURCE, Sepharose High Performance and Sepharose Fast Flow media are all available as BioProcess media for large-scale production. Please contact your local GE Healthcare representative for details.

Product	Quantity	Code No.
RESOURCE HIC Test Kit	3 x 1 ml	17-1187-01
RESOURCE PHE; RESOURCE PHE; RESOURCE ISO		
HiTrap HIC Selection Kit	6 x 1 ml	11-0034-53
Phenyl Sepharose High Performance, Phenyl Sepharose 6 Fast Flow 6 (high sub), Phenyl Sepharose 6 Fast Flow (low sub), Butyl Sepharose 4 Fast Flow, Butyl-S Sepharose 6 Fast Flow Octyl Sepharose 4 Fast Flow		
<b>SOURCE</b>		
RESOURCE PHE	1 x 1 ml	17-1186-01
SOURCE 15PHE 4.6/100 PE	1 x 1.7 ml	17-5186-01
SOURCE 15PHE	50 ml	17-0147-01
SOURCE 15PHE	200 ml	17-0147-02
RESOURCE ETH	1 x 1 ml	17-1184-01
SOURCE 15ETH	50 ml	17-0146-01
SOURCE 15ETH	200 ml	17-0146-02
RESOURCE ISO	1 x 1 ml	17-1185-01
SOURCE 15ISO	50 ml	17-0148-01
SOURCE 15ISO	200 ml	17-0148-02
<b>Sepharose High Performance</b>		
HiTrap Phenyl HP	5 x 1 ml	17-1351-01
HiTrap Phenyl HP	5 x 5 ml	17-5195-01
HiLoad 16/10 Phenyl Sepharose HP	1 x 20 ml	17-1085-01
HiLoad 26/10 Phenyl Sepharose HP	1 x 53 ml	17-1086-01
Phenyl Sepharose High Performance	75 ml	17-1082-01
<b>Sepharose Fast Flow</b>		
HiTrap Phenyl FF (high sub)	5 x 1 ml	17-1355-01
HiTrap Phenyl FF (high sub)	5 x 5 ml	17-5193-01
HiPrep 16/10 Phenyl FF (high sub)	1 x 20 ml	17-5095-01
Phenyl Sepharose 6 Fast Flow (high sub)	25 ml	17-0973-10
Phenyl Sepharose 6 Fast Flow (high sub)	200 ml	17-0973-05
HiTrap Phenyl FF (low sub)	5 x 1 ml	17-1353-01
HiTrap Phenyl FF (low sub)	5 x 5 ml	17-5194-01
HiPrep 16/10 Phenyl FF (low sub)	1 x 20 ml	17-5094-01
Phenyl Sepharose 6 Fast Flow (low sub)	25 ml	17-0965-10
Phenyl Sepharose 6 Fast Flow (low sub)	200 ml	17-0965-05

Product	Quantity	Code No.
<b>Sepharose Fast Flow (continued)</b>		
HiTrap Butyl FF	5 x 1 ml	17-1357-01
HiTrap Butyl FF	5 x 5 ml	17-5197-01
HiPrep 16/10 Butyl FF	1 x 20 ml	17-5096-01
Butyl Sepharose 4 Fast Flow	25 ml	17-0980-10
Butyl Sepharose 4 Fast Flow	200 ml	17-0980-01
HiTrap Butyl-S FF	5 x 1 ml	17-0978-13
HiTrap Butyl-S FF	5 x 1 ml	17-0978-14
Butyl-S Sepharose 6 Fast Flow	25 ml	17-0978-10
Butyl-S Sepharose 6 Fast Flow	200 ml	17-0978-02
HiTrap Octyl FF	5 x 1 ml	17-1359-01
HiTrap Octyl FF	5 x 5 ml	17-5196-01
HiPrep 16/10 Octyl FF	1 x 20 ml	17-5097-01
Octyl Sepharose 4 Fast Flow	25 ml	17-0946-10
Octyl Sepharose 4 Fast Flow	200 ml	17-0946-02

## Reversed phase media

SOURCE 15 and SOURCE 30 are available as BioProcess media for large-scale production. Please contact your local GE Healthcare representative for details.

Product	Quantity	Code No.
<b>µRPC</b>		
µRPC C2/C18 ST 4.6/100	1 x 1.7 ml	17-5057-01
µRPC C2/C18 SC 2.1/10 (requires Precision Column Holder 17-1455-01 for attachment to chromatography systems)	1 x 0.35 ml	17-0704-01
µRPC C2/C18 ST 1.0/150	1 x 120 µl	17-6002-88
µRPC C2/C18 ST 300µm/150	1 x 11 µl	17-6002-89
<b>SOURCE 5RPC</b>		
SOURCE 5RPC ST 4.6/150	1 x 2.5 ml	17-5116-01
<b>SOURCE 15RPC</b>		
SOURCE 15RPC ST 4.6/100	1 x 1.7 ml	17-5068-01
RESOURCE RPC, 1 ml	1 x 1 ml	17-1181-01
RESOURCE RPC, 3 ml	1 x 3 ml	17-1182-01
SOURCE 15RPC	10 ml	17-0727-20
SOURCE 15RPC	200 ml	17-0727-02
SOURCE 15RPC	500 ml	17-0727-03
<b>SOURCE 30RPC</b>		
SOURCE 30RPC	10 ml	17-5120-20
SOURCE 30RPC	200 ml	17-5120-02
SOURCE 30RPC	500 ml	17-5120-03

## Other columns and accessories

Product	Quantity	Code No.
<b>Desalting columns</b>		
HiTrap Desalting	5 × 5 ml	17-1408-01
HiTrap Desalting (available by special order)	100 × 5 ml	11-0003-29
HiPrep 26/10 Desalting	1 × 53 ml	17-5087-01
HiPrep 26/10 Desalting	4 × 53 ml	17-5087-02
PD-10 Desalting columns	30	17-0851-01
<b>Column Packing CD</b>		
Column Packing–The Movie	1	18-1165-33
<b>Empty Columns</b>		
Complete information on the range of Tricorn columns is available at <a href="http://www.gehealthcare.com/protein-purification-labresearch">www.gehealthcare.com/protein-purification-labresearch</a>		
Tricorn 5/100 column	1	18-1163-10
Tricorn 5/150 column	1	18-1163-11
Tricorn 5/200 column	1	18-1163-12
Tricorn 10/100 column	1	18-1163-15
Tricorn 10/150 column	1	18-1163-16
Tricorn 10/200 column	1	18-1163-17
Tricorn columns are delivered with a column tube, adaptor unit, end cap, a filter kit containing adaptor and bottom filters and O-rings, two stop plugs, two fingertight fittings, adaptor lock and filter holder, and two M6 connectors for connection to FPLC System, if required.		
XK 16/20 column	1	18-8773-01
XK 26/20 column	1	18-1000-72
XK 50/20 column	1	18-1000-71
XK columns are delivered with one AK adaptor, T EFZEL tubing (0.8 mm i.d. for XK 16 and XK 26 columns, 1.2 mm i.d. for XK 50 columns, with M6 connectors, thermostatic jacket, support snap-on net rings, dismantling tool (XK 16 and XK 26 only), and instructions.		
HR 16/5 column	1	18-1000-98
HR 16/10 column	1	19-7403-01
HR 16/50 column	1	18-1460-01
HR columns are delivered with a column tube, adaptor unit, end cap, a filter kit containing adaptor and bottom filters and O-rings and M6 male fittings for connection to FPLC System.		
Empty disposable PD-10 Desalting columns	50	17-0435-01

Product	Quantity	Code No.
<b>Accessories and spare parts</b>		
For a complete listing refer to GE Healthcare BioDirectory or <a href="http://www.gehealthcare.com/protein-purification">www.gehealthcare.com/protein-purification</a>		
LabMate PD-10 Buffer Reservoir	10	18-3216-03
Packing Connector XK 16	1	18-1153-44
Packing Connector XK 26	1	18-1153-45
Tricorn packing equipment 10/100 includes Tricorn packing connector 10-10, Tricorn 10/100 glass tube, bottom unit and stop plug.	1	18-1153-25
Tricorn packing connector 10-10*	1	18-1153-23
Connects extra glass column to a Tricorn 10 column to act as a packing reservoir for efficient packing.		





[www.gehealthcare.com/protein-purification](http://www.gehealthcare.com/protein-purification)

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