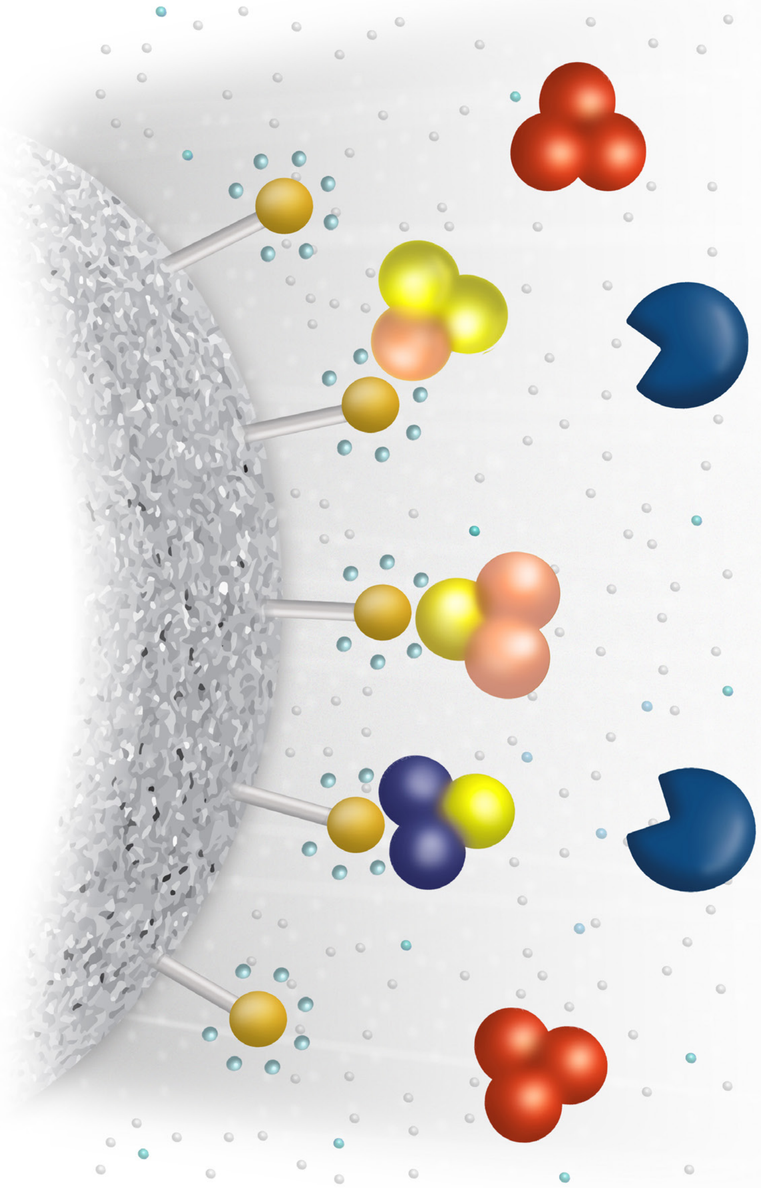
Principles and methods

Hydrophobic interaction and reversed phase chromatography



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MZ ANALYSENTECHNIK

AUTHORIZED DISTRIBUTOR
MZ-Analysentechnik GmbH, Barcelona-Allee 17 • D-55129 Mainz
Tel +49 6131 880 96-0, Fax +49 6131 880 96-20
e-mail: info@mz-at.de, www.mz-at.de

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Introduction

To purify biomolecules, scientists use chromatography techniques that separate them according to differences in their specific properties, as shown in Figure I1.1. There are different chromatographic techniques available according to your requirements (see Table I1.1). Hydrophobic interaction chromatography (HIC) separates biomolecules in relatively mild conditions according to differences in their hydrophobicity.

Table I1.1. Types of chromatographic techniques, and their properties

Technique	Property
Hydrophobicity interaction chromatography (HIC)	Hydrophobicity
Reversed phase chromatography (RPC)	Hydrophobicity
Ion exchange chromatography (IEX)	Charge
Size exclusion chromatography (SEC, also called gel filtration)	Size
Affinity chromatography (AC)	Biorecognition (ligand specificity)
Multimodal chromatography (MM, also called mixed-mode chromatography)	A combination of several protein properties

Many scientists use HIC in protein purification as a complement to other techniques that separate according to charge, size, or biospecific recognition. HIC is a good next step after subjecting your samples to ammonium sulfate precipitation (often used for initial sample concentration, and clean-up), or after separation by ion exchange chromatography. In both situations, the sample has a high salt concentration, and you can directly apply it 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 resin.

During separation, scientists purify, and elute samples in smaller volumes — this concentrates the sample so that it can go directly to size exclusion chromatography or, after a buffer exchange, to an ion exchange separation. You can use HIC for capture, intermediate purification, or polishing steps in a purification protocol. Cytiva has resins that you can use for small-scale separations in the laboratory, through to the production of kilogram quantities of product.

This handbook describes both theoretical, and practical aspects of the technique, the resins available, and how to select them, together with application examples, and detailed instructions for the most common procedures. You will find practical information with many hints and tips drawn from our over 60 yr of experience in chromatography purification — the handbook will help beginners and experts to obtain optimal results from the latest chromatography resins.

Chapter 6 focuses on RPC, a technique that provides good resolution separations. You can also use RPC as a final polishing step in a purification strategy, but you should ensure that the presence of organic solvents does not compromise the recovery of biological activity or tertiary structure.

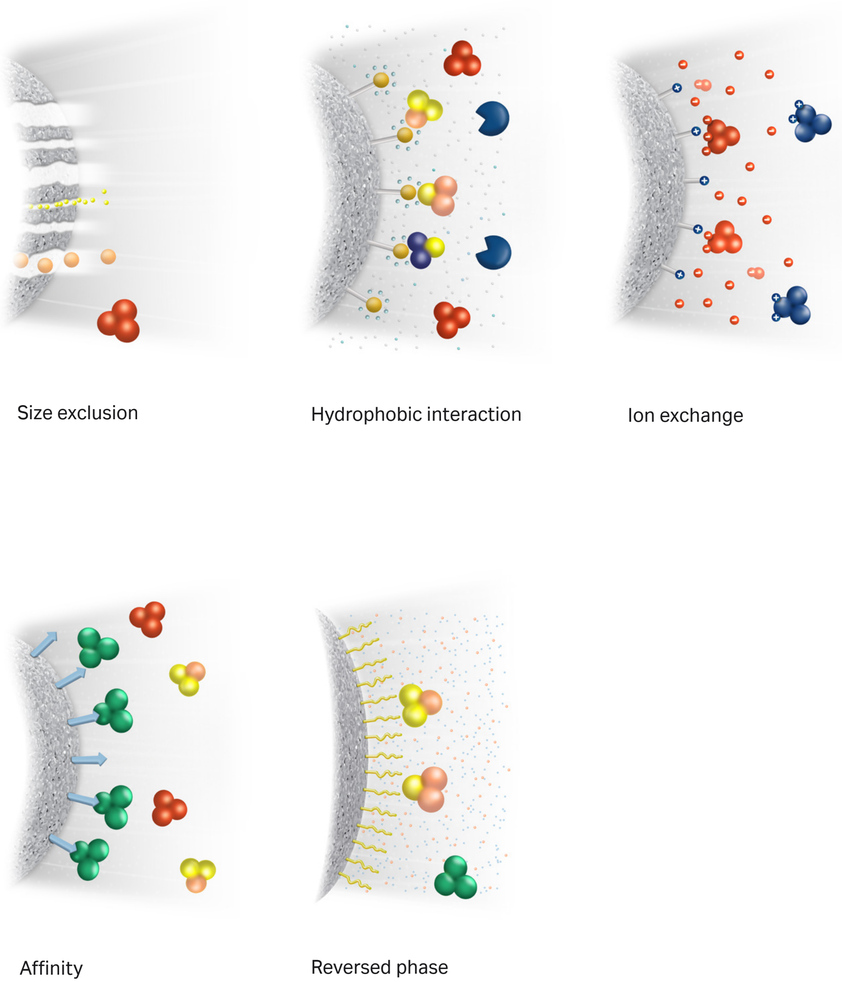


Fig I.1. Separation principles in chromatographic purification.

Symbols

Highlights troubleshooting advice to help analyze, and resolve difficulties.

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

Highlights chemicals, buffers, and equipment.

Provides an outline of the experimental protocol.

Common abbreviations in chromatography

$A_{280nm,}$ A_{214nm}	UV absorbance at a specified wavelength
AC	affinity chromatography
CF	chromatofocusing
CIPP	capture, intermediate purification, polishing
CIP	cleaning in place
CV	column volume
DBC	dynamic binding capacity
HIC	hydrophobic interaction chromatography
IEX	ion exchange chromatography
MM	mixed mode chromatography (also referred to as multimodal chromatography)
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
SEC	size exclusion chromatography sometimes referred to as GF: (gel filtration)

Abbreviations found in product names

FF	Fast Flow
HP	High Performance
i.d.	inner diameter
PE	PEEK
ST	column manufactured in stainless steel

01

Principles of hydrophobic interaction chromatography

This chapter provides a brief introduction to hydrophobic interaction chromatography (HIC), and mainly focuses on the basic principles of separation. We cover the practical aspects of performing a separation in Chapter 2.

HIC separates proteins according to differences in their surface hydrophobicity through a reversible interaction between the proteins, and the hydrophobic surface of a HIC resin. Even though you can find several suggestions in the scientific literature, there is no universally accepted theory on the mechanisms involved in HIC.

You can separate standard proteins with different degrees of surface hydrophobicity. Certain salts in the running buffer can significantly influence the way that proteins and a HIC resin interact. A high salt concentration enhances the interaction, and a low salt concentration weakens the interaction.

In the example presented on Fig 1.1, all four proteins interact with the hydrophobic surface of the HIC resin. But as you reduce the ionic strength of the buffer, the interaction reverses, and the protein with the lowest degree of hydrophobicity elutes 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, α-chymotrypsin
Start buffer:	1.7 M ammonium sulfate, 0.02 M Tris-HCl, pH 7.5
Flow rate:	0.8 mL/min (24 cm/h)
Elution buffer:	0.02 M Tris-HCl, pH 7.5
Flow rate:	1 mL/min, 76 cm/h
Gradient:	0% to 100% elution buffer in 10 CV

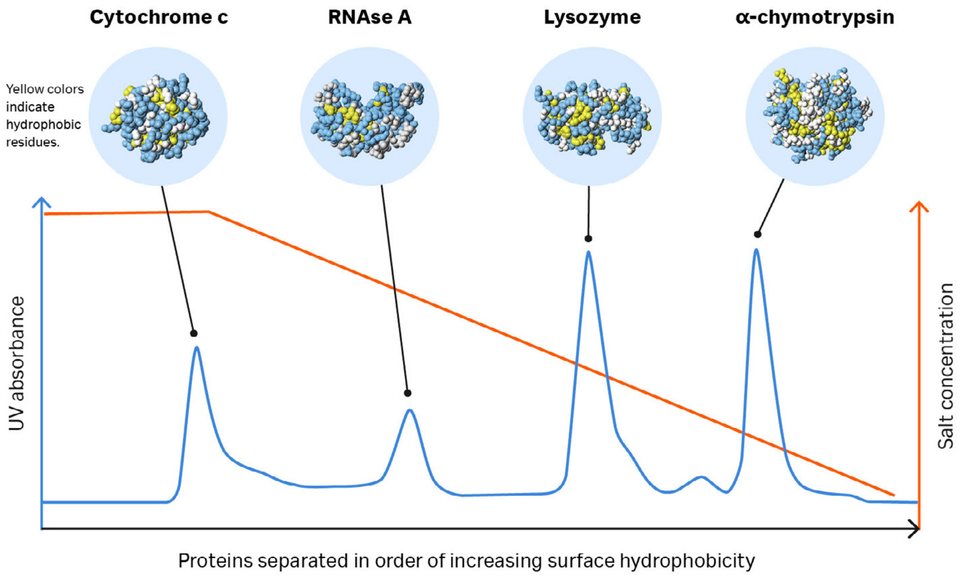


Fig 1.1. Proteins separate according to differences in their surface hydrophobicity (yellow indicates hydrophobic and blue hydrophilic amino acid residues), as shown in this separation of standard proteins on Phenyl Sepharose™ High Performance.

Hydrophobic interaction chromatography in theory

The role of water

Water is a good solvent for polar substances, but a poor solvent for nonpolar substances. In any liquid water, most of the water molecules occur in clusters due to hydrogen bonding between themselves (Fig 1.2). The half-life of water clusters is short, but the net effect is a strong cohesion between the water molecules. this is shown, for example, by a high boiling point.

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

When you immerse a hydrophobic substance like a protein or hydrophobic ligand 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 decreases the number of ordered water molecules – that is, a thermodynamically more favorable situation in which entropy increases. To gain entropy, hydrophobic substances need to merge and minimize the total area of shells. This means that hydrophobic interaction depends on the behavior of the water molecules rather than on direct attraction between the hydrophobic molecules (Fig 1.2).

Protein structure

The three-dimensional structure of a protein is a result of intramolecular interactions as well as interactions with the surrounding solvent. For readily soluble proteins, this solvent is water, which typically drives hydrophobic side chains to the interior of the protein. The final structure is a result of a thermodynamic compromise that best suits the surrounding solution. So although the interior of globular proteins buries most hydrophobic amino acid residues, some are exposed, and this results in hydrophobic patches on the protein surface.

Because proteins carry both hydrophilic and hydrophobic areas on their surfaces, they might precipitate at high concentrations of certain salts due to 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, like those in HIC resins.

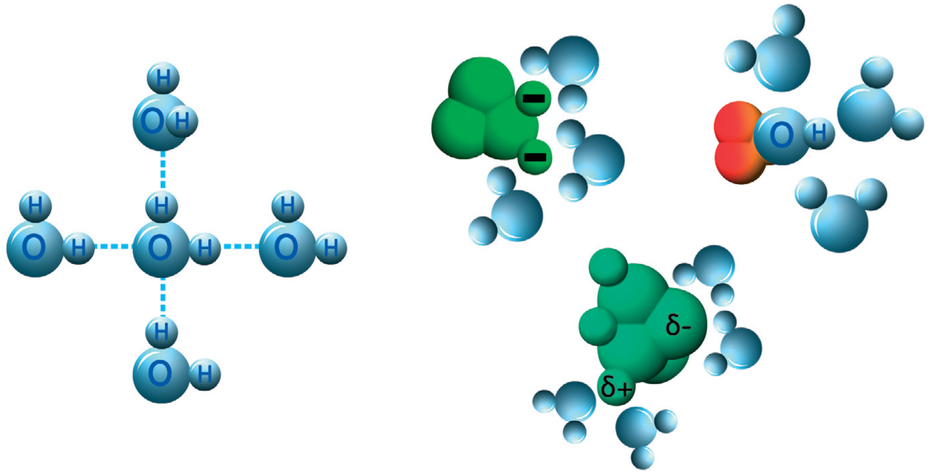


Fig 1.2. The solubilizing properties of water reside in its ability to interact with dipoles and form hydrogen bonds.

Reversible interactions

The hydrophobic ligands on HIC resins 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 resins. For selective elution (desorption), scientists lower the salt concentration gradually, and the sample components elute in order of hydrophobicity (Fig 1.3).

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

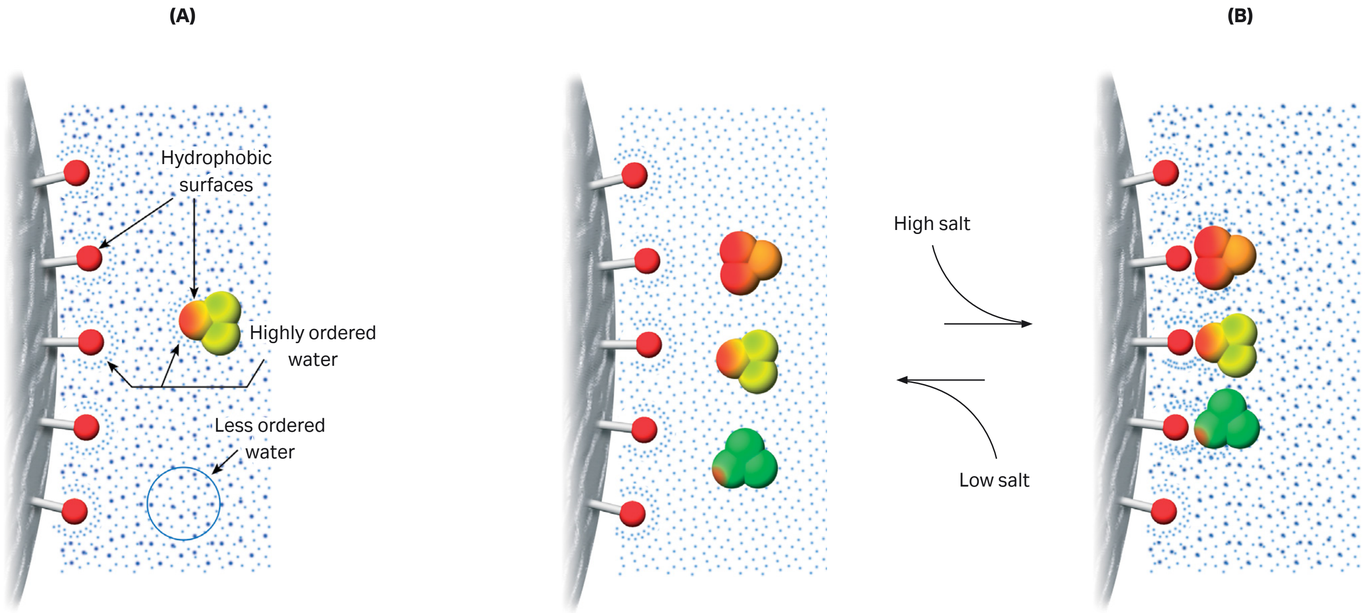


Fig 1.3. (A) Highly ordered water shells surround the hydrophobic surfaces of ligands and proteins. Hydrophobic substances are forced to merge to minimize the total area of such shells (maximize entropy). Salts enhance the hydrophobic interaction. (B) The equilibrium of the hydrophobic interaction is controlled predominantly by the salt concentration.

Steps in a HIC separation

HIC resins are composed of ligands that contain alkyl or aryl groups coupled to an inert matrix of spherical particles. The matrix is porous to provide a high internal surface area, while the ligand plays a significant role in the final hydrophobicity of the resin. Packing the resin into a column forms a packed bed. For more details on column packing, *see* Appendix 2. Equilibrating the bed with buffer fills the pores of the matrix and the space in between the particles. Figure 1.4 illustrates the separation process that follows.

Moderately high salt concentrations promote interaction between the protein and the resin, typically 1 to 2 M ammonium sulfate or 3 M NaCl. Scientists select the type of salt, and the concentration required in the start buffer to ensure that the proteins of interest bind to the resin, and that other less hydrophobic proteins, and impurities pass directly through the column.

Binding conditions are a key factor in any HIC separation. At this stage, they can significantly influence the final selectivity, resolution, and binding capacity of the target protein(s). 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 plays a less important role. You can adjust the pH directly if necessary.

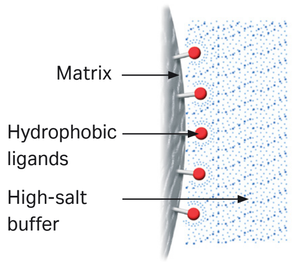
Since many proteins can precipitate in raised salt concentrations, you should check the "stability window" of the target protein(s) at different salt concentrations before optimizing binding conditions. Precipitation of the target protein might make separation impossible or, at best significantly reduce yield. The simplest approach to determine a "stability window" can be to observe the samples 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 unbound proteins have passed through (i.e., the UV signal has returned to baseline), conditions are altered to begin elution. To elute proteins, you can decrease the salt concentration in the elution buffer. As the level of salt decreases, proteins with the lowest hydrophobicity begin to elute from the column. You can use gradients to control changes in salt concentration and elute proteins differentially in a purified, concentrated form. 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 you have correctly judged the hydrophobicity of the resin and the proteins in the sample, all proteins will be eluted by this stage. Then you can reequilibrate the column in the start buffer before applying more samples in the next run.

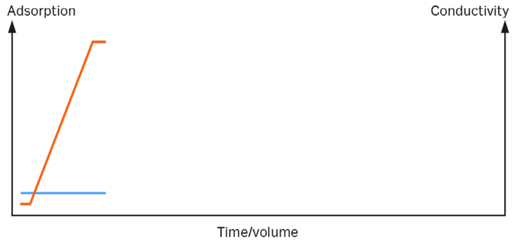
Occasionally the hydrophobic interaction is so tight that you might need harsher conditions to remove all bound material, for example, 0.5 to 1.0 M NaOH, 70% ethanol, or 30% isopropanol. You will need to follow these wash steps with water or salt-free buffer wash before reequilibrating the column with a high-salt start buffer. *See also* Column cleaning, Appendix 9.

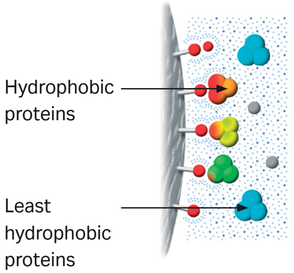
Alternatively, you can choose conditions that maximize the binding of hydrophobic contaminants and allow the target protein(s) to pass through the column.



Equilibration

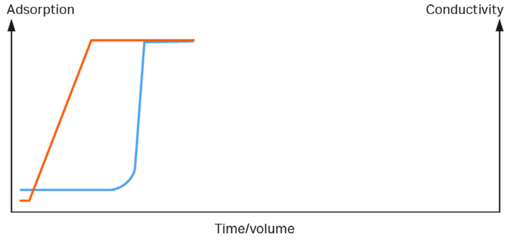
HIC resin equilibrated with high-salt start buffer.

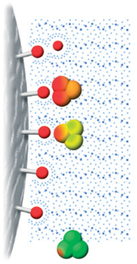




Sample application

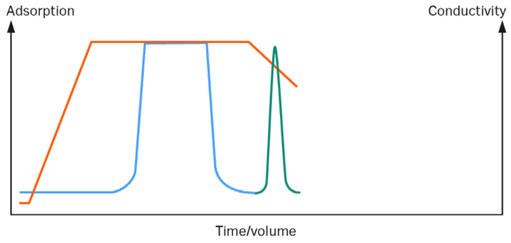
Start buffer allows binding of hydrophobic proteins to hydrophobic ligands on the resin, which leads to concentration of proteins on the ligand. Proteins with insufficent hydrophobic proteins elute during or just after sample application.

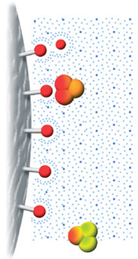




Elution 1

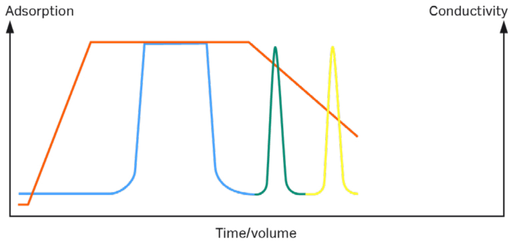
Decreasing salt content (using a linear gradient) causes hydrophobic proteins to elute. The least hydrophobic proteins elute first.

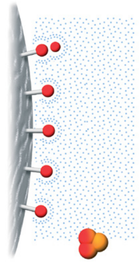




Elution 2

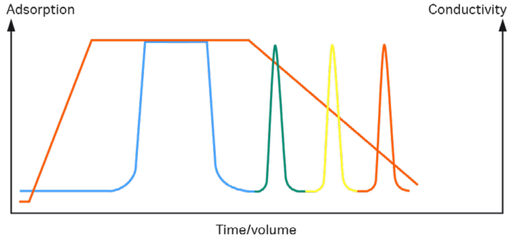
Further decreases in salt displace the more hydrophobic proteins (more tightly bound).

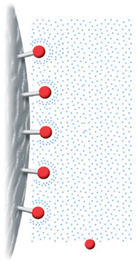




Elution 3

More proteins are displaced as the salt content continues to decrease.





Wash

Final “salt-free” wash removes any hydrophobically bound proteins before re-equilbration.

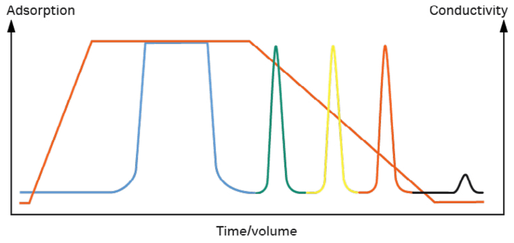


Fig 1.4. Steps in a HIC separation

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 like matrix properties, binding and elution conditions, column packing, and flow rates; we cover these in detail in Chapter 2. 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 1.5.

Measuring elution volumes and peak widths with the same units give a dimensionless resolution value. R_s gives a measure of the relative separation between two peaks, and you can use it to determine whether you need to further optimize the chromatographic procedure.

If R_s = 1.0 (Fig 1.6), then 98% purity has been achieved at 98% of peak recovery, provided the peaks are symmetrical and approximately equal in size. Baseline resolution requires R_s >1.5. At this value, peak purity is 100%.

A single, well-resolved peak is not necessarily a pure substance, but it might represent a series of components that could not separate in the chosen elution conditions. You might need further purification using an alternative chromatography resin. *See* Chapter 4 for advice on purification strategies.

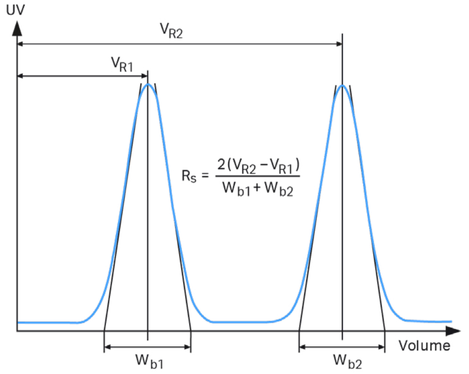


Fig 1.5. Determination of the resolution (R_s) between two peaks.

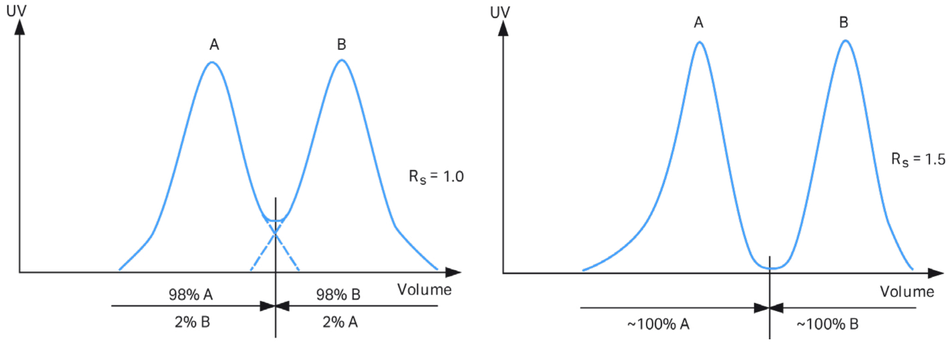


Fig 1.6. Separation results with different resolutions.

Efficiency

Column efficiency is the ability to elute narrow, symmetrical peaks from a packed bed. It relates to the zone broadening that occurs on the column, and scientists often describe it 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 the longitudinal diffusion of the solute molecules (proteins). You can minimize zone broadening if you minimize the distances available for diffusion. In all situations, a well-packed column significantly contributes to resolution. Columns that are packed unevenly, too tightly, too loosely, or that contain air bubbles, will lead to channeling (uneven passage of buffer through the column), and zone broadening, resulting in loss of resolution. Figure 1.7 illustrates the parameters that contribute to good column efficiency. Particle size is a significant factor in resolution and, in general, the smallest particles will produce the narrowest peaks in the correct elution conditions, in a well-packed column.

Although you can improve resolution by decreasing the particle size of the matrix, using smaller particles often creates an increase in backpressure. That means you would need to decrease flow rates to compensate, which lengthens the run time. Because of this, you should match the resin with the requirements for purification (speed, resolution, and purity).

If you run columns packed with small particles, the viscosity of large volumes of highly concentrated samples can reduce the resolution. You might need to dilute your samples or use larger particles.

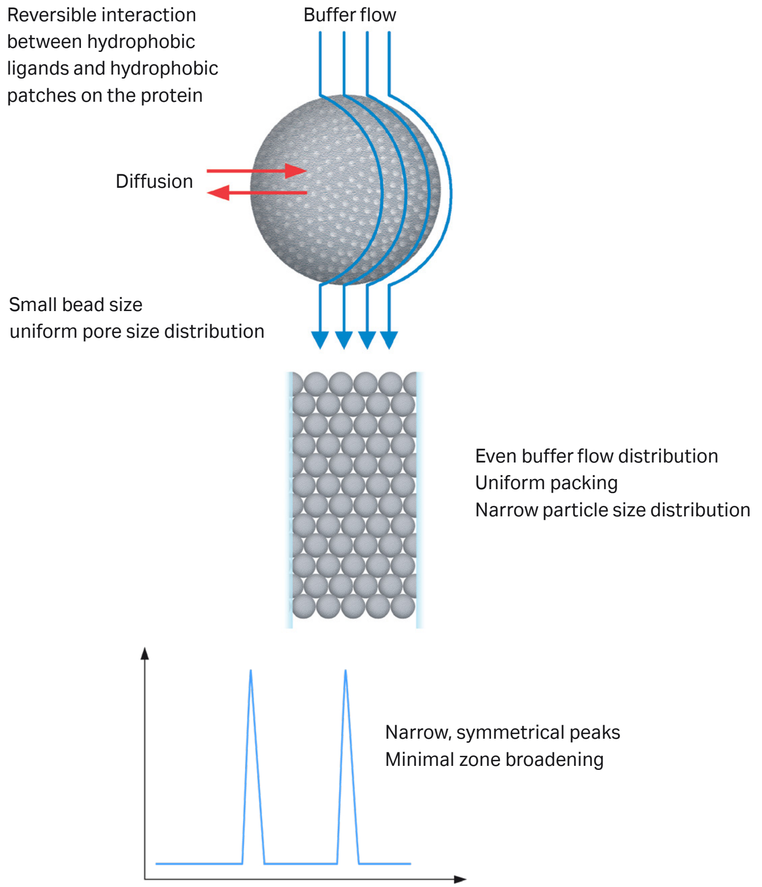


Fig 1.7. Factors that affect column efficiency.

Selectivity

When determining resolution, good selectivity (the degree of separation between peaks) is a more important factor than high efficiency (Fig 1.8).

In HIC, selectivity depends largely on the nature of the ligand, the ligand's 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 resins are described according to the type of ligand (and sometimes ligand density), the actual amount of protein that you can bind to a HIC resin in your experimental conditions is more relevant. This is referred to as the available capacity of a resin for a specific protein. If the defined conditions include the flow rate of the resin, you can refer to the amount bound as the dynamic capacity of the resin. The dynamic capacity of a HIC resin is dependent on the properties of the resin, 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 resin that you need to determine and optimize these parameters by experimentation. That's unlike techniques like ion exchange or affinity chromatography where you can use "standard proteins" as a guideline to predict selectivity and capacity.

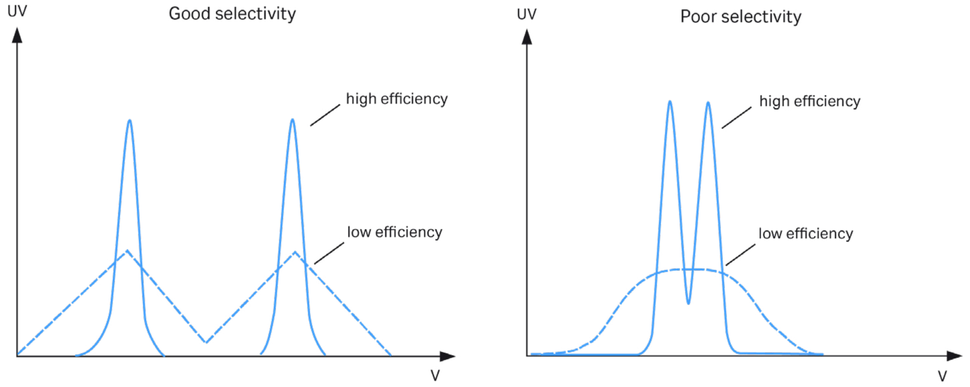


Fig 1.8. Effect of selectivity, and efficiency on resolution.

Selectivity and salt selection

When using HIC resins, the ability of a particular salt to promote hydrophobic interaction depends on the ionic species present and their concentration (Fig 1.9). Protein precipitation has the same driving force that you see when hydrophobic proteins interact with a hydrophobic resin. It is also enhanced by increasing the ionic strength (concentration) of the surrounding buffer.

The Hofmeister series describes the elution and precipitation strength of an ion. Small, highly charged ions are strong precipitators (anti-chaotropic), whereas organic acids and bases have a more stabilizing effect (chaotropic) on the proteins in a solution. The term chaotropic refers to the ability of the ion to produce order or chaos in the water structure. Calcium and magnesium salts are not the strong precipitators that you might expect from the Hofmeister series because these ions can bind to specific sites on the protein surface.

Anion: $SO_4^{2-} > HPO_4^{2-} > CH_3COO^- > Cl^- > NO^{3-} > Br^- > ClO^{3-} > I- > ClO^{4-} > SCN^-$

Cation: $NH_4^+ > K^+ > Na^+ > Li^+ > Mg^{2+} > Ca^{2+} >$ guanidinium

Sodium, potassium, and ammonium sulfates produce relatively high precipitation. These salts effectively promote hydrophobic interaction and have a stabilizing influence on protein structure. In practice, these sulfates effectively promote ligand-protein interactions in HIC and have a stabilizing influence on protein structure. Therefore, scientists most commonly use $(NH_4)_2SO_4$, Na_2SO_4, NaCl, KCl, and CH_3COONH_4.

The amount of protein that you can bind to HIC resins increases almost linearly up to a specific salt concentration. At higher salt concentrations, the amount bound continues to increase exponentially. If the protein is unstable or its stability is unknown, we recommend performing protein binding in the region where the amount of bound protein increases linearly with the salt concentration.

Selectivity and the properties of a HIC resin

While ligands contribute significantly to a resin's hydrophobicity, the matrix can also influence the final selectivity. Chromatography resins for hydrophobic interaction are made from porous matrices. These matrices are chosen for their physical stability, their chemical resistance to stringent cleaning conditions, and their low level of nonspecific interaction.

$$Na_2SO_4 > K_2SO_4 > (NH_4)_2SO_4 > Na_2HPO_4 > NaCl > LiCl ..... > KSCN$$

Fig 1.9. Relative effects of some salts on protein precipitation.

Matrix

1. A good balance between porosity and particle size offers a large surface area covered by ligands and ensures a high binding capacity. When separating large biomolecules, high porosity with an open pore structure is an advantage.
2. An inert matrix minimizes nonspecific interactions with sample components.
3. High physical stability ensures that the volume of the packed resin remains constant despite extreme changes in salt concentration or pH, helping to improve reproducibility and avoid the need to repack columns.
4. High physical stability and uniformity of particle size facilitate high flow rates, particularly during cleaning, and re-equilibration steps, to improve throughput, and productivity.
5. High chemical stability ensures that you can clean the matrix using stringent cleaning solutions if needed.
6. Modern HIC resins use agarose-based matrices to fulfill the requirements for chemical, and physical stability, high binding capacity, and different particle sizes (Table 1.1).

We based our Capto™, and Capto™ ImpRes modern resins on a chemically modified, highly cross-linked agarose matrix (Table 1.1). This matrix provides particle rigidity without compromising pore size, outstanding pressure-flow properties, and high chemical stability to support cleaning-in-place procedures. You can use Capto™ and Capto™ ImpRes resins for scaling up, and in large-scale bioprocess purifications, but also at lab scale, for research, and process development needs. To select the most suitable matrix, consider the degree of resolution, binding capacity, and flow rates you need. For example, gradient elution on Capto™ ImpRes resins (40 µm) will give a high-resolution separation, whereas the larger particles of Capto™ resins (75 µm) are best suited for high capacity, step elution at a high flow rate.

We offer our legacy Sepharose™ High Performance (34 µm) and Sepharose™ Fast Flow (90 µm) as alternatives to our modern resins. Sepharose™ matrices are based on hydrophilic chains of agarose, arranged in bundles, and with different degrees of intra-chain crosslinking. To choose between Sepharose™ High Performance (34 µm) and Sepharose™ Fast Flow (90 µm), consider the degree of resolution, binding capacity, and flow rates that you need.

Table 1.1. Matrices used for HIC resins

	Resin	Matrix and form	Particle size[1]
Modern resins	Capto™ ImpRes	Highly cross-linked agarose, spherical	40 µm
	Capto™	Highly cross-linked agarose, spherical	75 µm
Legacy resins	Sepharose™ High Performance	Cross-linked agarose, spherical	34 µm
	Sepharose™ 6 Fast Flow	Cross-linked agarose 6%, spherical	90 µm
	Sepharose™ 4 Fast Flow	Cross-linked agarose 4%, spherical	90 µm
	SOURCE™ 15	Spherical and monodisperse, porous, rigid, polystyrene/divinylbenzene particles	15 µm[2]

[1] Median particle size of the cumulative volume distribution.

[2] Mean particle diameter (monodisperse size distribution).

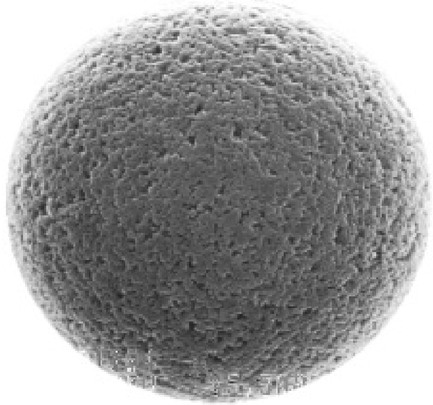


Fig 1.10. Highly cross-linked agarose, spherical bead (Capto™ resin).

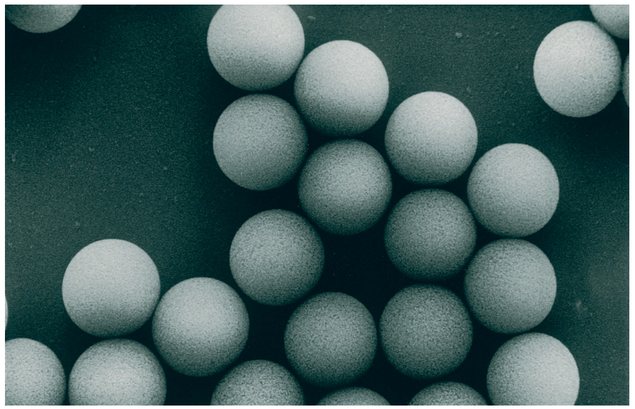


Fig 1.11. Electron micrograph of SOURCE™ showing spherical, monodispersed particles.

Over the years, scientists have used different matrices for HIC resins, for example, Sepharose™ Fast Flow or High Performance substituted with various ligands, and you can still find references to them in scientific literature. Some established processes still include them today. However, more recently developed Capto™, and Capto™ ImpRes resins are based on a very rigid, high-flow agarose base matrix with an optimized pore structure that offers outstanding pressure, and flow properties. If you want to benefit from significantly faster separations, and improved performance, you can test for selectivity on Capto™, and Capto™ ImpRes resins, and reoptimize old protocols.

A SOURCE™ matrix is made from polystyrene with divinylbenzene to produce highly spherical (monodispersed), small (15 µm), porous particles (Fig 1.11) that facilitate high-resolution separations at high flow rates.

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 resin and its selectivity. Figure 1.12 shows an example of a protein mixture separated on the same base matrix, but with four different ligand conditions; phenyl (high substitution), phenyl (low substitution), butyl, and octyl.

Ligands:	(A) Phenyl (high sub) (B) Phenyl (low sub) (C) Butyl (D) Octyl
Sample:	cytochrome C (1) 10 mg/mL, ribonuclease A (2) 30 mg/mL, lysozyme (3) 10 mg/mL, α-chymotrypsinogen (4) 10 mg/mL
Sample load:	2 mL in start buffer
Start buffer:	100 mM sodium phosphate, 1.5 M ammonium sulfate, pH 7.0
Elution buffer:	100 mM sodium phosphate,
Flow:	2 mL/min, 60 cm/h
Gradient:	0% to 100% elution buffer in 10 CV
Detection:	280 nm

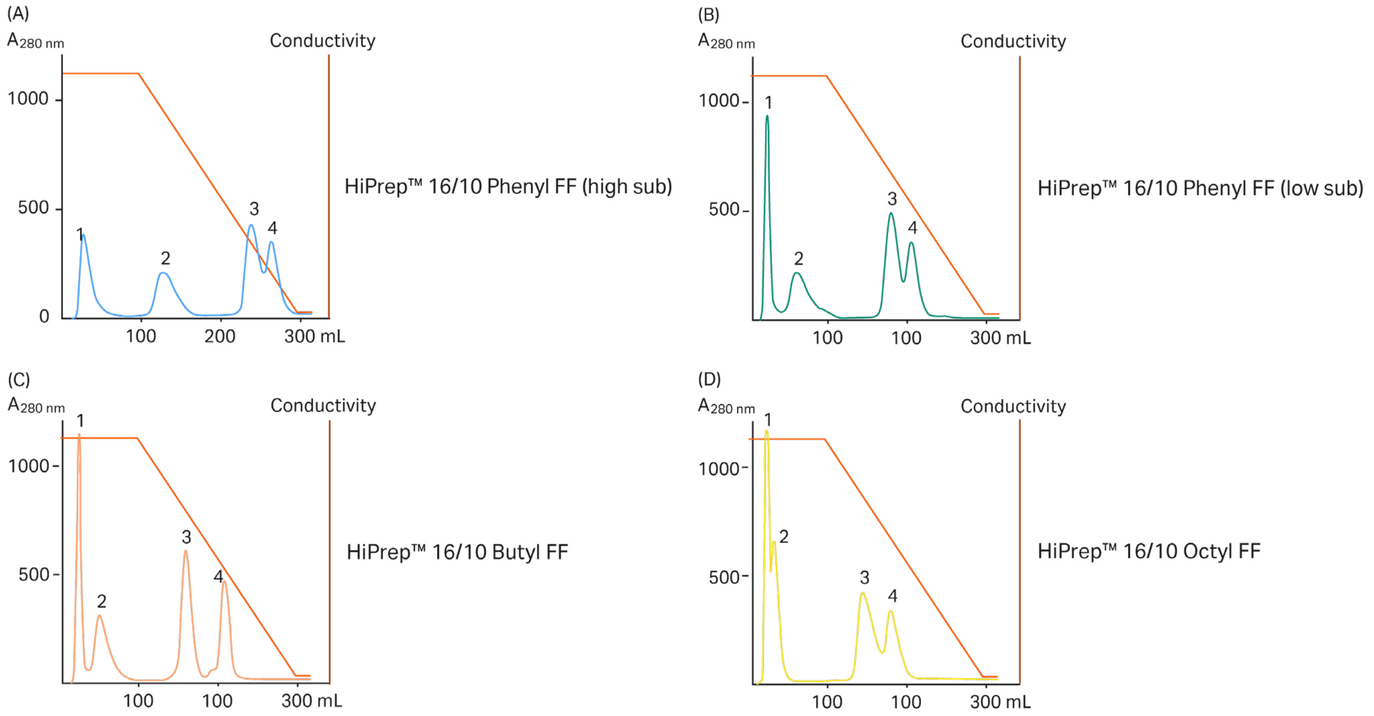


Fig 1.12. Different ligands and differences in ligand density influence the selectivity of a HIC resin.

Up to a certain level, the binding capacity of HIC resins increases as ligand density increases. Simultaneously, the strength of the interaction increases, which can lead to difficulties eluting bound components. Selecting a resin substituted with the same ligand, but at a lower ligand density can solve this problem. Figure 1.12 A and B show how the difference in ligand density between two resins can influence selectivity.

The most common hydrophobic ligands are shown in Table 1.2. In general, HIC resins 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 where both aromatic, and hydrophobic interactions, as well as lack of charge, play a role in their final chromatographic properties.

If your protein of interest does not bind in high salt conditions, use a more hydrophobic resin. If the protein of interest binds so strongly that you need nonpolar additives for elution, reduce the salt concentration in the start buffer or use a less hydrophobic resin.

Table 1.2. Ligands substituted on HIC resins

Phenyl	—O— 
Butyl-S	—S—$(CH_2)_3$—CH_3
Butyl	—O—$(CH_2)_3$—CH_3
Octyl	—O—$(CH_2)_7$—CH_3
Ether	—O—CH_2—CHOH—CH_2—OH
Isopropyl	—O—CH—$(CH_3)_2$

Selectivity and elution

Figures 1.13 and 1.14 show the most common forms of HIC separation where you elute proteins 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 (CV). For example, 5 CV = 5 mL for a column with a 1 mL bed volume. Using CV to describe a separation profile supports method development and method transfer to columns with different dimensions when scaling up.

We often use *gradient elution* (Fig 1.13) 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 and analysis.

We use *step elution* (Fig 1.14) in several ways. After optimizing a HIC separation using gradient elution, changing to a step elution speeds up separation times, and reduces buffer consumption while retaining the required purity level.

You can also use the same step elution for group separation to concentrate your proteins of interest, and rapidly remove them from unwanted substances. In this method, you elute the target protein(s) in an enriched, concentrated form.

Occasionally, you can use step elution to remove contaminants by choosing conditions that maximize contaminant binding and allow the target protein(s) to pass through the column. If you use this method, you should ensure that the binding capacity of the column is sufficient to bind all contaminants.

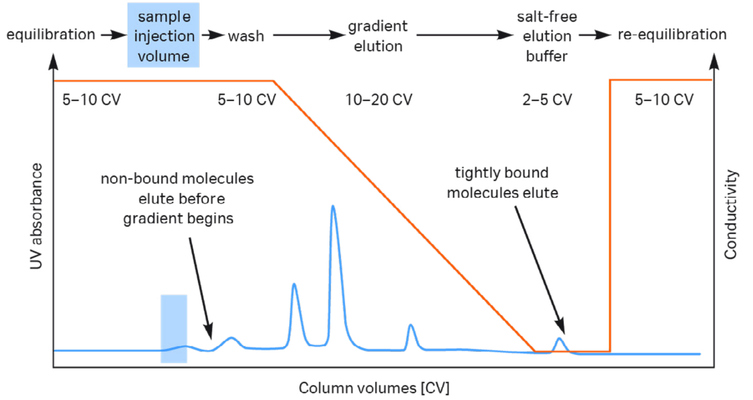


Fig 1.13. Typical high-resolution HIC separation using linear gradient elution.

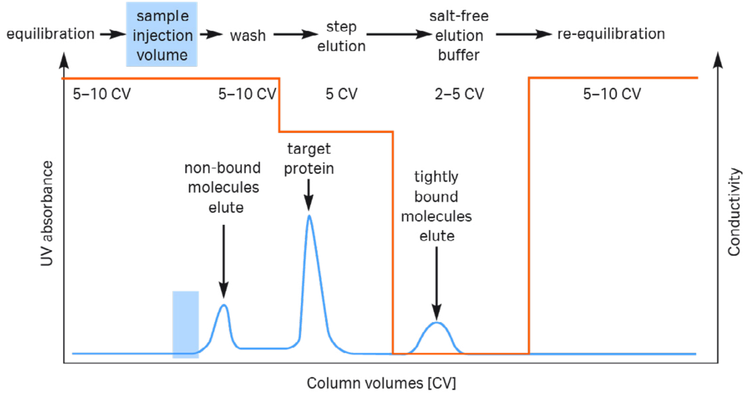


Fig 1.14. Typical HIC separation using step elution.

02

Hydrophobic interaction chromatography in practice

This chapter has practical advice on how to control experimental conditions for successful separation, and guidelines for selecting the good resin or prepacked column for each application. You can find additional application examples, and product-related information in Chapter 3.

Resin selection

We explain the origin, and differences between HIC resins in Chapter 1. Overall, many parameters influence the performance of a HIC resin (selectivity, resolution, and binding capacity): 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 will help you to perform every separation with the required resolution, throughput, and speed. With so many parameters to consider, we recommend prioritizing separation development steps as follows:

1. Screen to select resin with correct selectivity.*
2. Select the 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 the resolution or recovery is not optimal.

*Selectivity is the most important parameter when choosing a HIC resin. If more than one resin appears suitable, reselect according to the intended final scale of purification and purpose of the separation (capture, intermediate purification, or polishing; *see* Fig 2.1). Your choice of the matrix will often depend on the availability of a resin with the correct selectivity.

Keep the temperature constant throughout all separations.

HIC in a purification strategy (CIPP)

To ensure an efficient and reproducible protein purification that gives the required degree of purity, you should develop a multistep process that follows the strategy of capture, intermediate purification, and polishing (CIPP). We show this strategy in Figure 2.1 and describe it in more detail in Chapter 4.

Each phase has a specific objective that largely depends on the properties of the starting material. You can select a HIC resin according to the aim of the purification step and the condition of your starting material. The pharmaceutical industry and research laboratories often use CIPP for faster method development, to obtain a shorter time to pure product, and for a good economy.

You can use HIC for capture, intermediate purification, or polishing steps. Since your 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. HIC is also highly suitable for intermediate steps directly after an ion-exchange separation. In both situations, the sample is already in a high-salt solution and requires only the addition of more salt to complete preparation. Since a HIC separation concentrates the protein of interest into a reduced volume, you can also transfer fractions directly to size exclusion chromatography. You can use HIC with stepwise elution for a rapid capture step, or gradient elution to achieve the highest resolution in a polishing step.

An important first step for any purification is correct sample preparation. We cover this in more detail in Appendix 1, and Chapter 2. *See* Chapter 4 for more details on the use of CIPP steps in a purification strategy.

Capture

Resins for primary capture steps where the aim is to isolate, concentrate, and stabilize the target products, should offer high speed, and high capacity. For capture , we recommend HIC resins based on the Capto™ base matrix (75 µm particle size) — Capto™ Phenyl, Capto™ Butyl, or Capto™ Octyl — as it possesses good pressure, and flow properties (flows up to 600 cm/h).

Select Capto™ PlasmidSelect (40 µm particle size) for purification of supercoiled DNA.

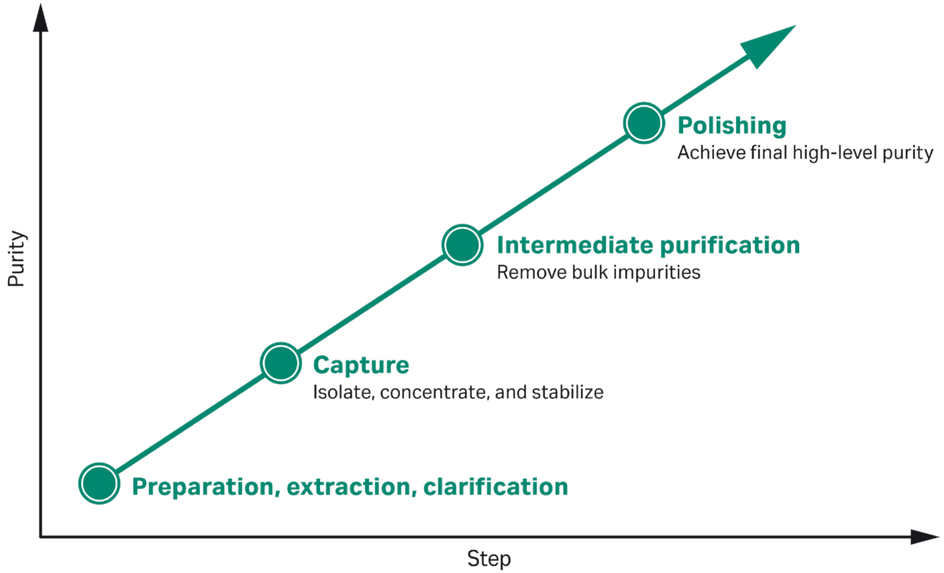


Fig 2.1. Sample preparation, extraction, clarification, and CIPP.

Intermediate purification

Resins for intermediate purification, where the aim is to remove most of the bulk impurities, should offer high capacity and high resolution. We recommend Capto™ ImpRes resins (butyl- or phenyl-based) with a smaller particle size (40 µm) and good pressure-flow properties.

Polishing

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

- Capto™ ImpRes (40 µm particle size): Select for polishing in the laboratory or large-scale applications that require high resolution, and throughput.
- If the required selectivity is not available in a resin of larger particle size, try SOURCE™ 15 (15 µm particle size) for HIC separation.

Practical considerations for HIC separation

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

Practical considerations

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

We highlight these steps along with more detailed hints, and advice throughout this section.

Buffer volumes are expressed in CV — for example, 3 CV = 3 mL for a column with a 1 mL bed volume. Using CV to describe a separation profile facilitates method development and transfer of methods to columns of different dimensions. Often, you can reduce the number of CVs at each stage of separation with optimization. For example, you can reduce the gradient volume if you can maintain the resolution, and you might need less buffer for washing when separating reasonably clean samples.

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

Fig 2.2. HiTrap™ Capto™ HIC Selection Kit and PreDictor™ Capto™ HIC Screening Kit.

Screening for selectivity

In the initial stages of development, you can save time, and sample by using small (1 mL), prepacked columns like those in the HiTrap™ Capto™ HIC Selection Kit (Fig 2.2), or 96-well plates prepacked with different Capto™ HIC resins like the PreDictor™ Capto™ HIC Screening Kit. You can quickly, and efficiently screen various resins for the required selectivity. This approach is helpful because — even if you know the properties of the target protein(s) — the final selectivity, binding capacity, and recovery depend largely on the resin's interaction with the specific protein of interest.

HiTrap™ Capto™ HIC Selection Kit contains resins based on high-flow agarose (Capto™ and Capto™ ImpRes):

- HiTrap™ Capto™ Phenyl (high sub)
- HiTrap™ Capto™ Butyl
- HiTrap™ Capto™ Octyl
- HiTrap™ Capto™ Phenyl ImpRes
- HiTrap™ Capto™ Butyl ImpRes

You can use all HiTrap™ columns for small-scale purification, and each includes detailed protocols for use. The resins in these test kits are available for large-scale production so that you can easily transfer optimized methods to the scale of operation you need.

PreDictor™ Capto™ HIC Screening Kit (Fig 2.2) supports high-throughput process development (HTPD) by allowing parallel screening of multiple chromatographic conditions using a 96-well plate format. It contains resins based on high-flow agarose Capto™ and Capto™ ImpRes:

- Capto™ Phenyl (high sub)
- Capto™ Butyl
- Capto™ Butyl-S
- Capto™ Octyl
- Capto™ Phenyl ImpRes
- Capto™ Butyl ImpRes

Before starting any HIC separation, establish the "salt stability window" for the sample. For example, add increasing amounts of salt to the crude sample to establish the concentration at which precipitation occurs. To avoid precipitation, make sure that the sample is below this salt concentration when applied to a column. *See* Appendix 1 for a guide to using ammonium sulfate for sample clean-up by precipitation. When possible, test the biological activity of the target protein to establish the concentration range over which activity can be maintained, and remember that you might need to desalt the sample before an activity test.

Automated HIC resin screening, method development, and optimization

AKTA™ chromatography system users can select method templates, and program the system to automatically perform separations using a range of columns and 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. To avoid precipitation caused by the high salt concentrations that can occur locally when adding salt as a solid, add salt from a high-concentration stock solution. Adjust the pH of the sample directly. HIC is not very sensitive to pH conditions, so 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 salt precipitation when storing columns in 20% ethanol.
4. Scout for optimum selectivity, applying the sample to each column under a range of salt concentrations. Collect eluate throughout the run. Choose a resin in which the target protein(s) elute within the gradient.
5. Scout for the lowest salt concentration in the start buffer that maximizes the binding capacity for the target protein, maintains or improves resolution, and minimizes contamination from other bound proteins.
6. Optimize for the steepest gradient that gives an acceptable resolution.
7. Optimize for the highest flow rate that maintains resolution and minimizes separation time. Check the recommended flow rates for the specific resin.
8. Optimize for the maximum sample load that you can apply while maintaining satisfactory resolution. In general, loading 20% to 30% of the total binding capacity of the column gives optimal resolution with gradient elution.

Once you optimize separation conditions, reduce separation time and buffer consumption by transferring to a step elution. You can often increase sample loads when using a step elution.

Figure 2.3 shows an example of resin screening on different HIC resins with various ligands and bead sizes prepacked in HiTrap™ 1 mL columns. To minimize the need for sample conditioning after capture, we kept the buffer pH at pH 5.0. We selected the resin with Phenyl (high sub) because this resin showed the best selectivity for the target protein. We eluted the protein within the gradient and separated it from the bulk contaminants. Then, we optimized the conditions so that we could use a step elution to maximize the throughput, and concentration of the target protein before scaling up (Figure 3.23, Chapter 3 shows the optimized elution scheme and subsequent scale-up).

Manual resin screening, method development, and optimization

You can use HiTrap™ columns with a syringe or peristaltic pump for manual resin screening, method development, and method optimization. However, using a syringe limits the degree to which you can develop a HIC separation because the separation mechanism is not a simple "on/off" process — to achieve a satisfactory separation, it requires some degree of gradient elution.

The methods here are optimized for use with 1 mL HiTrap™ columns, and you should adjust them if you are using another CV. Also, note that you might need to reduce flow rates depending on the viscosity of the sample or buffers.

Column:	HiTrap™ Phenyl HP HiTrap™ Phenyl FF (low sub) HiTrap™ Phenyl FF (high sub) HiTrap™ Butyl FF HiTrap™ Octyl FF
Sample:	Fab fraction from ion-exchange run,
Sample load:	2 mL
Column volume:	1 mL
Start buffer:	1 mL $(NH_4)_2SO_4$, 50 mM NaAc, pH 5.0
Elution buffer:	50 mM NaAc, pH 5.0
Flow:	2 mL/min, (300 cm/h)
Gradient:	0% to 100% elution buffer in 20 CV
System:	ÄKTA™ chromatography system

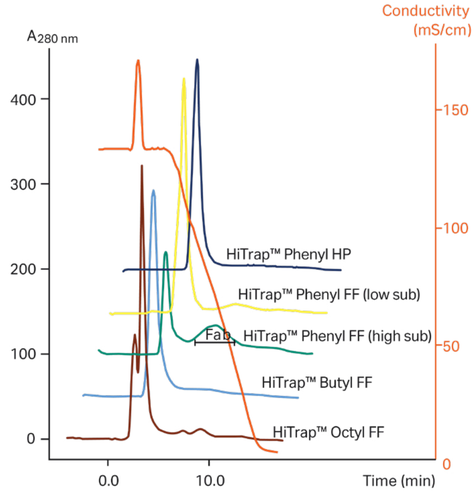


Fig 2.3. Resin screening on different HIC resins prepacked in HiTrap™ 1 mL columns.

Screening for selectivity using HiTrap™ Capto™ 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 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, 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 to 10 mL salt-free elution buffer at 1 mL/min. This avoids salt precipitation when columns have been stored in 20% ethanol.
4. Equilibrate the column(s) with 5 to 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 to 5 mL is usually sufficient, but other volumes might be required depending on the experiment conditions). Collect eluate.
8. Analyze all eluates (e.g., by activity assay), and determine purity and the amount bound to the column.
9. Select the resin to which the target protein(s) binds and can be eluted. If running a gradient, select the resin that gives optimal selectivity and resolution.

Screening for binding (salt) conditions

1. Using the selected resin 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 the lowest concentration of 0.05 M).
2. Repeat steps 2 to 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 you have gradient-making equipment, determine the steepest gradient that gives an acceptable resolution.
2. Begin with a gradient of 10 CV. 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 you determined during screening. Alternatively, begin with a gradient of 0% to 50% elution buffer and a 10 to 20 CV gradient volume.
3. To save time, determine the highest flow rate that maintains resolution, and minimizes separation time. Check the recommended flow rates for the specific resin.
4. Determine the maximum sample load that you can apply while maintaining resolution. In general, loading 20% to 30% of the total binding capacity of the column gives optimal resolution with gradient elution. You can often increase sample loads if the resolution is satisfactory or when using a step elution.

To reduce separation time and buffer consumption, you can transfer to a step elution after establishing optimized separation conditions. You can usually increase sample loads 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 when determining the selectivity of a HIC resin. This means you need to empirically determine the most suitable ligand through screening experiments, preferably using the target protein. Even if you assume certain proteins have remarkably similar properties, they can interact quite differently in identical experimental conditions in a HIC separation, as demonstrated by the behavior of three monoclonal antibodies in Figure 2.4.

Column:	HiTrap™ Phenyl HP, 1 mL
Samples:	1) Monoclonal antibody anti-lac cl 507, 8.8 mg/mL, 100 µL 2) Monoclonal antibody anti-trn cl 739, 1.0 mg/mL, 500 µL 3) Monoclonal antibody anti-TSH cl 79, 6.7 mg/mL, 100 µL (All three monoclonal antibodies are pure and a kind gift from Pharmacia Diagnostics AB, Uppsala, Sweden)
Sample preparation:	Dilution 1:1 (v/v) with start buffer
Start buffer:	50 mM NaH_2PO_4, 1.0 M $(NH_4)_2SO_4$, pH 7.0
Elution buffer:	50 mM NaH_2PO_4, pH 7.0
Gradient:	0% to 100% elution buffer in 15 CV
Flow:	1.0 mL/min (156 cm/h) at room temperature

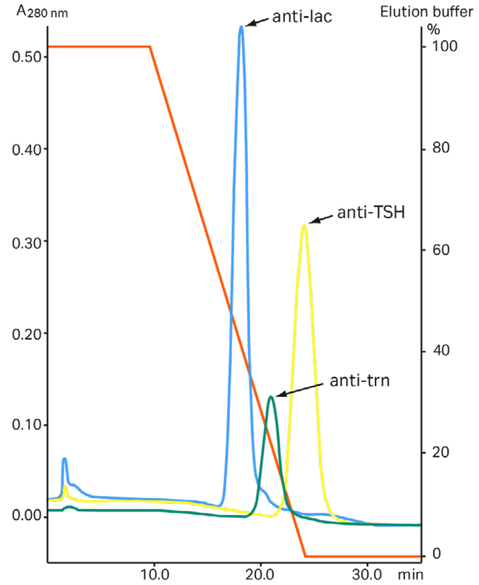


Fig 2.4. Three monoclonal antibodies interact differently under identical running conditions using a phenyl ligand. A suitable ligand must be determined empirically.

Salt selection and buffer preparation

Salts

In HIC the binding process is more selective than the elution process, making it essential to optimize the conditions of your start buffer. The right 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 most 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 commonly used salts are $(NH_4)_2SO_4$, Na_2SO_4, NaCl, KCl, and CH_3COONH_4. Figure 2.5 shows an example of how different salts can affect selectivity. Here we obtained the best resolution of four standard proteins using 1.7 M ammonium sulfate in the start buffer.

As with resin selection, the choice of salt for a HIC separation is 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 exponentially at higher concentrations.

- At a given concentration, ammonium sulfate often gives a good resolution when compared to other salts, and you can use it at concentrations up to 2 M.
- You usually need concentrations up to 3 M when using sodium chloride.
- Sodium sulfate is a good salting-out agent, but problems with protein solubility can exclude its use at high concentrations.
- We do not recommend ammonium sulfate when working above pH 8.0.

Column: HiTrap™ Butyl FF, 1 mL
Sample: 1 mg/mL cytochrome C, 1 mg/mL lysozyme, 1 mg/mL α-chymotrypsinogen, 3 mg ribonuclease A
Sample load: 6 mg in 1 mL start buffer
Start buffer: (A) 0.1 M sodium phosphate, 1.7 M ammonium sulfate, pH 7.0
(B) 0.1 M sodium phosphate, 1 M sodium sulfate, pH 7.0
(C) 0.1 M sodium phosphate, 3 M NaCl, pH 7.0
Elution buffer: 0.1 M sodium phosphate, pH 7.0
Flow: 0.5 mL/min, 75 cm/h
Gradient: 0% to 100% elution buffer in 10 CV
System: AKTA™ chromatography system
Detection: 280 nm

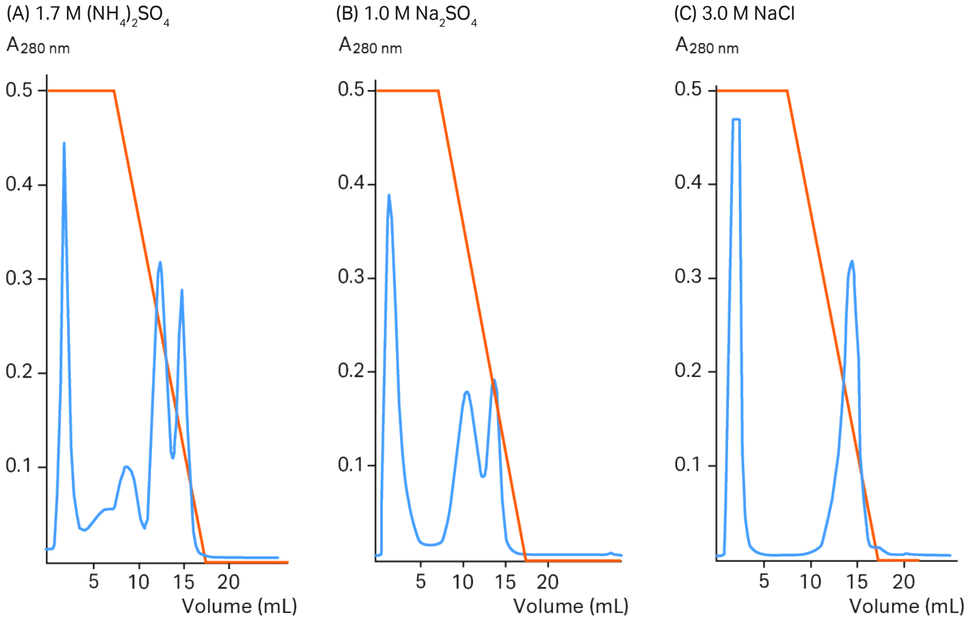


Fig 2.5. Different salts influence selectivity: elution takes place in the order of increasing elution volumes; cytochrome C, lysozyme, ribonuclease A, α-chymotrypsinogen.

Salt concentration

Figure 2.6 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 resin is satisfactory because 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 a similar resolution but ensures that contaminants that bound during earlier runs (with a higher salt concentration) 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 does not bind strongly enough during sample application, resulting in significant peak broadening during elution.

If the target molecule elutes too late or not at all, and you cannot switch to a different resin, try binding in 50% less salt.

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

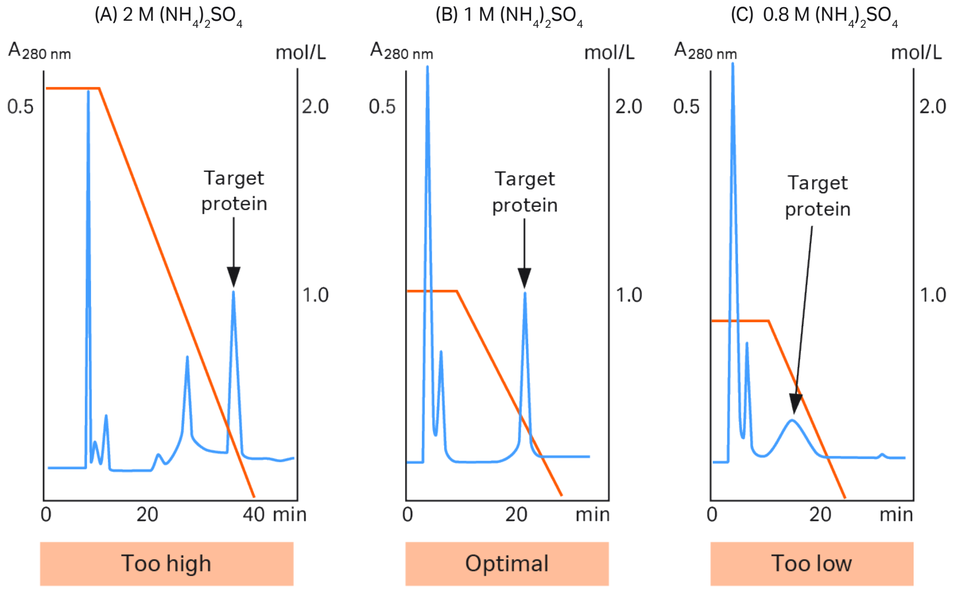


Fig 2.6. Salt concentration in the start buffer influences selectivity and resolution.

Buffer ions and pH

Your choice of buffering ions is not critical to hydrophobic interaction. Most often, you may use phosphate buffers.

The pH chosen must be compatible with protein stability and activity and you should check for optimum pH conditions for each of your applications. However, between pH 5.0 to 8.5, pH values have less impact 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 you are using during the separation, especially if recovery of biological activity is a priority. Avoid extreme changes in pH or other conditions that can cause inactivation or even precipitation.
- Use a buffer concentration, typically 20 to 50 mM, that is sufficient to maintain buffering capacity pH during sample application, and changes in salt concentration.
- Transfer the purified protein into a volatile buffer if you are planning to lyophilize the product (Table 2.1).
- Prepare buffers at the same temperature at which they will be used to ensure the correct pH.
- Filter buffers, and samples after including all salts, and additives. Use high-quality water, and chemicals. Use 1 µm filters for resins with particle sizes above 75 µm, 0.45 µm filters for 40 µm particles, and 0.22 µm filters for particles below 15 µm or when sterile or extra-clean samples are required. To avoid forming air bubbles in a packed column, and to ensure reproducible results, keep the column, and buffers sat 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

Table 2.1. Suitable volatile buffer systems

pH	Buffer system	pK_a values for buffering[1]
3.3 to 4.3	Formic acid	3.75
3.3 to 4.3; 4.8 to 5.8	Formic acid / Pyridine	3.75; 5.25
3.3 to 4.3; 8.8 to 9.8	Formic acid / Ammonia	3.75; 9.25
3.3 to 4.3; 9.3 to 10.3	Formic acid / Trimethylamine	3.75; 9.81
4.3 to 5.8	Acetic acid / Pyridine	4.75; 5.25
4.3 to 5.3; 7.2 to 8.2	Acetic acid / N-ethylmorpholine	4.75; 7.72
4.3 to 5.3; 8.8 to 9.8	Acetic acid / Ammonia	4.75; 9.25
4.3 to 5.3; 9.3 to 10.3	Acetic acid / Trimethylamine	4.75; 9.81
5.9 to 6.9; 8.8 to 9.8	Hydrogen carbonate / Ammonia	6.35; 9.25

[1] Ref: Handbook of chemistry and physics, 83rd edition, CRC, 2002–2003.

Buffer additives

You can use additives to improve selectivity and resolution, for example, when a protein binds too strongly to a HIC resin. However, if you use them at high concentrations, you risk 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. You can find a list of typical additives in Table 2.2.

Run blank elution gradients with additives including checking their effect on the elution profile. That is, perform a run without loading any sample.

Column and resin preparation

Equilibrate the column with 5 to 10 CV of start buffer or until UV baseline and conductivity is stable.

Use prepacked columns to ensure good performance and reproducible results. An evenly packed column ensures that component peaks do not broaden too much as the sample passes down the column, helping you get a good resolution.

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

Wash away storage solutions and/or preservatives before using any HIC resin. Wash columns with 10 CV of salt-free elution buffer before equilibrating with start buffer to avoid the risk of ethanol in the storage solution that leads to salt precipitation.

See Appendix 2 for details on column packing. The volume required for the packed bed depends on the amount of sample you are purifying and the resin's binding capacity. Pack a column with a five 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).

Table 2.2. 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 to 80 v/v % ethylene glycol	Alter buffer polarity. Decrease the surface tension of water to weaken the interaction, and cause dissociation. Nonpolar regions compete with the proteins for the hydrophobic ligands, causing dissociation.
Chaotropic salts	$MgCl_2$ $CaCl_2$ KI NaCNS up to 8 M urea	Decrease the hydrophobic effect in solution to weaken the interaction, and cause dissociation. Can also affect the conformation of the protein. Ca^{2+} increases stability during the purification of calcium-binding proteins; Mg^{2+} decreases stability.

Sample preparation

Correct sample and buffer preparation are essential to achieving optimal separation and maintaining column performance. There are simple steps you can take to clarify any sample before applying it to a column that will help avoid the risk of blockage, reduce the need for stringent washing procedures, maintain column performance, and avoid increases in backpressure. Appendix 1 outlines sample preparation techniques in more detail.

In HIC, the initial binding conditions also affect the final selectivity of the separation. Before starting any separation, establish the salt-stability window of the sample. For example, add increasing amounts of salt to the crude sample to determine the concentration where precipitation occurs. To avoid blockage, ensure that the sample is below the salt precipitation concentration when applying it to the column. *See* Appendix 1 for a guide to using ammonium sulfate in precipitation experiments.

When possible, test for the target protein's biological activity to establish the concentration range (remember that you might need to reduce high salt concentrations before assaying for activity). After establishing the salt stability window, begin with the highest salt concentration that retains biological activity without causing precipitation problems. You should keep the salt content and pH of the sample the same as those of the start buffer to ensure optimal binding conditions.

HIC requires little sample preparation work. Binding mostly takes place because of the high salt conditions, and the technique is fairly insensitive to pH conditions. You do not need to exchange the sample buffer before applying the sample to a HIC column; just ensure you have enough salt, and directly adjust the pH if needed. Adding salt as a solid can lead to local, high salt accumulations and precipitation – to avoid this risk, add salt from a high concentration stock solution.

- Make sure your samples are clear, and free from particulate matter, especially if you are using resins with particle sizes 40 µm or less. To filter small sample volumes, you can use a syringe-tip filter of cellulose acetate, PVDF, or regenerated cellulose. For example, use Protein Prep syringe filters for AKTA™ systems. Filter your samples after adding salt and any other additives.
- Ensure that the sample is at the same temperature as buffers, columns, and chromatographic equipment.
- Use buffer exchange (*see* Buffer exchange in Appendix 1) to remove chaotropic agents like guanidine hydrochloride and urea as they will inhibit hydrophobic interaction.
- If the 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, but keep the concentration of the start buffer.
- Lipids and other hydrophobic substances in the sample can interact with the resin, reducing binding capacity during the run, and in subsequent runs. Using a slightly less hydrophobic resin (such as Capto™ Butyl-S) as a "precolumn" is one way to remove these contaminants before the main separation.

Sample concentration and viscosity

Viscosity varies with temperature and will increase at very high salt concentrations. A sample's solubility or viscosity can limit the quantity that you can apply to a column. High sample viscosity can cause an irregular flow pattern that results in broad, distorted peaks, and problems with backpressure. The critical parameter is the viscosity of the sample relative to the viscosity of the eluent.

Dilute viscous samples. If you cannot use dilution, try using a lower salt concentration or a resin with a larger particle size. If nucleic acid contaminants are causing high viscosity, *see* Appendix 9 for advice on their removal.

Samples should generally not exceed 50 mg/mL protein, but this can vary depending on your type of sample, and chromatography resin.

Sample application and wash

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

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

For efficient binding, you should keep the sample at the same salt concentration as the start buffer. Since the sample binds near the top of the column, you can use a relatively large sample volume without affecting the separation if the application conditions are correct.

Apply the samples directly to the column using a chromatography system, peristaltic pump, or syringe. The choice of equipment depends on your sample volume, size, type of column, type of HIC resin, 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 you can apply to a column depends on the resin's binding capacity and the degree of resolution you need. Binding capacity is determined by the resin, protein properties, 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.

The width of the peaks is directly related to the amount of substance present, making sample load a major influence on resolution. To achieve good resolution, keep the total amount of protein bound to the resin below the total binding capacity of the packed column.

For optimal resolution with gradient elution, you can apply up to 30% of the total binding capacity of the column. You can increase the sample loads if the resolution is satisfactory or when you are using a step elution.

For example, you can increase capacity by decreasing flow rates, or by optimizing the start conditions to favor target protein(s) binding and minimize contaminant binding.

Capacity decreases with increasing flow rates, so you need to find a balance between the maximum dynamic binding capacity (DBC), and a fast separation, especially with large sample volumes.

Capacity also decreases for molecules with a very large diameter or length – for example, protein complexes > M_r 400 000, asymmetric proteins, and DNA. These molecules cannot penetrate the matrix pores, which limits 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 depending on the buffer conditions, there is no distinct molecular weight cut-off point when molecules can or cannot penetrate the matrix pores.

Sample volume

As long as the salt content of your sample and start buffer ensure adequate binding conditions, the binding technique of HIC is independent of sample volume. You can apply large volumes of dilute solutions like fractions from a cell culture supernatant directly to a HIC resin without prior concentration.

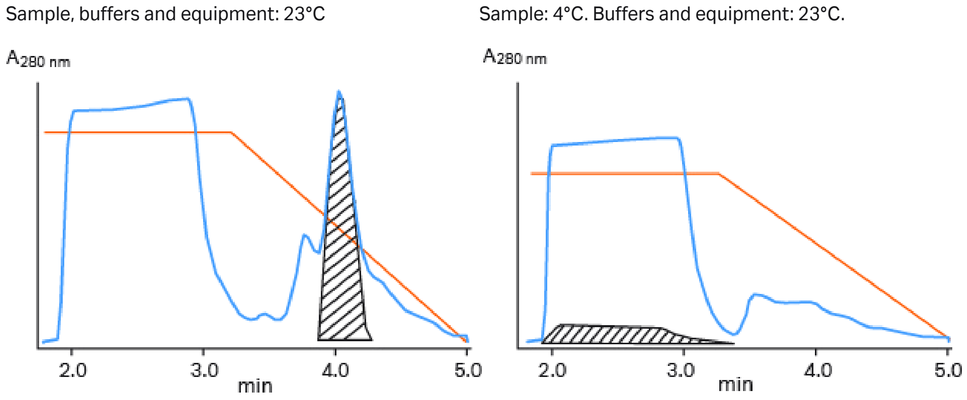
Temperature

The role temperature plays is complex. In practice, this means that working at a constant temperature improves reproducibility and that you might not be able to reproduce a separation developed at room temperature in cold-room conditions or *vice versa*.

Figure 2.7 demonstrates the importance of having your sample, start and elution buffers, columns, and chromatographic equipment at the same temperature. We performed both separations at room temperature (23°C) in identical conditions except for the temperature of the sample (23°C or 4°C). With all components at the same temperature, we bound the target protein and eluted it in the middle of the gradient. With the samples at 4°C, the target protein eluted in the flowthrough.

In most cases, increasing the temperature enhances hydrophobic interactions. This means that working at lower temperatures (typically below 10°C) can minimize the aggregation caused by hydrophobic interactions between sample components. To improve solubility, you can try lowering the temperature instead of adding detergents.

Ensure that the sample, column, start and elution buffers are at the same temperature. Note that temperature also affects the viscosity of your sample and buffers.



Column: RESOURCE™ ISO, 1 mL
Sample: Monoclonal antibody in crude ascites fluid
Start buffer: 0.05 M sodium phosphate, 1.25 M ammonium sulfate, pH 7.0
Elution buffer: 0.05 M sodium phosphate, pH 7.0
Detection: 280 nm

Fig 2.7. Influence of temperature on a HIC separation.

Elution

Researchers elute bound proteins with controlled decreases in salt concentration, using a linear or step elution selected according to the aim of the separation:

1. Linear gradient elution
    a. High-resolution separation or analysis
    b. Determination of conditions for a step elution
    c. Optimized gradient elution at an increased speed while retaining required resolution
2. Step elution
    a. Faster separation time, reduced buffer consumption
    b. Group separation

Linear gradient elution

Aim: High-resolution separation, resin screening, screening for optimal salt conditions

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

You can usually use linear salt gradients for elution, as shown in Figure 2.8. 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 the 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.

We recommended using linear gradient elution during method development. With a suitable chromatography system, you can easily prepare reproducible linear salt gradients. After, you can use the results as a basis for optimizing the separation.

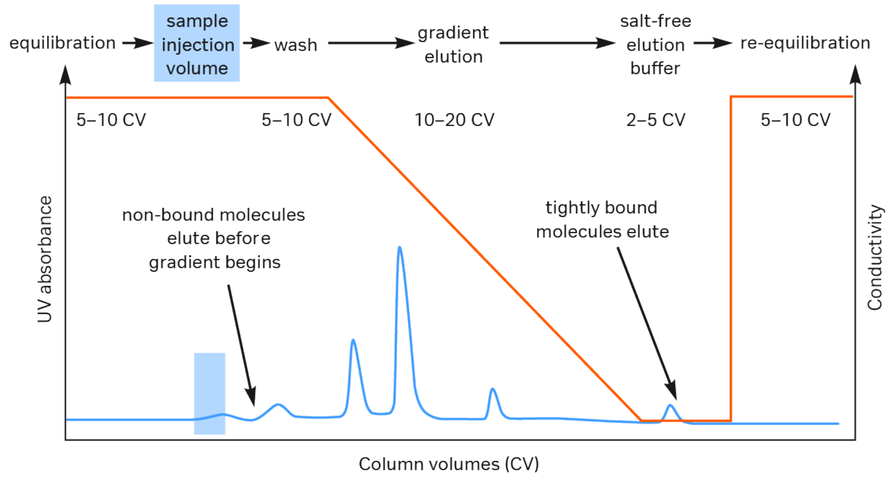


Fig 2.8. Typical HIC separation using linear gradient elution. The UV (protein), and conductivity (salt) traces show the elution of protein, peaks, and the changes in salt concentration during elution.

There are a few ways that changes in gradient elution can alter the retention of hydrophobic proteins on resins:

- 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 that elute earlier.

Select the steepest gradient to give an acceptable resolution. Figure 2.9 shows the effects of changing gradient slope.

If you decrease gradient elution volumes, you might need to proportionally decrease the sample load to maintain the same resolution. If you increase the sample load (within the total binding capacity of the column), you might need to increase gradient volumes to maintain resolution.

You can easily form gradients using purpose-designed equipment like ÄKTA™ chromatography systems that automatically control the mixing of solutions you supply to a column. Alternatively, some systems 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 you form gradients accurately.

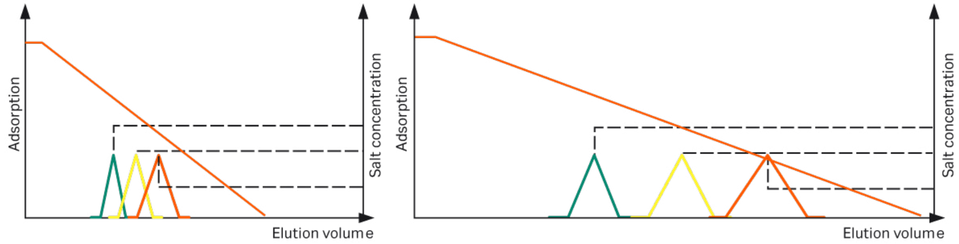


Fig 2.9. Schematic chromatograms show the effect of decreasing gradient slope.

Step elution

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

As shown in Figure 2.10, you may perform step elution by sequentially adding the elution buffer with decreasing salt concentration:

Step 1: Optimize the salt concentration, and volume of elution buffer to elute all compounds that bind less strongly to the resin than the target protein(s). Note: The salt concentration and buffer volume should be large enough to elute the contaminating weaker binding substances but keep salt concentration low enough to coelute the peak of interest.

Step 2: Decrease the salt concentration until the target protein(s) elutes.
Note: You should keep the salt concentration low enough to elute the target protein(s) without excessive dilution but keep it high enough to prevent the coelution of strongly bound contaminants.

Step 3: Further decrease the salt concentration to elute all remaining contaminants. You can also use water at this step.

Step 4: Re-equilibrate the column in the start buffer to prepare for the next run.

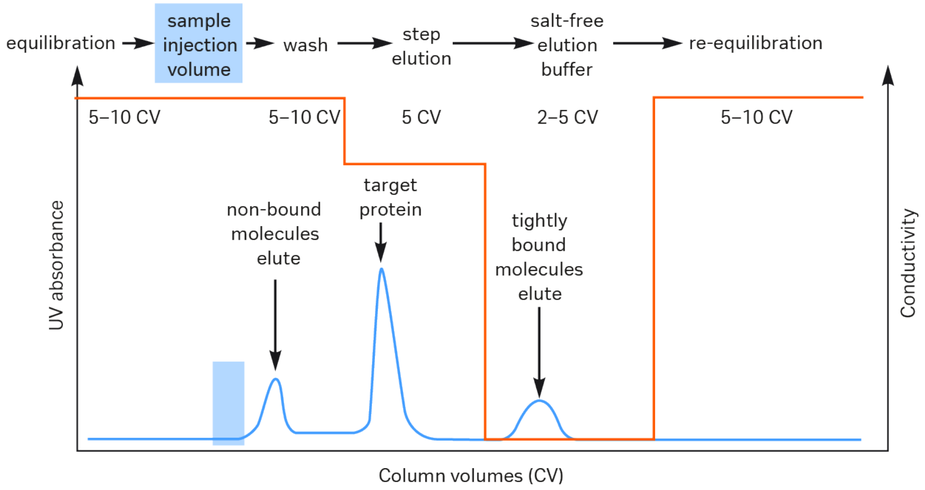


Fig 2.10. Typical HIC separation using step elution. The UV (protein), and conductivity (salt) traces show the elution of protein peaks and the changes in salt concentration during elution.

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

After you have optimized a HIC separation using gradient elution, changing to a step elution can reduce the total number of CVs needed for separation. This can speed up separation times and reduce buffer consumption while helping you get the purity level you need. Scientists often use step elution like this for routine, large-scale separation.

Aim of a step elution: group separation

In a group separation, you can separate target proteins, and contaminants in one step elution. Due to their hydrophobic properties, you can bind proteins and contaminants to the column, concentrate them, and rapidly separate them.

As in a normal step elution, you should choose conditions that will maximize the binding of the target proteins (or known contaminants) during sample application. You can either collect the fraction of interest in the wash or elute it in an enriched, concentrated form. In both cases, you only need a single buffer change.

Stepwise elution can be good for small-scale applications. If you keep the eluting strength of the buffer high enough to avoid co-elution with more strongly bound contaminants, you can elute the target protein in a more concentrated form.

Using a step elution with optimized salt concentration during sample application (to minimize binding of contaminants) often significantly improves the purity level of your final product.

Step elution can be a technically simple alternative if you do not have gradient-making equipment available. However, you should take care when designing the steps and interpreting your results. Substances eluted by a sharp change in salt conditions can elute close together, giving a false peak that contains several components. Since they frequently have more than one component, peaks tend to have sharp fronts and pronounced tailing. Because of this tailing, false peaks can appear if you introduce a change in salt concentration too early.

We recommend using a linear salt gradient when developing a new method. After characterizing the chromatographic behavior of the target protein(s), it will be easier for you to use a step elution to increase resolution in the area where the peak of interest elutes.

Flow rates

You can vary the maximum flow rate depending on the stage of the separation. For example, lower flow rates allow time for binding or elution, while higher flow rates can save time during equilibration, washing, and re-equilibration. The rigidity of the resins and pressure specifications of your equipment will primarily limit the flow rates that you can use.

We recommend flow rates for each HIC resin (*see* Chapter 3). During separation, aim for the highest flow rate that maintains resolution, and minimizes separation time. For example, if the peaks are well separated at a low flow rate, increase the flow rate to save time. Alternatively, if the peaks are well resolved, you might be able to load more samples and achieve a higher capacity without losing significant resolution. Figure 2.11 shows an example of how increasing flow rate can influence resolution on a HIC column.

You may measure flow rate in simple linear terms like mL/min — but when scaling up or comparing results between columns of different sizes, it is useful to use linear flow rate (flow velocity): cm/h (*see* Appendix 4). As far as the effects of flow rate are concerned, you can compare results obtained at the same linear flow on different size columns.

Do not exceed the maximum recommended flow for the resin or prepacked column.

Higher flow rates and viscous buffers increase operating pressure and 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 (instead of a peristaltic pump) to take full advantage of the rigidity, and excellent flow properties of resins like Capto™ and Capto™ ImpRes.

Always pump buffer onto a column instead of drawing the buffer through the column with the pump below. This reduces the risk of bubble formation that suction can cause.

If you are using a column that was packed in normal laboratory conditions, always use a lower flow rate for separation than the flow rate you used for column packing. This helps you apply your sample without increasing the pressure and shrinking the column bed. *See* Appendix 2 for column packing information.

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% to 100% B, 20 CV

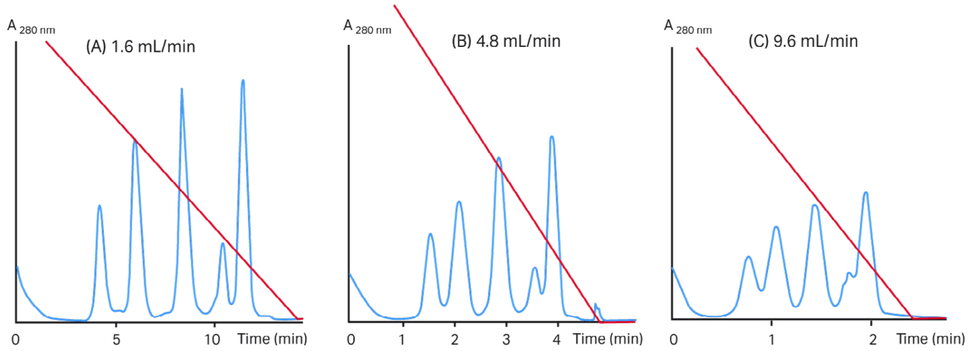


Fig 2.11. Influence of increasing flow rate on resolution when separating a model protein mixture on RESOURCE™ PHE column.

Wash and re-equilibration

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

You can effectively elute most bound proteins by washing the HIC resins with a salt-free buffer solution. Include a salt-free wash step at the end of each run to remove any molecules that are still bound to the resin. Keep an eye on the UV absorbance so that you can shorten or lengthen the wash step as needed.

Sometimes, the hydrophobic interaction is so tight that you might need to use harsher conditions to remove all bound material — for example, 0.5 to 1.0 M NaOH, 70% ethanol, or 30% isopropanol. You need to follow these wash steps with water or salt-free buffer wash before re-equilibrating the column with a high-salt start buffer.

Re-equilibrate with 5 to 10 CV 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 the conductivity to see when your start conditions are reached. Then you can shorten or lengthen the re-equilibration step as needed.

You might need to clean your resins if you observe a colored band at the top of the column, a space between the upper adapter and the bed surface, a loss in resolution, or a significant increase in backpressure. We provide a general cleaning procedure for each HIC resin in Chapter 3 and outline procedures for removing severe contamination (*see* Appendix 9). In all cases, prevention is better than cure — we recommend cleaning your resins routinely.

Analyzing results and further steps

Analyzing the results of your first separation will help you see if you can improve the conditions 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 contain a range of salt concentrations. If your assay is sensitive to changes in salt concentration, be sure to dilute or desalt fractions before analysis. We outline common analytical in Appendix 7.

Scaling up

For fast separations, it can be easier to repeat separation several times on a small column and pool the fractions of interest instead of scaling up to a larger column. However, you might want to use a larger column if you are routinely processing large sample volumes. Table 2.3 shows general guidelines for scaling up and Fig 2.12 shows available columns and other formats from Cytiva.

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 a small scale.
2. Maintain the bed height, sample concentration, and ratio of sample volume: volume of resin.
3. Increase the column volume by increasing the diameter of the column.
4. Run the separation at the same flow velocity (*see* Appendix 4) that you used on the smaller column with the same ratio of gradient volume: column volume.

Whenever possible, develop your separation method on the resin that you are planning to use at a larger scale.

If you are performing production-scale separations, note that all the HIC resins mentioned in this handbook (Capto™, Capto™ ImpRes, Sepharose™ High Performance, Sepharose™ Fast Flow, and SOURCE™) meet the needs for throughput and cleaning-in-place (CIP) at industrial scale.

See Appendix 2 for column selection and column packing.

Equipment selection

See Appendix 3 for a guide to selecting systems for HIC.

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

Table 2.3. Guidelines for scaling up

Maintain	Increase
Column bed height	Column volume (column diameter)
Flow velocity (cm/h)	Volumetric flow rate (mL/min)
Sample concentration	Sample load
Gradient elution volume (column volumes used for the gradient)	

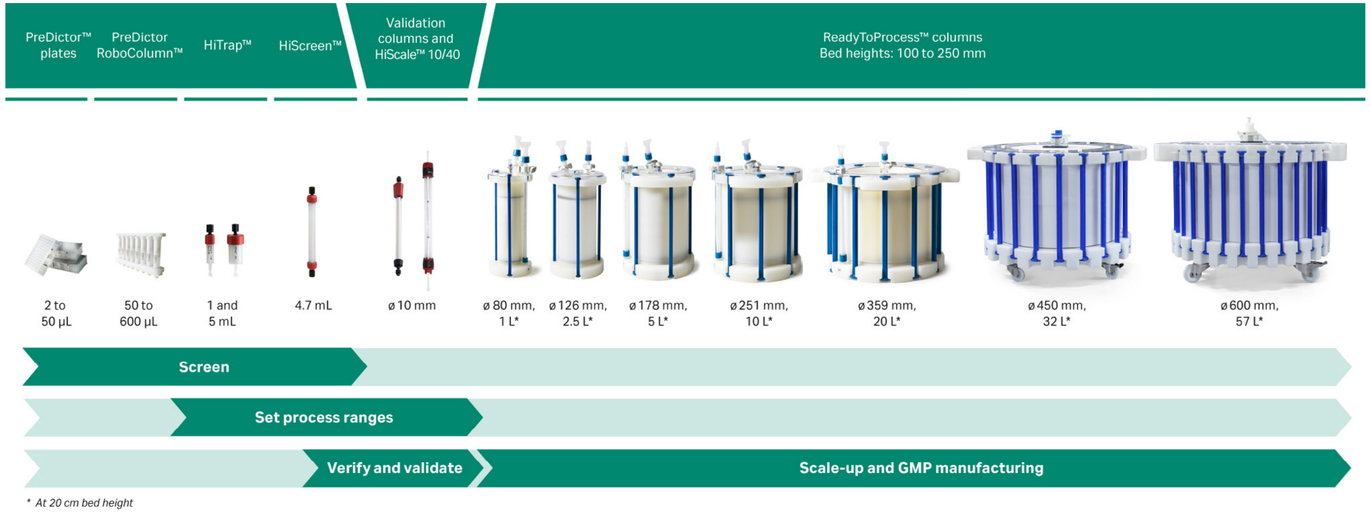


Fig 2.12. The variety of columns available in Cytiva is based on your manufacturing requirements.

Caring for HIC resins

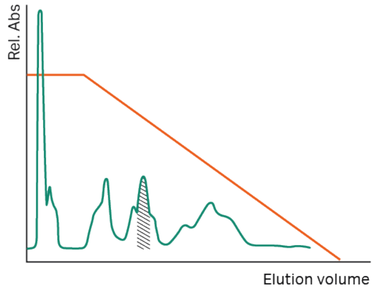
Washing between each separation with 5 to 10 CV of distilled water should be enough to keep your resin in good condition. However, using columns for some time can allow for precipitated proteins or other contaminants to build up.

To know if your columns need cleaning, look for a colored band at top of the column, a space between the upper adapter, and the bed surface, a loss in resolution, or a significant increase in backpressure. We provide a general cleaning procedure for each HIC resin (*see* Chapter 3), along with procedures we recommend for removing severe contamination, *see* Appendix 9.

Remember that prevention is better than cure — we always recommend cleaning your resin routinely. *See* Appendix 1 for more details on sample preparation.

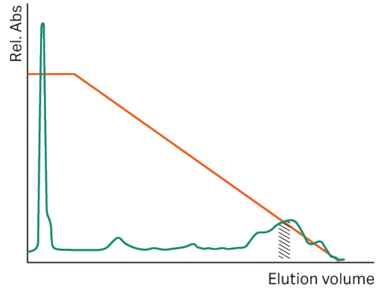
- Always use filtered buffers, samples, and cleaning solutions to reduce the need for additional column maintenance.
- Always degas buffers, and keep your buffers, columns, and samples at the same temperature to avoid bubble formation in the column and ensure reproducible results.
- If you see an increase in backpressure either on the pressure monitor or by the surface of the resin moving downward, check whether the column is causing the problem before starting the cleaning procedure. Starting at the fraction collector, disconnect one piece of tubing at a time, and check the pressure after disconnecting each piece. A blocked online filter can often cause back pressure to increase. Be sure to check the backpressure at the same stage of each run, because the value can vary within a single run or when changing to a different buffer.
- Store your resins and packed columns in 20% ethanol to prevent microbial growth.

Troubleshooting



The desired HIC separation: Gradient elution resolves the target protein.

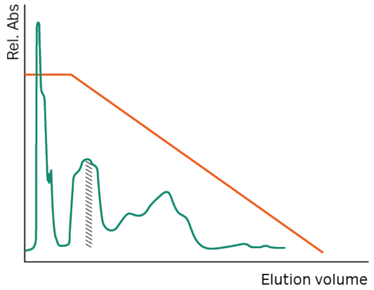
The rest of this section focuses on ways you can troubleshoot a suboptimal HIC separation.



Target protein elutes near the end of the gradient: Poor resolution.

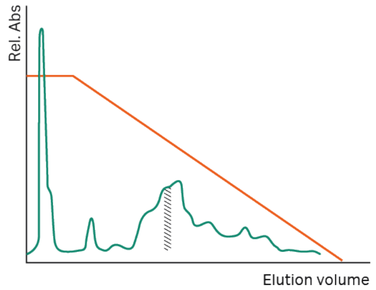
Repeat the separation with a lower salt concentration in the start buffer or use salt with a lower salting-out strength (*see* the Hofmeister series on Chapter1). If neither improves selectivity, try using a resin with a different ligand or with a lower degree of ligand substitution.

A decrease in the initial salt concentration weakens the binding, resulting in earlier elution of the protein. But since the contaminants elute very close to the protein of interest, this might not improve selectivity.



Target protein elutes early in the gradient: Poor resolution.

Repeat the separation at a higher salt concentration in the start buffer, or use a salt with a higher salting-out strength (*See* the Hofmeister series on Chapter1). If neither improves selectivity, try using a resin with a different ligand or with a higher degree of ligand substitution.



The target protein is eluted in the middle of the gradient: Poor resolution.

Optimize the gradient around the target protein. For example, you can use a segmented gradient with a shallower region around the target protein. Also, consider using additives (see additives in Chapter2). If this does not improve the resolution, try using an alternate chromatography technique like ion exchange for further purification.

Table 2.4. Overall guidance on various scenarios in handling HIC columns

Situation	Cause	Remedy
Reduced or no flow through the column.	The outlet is closed, or the pumps are not working.	Open the outlet. Check the pumps for signs of leakage. If you are using a peristaltic pump, check the tubing as well.
	Blocked filter, end-piece, adapter, or tubing.	Remove, and clean the blocked piece or replace it if possible. Always filter samples, and buffer before use.
	Lipoproteins or protein aggregates have precipitated.	Remove lipoproteins, and aggregates in sample preparation or use a scavenger column with lower hydrophobicity (*see* Appendix 1). Follow the cleaning procedures in Appendix 9.
	Protein precipitation in the column.	Reduce salt concentration in the start buffer. Follow cleaning procedures to remove precipitated proteins (*see* Appendix 9).
	The sample is too viscous.	Dilute with buffer. Keep protein concentration below 50 mg/mL.
	Microbial growth as occurred in the column.	Follow the cleaning procedures in Appendix 9. Always filter buffers. Store with 20% ethanol to prevent microbial growth when not in use.
The peak of interest resolves poorly from other major peaks.	Large mixing spaces at the top of the after column.	Adjust the top adapter to the resin surface if necessary. Reduce all post-column volume.
	Incorrect salt conditions.	Check binding conditions. Prepare new solutions.
	Suboptimal elution conditions (e.g., gradient too steep; flow rate too high).	Alter elution conditions by using a shallower gradient and reducing the flow rate.
	Poorly packed column.	Check column efficiency (*see* Appendix 2). Repack if needed. Use prepacked columns.
	Column overloaded with the sample.	Decrease sample load.
	Precipitation of proteins in the column.	Follow cleaning procedures (*see* Appendix 9). Reduce salt concentration in buffer or apply sample in aliquots at lower salt concentration while maintaining salt in the buffer.
Proteins do not bind or elute as expected.	Incorrect salt conditions.	Check conditions required. Prepare new solutions.
	Proteins or lipids have precipitated on the column or column filter.	Clean the column and replace or clean the filter. Check pH and salt stability of the sample.
	Sample changed during storage. Protein might be unstable or inactive in the elution buffer.	Prepare fresh samples. Determine the stability of the protein.
	Incomplete column equilibration.	Repeat or lengthen equilibration until conductivity, and pH is constant.
	Proteins are forming aggregates and bind strongly to the resin.	Use lower salt concentrations. Consider using additives to reduce hydrophobic interactions (*see* Buffer additives in Chapter 2).
	Sample, buffer, or temperature conditions are different from previous runs.	Check conditions.
Protein elutes later than buffer. expected or not at all.	Salt concentration too high.	Decrease salt concentration in the elution buffer.
	Hydrophobic interactions strong.	Use resin with lower hydrophobicity or lower too ligand density. Consider using an additive to reduce hydrophobic interaction (*see* Buffer additives in this chapter).
Protein elutes earlier than expected (during the wash phase).	Salt concentration of sample and buffer is too low.	Increase salt in the sample, and buffer.
	Column equilibration incomplete.	Repeat or prolong the equilibration step until conductivity is constant.

Situation	Cause	Remedy
Leading or very rounded peaks in the chromatogram.	Channeling in the column.	Repack column using thinner resin slurry. Check column packing (see Appendix 2).
	Column overloaded with sample.	Decreased sample load, and repeat.
	Column contaminated.	Clean the column.
Peaks are tailing.	Column packed too loosely.	Check column efficiency (*see* Appendix 2). Repack using a lower flow rate. Use prepacked columns.
Peaks have a leading edge.	Compressed column packing	Check column efficiency (*see* Appendix 2). Repack using a lower flow rate. Use prepacked columns.
Resin or beads appear in the eluent.	Compressed column packing.	Check column efficiency (*see* Appendix 2). Repack using a lower flow rate. Use prepacked columns.
	The bed support end piece is loose or broken.	Tighten or replace the bed support end piece.
	Column operated at too high pressure.	Do not exceed recommended operating pressure for resin or column.
	The resin was 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 yields are lower than expected.	Protein may be degraded by proteases.	Add protease inhibitors to the sample, and buffers to prevent proteolytic digestion. Run sample through a resin like 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 and adjust to improve sample solubility.
	Proteins are not eluting.	Consider using additives to reduce hydrophobic interactions (*see* Buffer additives in this chapter). Use a less hydrophobic resin.
Peaks too small.	The sample absorbs poorly at the chosen wavelength.	If appropriate, check the absorbance range on the 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 the same assay conditions for all assays.
	Excessive peak broadening.	Check the 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 resin. Check purity of the protein.
More activity is recovered than was applied to the column.	Different assay conditions have been used before and after the chromatography step.	Use the same assay conditions for all assays.
	Removal of inhibitors during separation.	Desalt the original sample before measuring activity, as cell lysates, and extracts often contain low molecular weight substances that can affect activity.

Situation	Cause	Remedy
Backpressure increases during a run or successive runs.	Bed compressed.	Repack or replace the column. Check sample preparation.
	Sample is too viscous.	Dilute with buffer. Keep protein concentration below 50 mg/mL.
	Sample not filtered properly.	Clean the column, filter the sample, and repeat.
	Microbial growth.	Follow cleaning procedures (*see* Appendix 9). Always filter buffers. Store with 20% ethanol to prevent microbial growth.
	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. Clean or replace the filter or use a new column. Reduce the salt concentration of the start buffer.
	Precipitation of lipoproteins at increased salt concentration.	Remove lipoproteins before chromatography by adding 10% dextran sulfate (final 0.2%), and 1 M calcium chloride (final 0.5 M).
Air bubbles in the bed.	Buffers not properly degassed.	Degas buffers thoroughly.
	Column packed or stored at a cool temperature, and then warmed up.	Remove small bubbles bypassing degassed buffer through the column. Take special care if buffers are used after storage in a fridge or cold room. Do not allow the column to warm up from sunlight or a heating system. Repack the column (*see* Appendix 2).
Cracks in the bed.	Large air leak in the column.	Check all connections for leaks.Repack the column (*see* Appendix 2).
Negative peaks at the solvent front.	Refractive index effects.	Exchange the sample into the start buffer.
Unexpected peaks in the	Buffer impurities.	Clean the buffer by running it through a precolumn. Use high-quality reagents.
Peaks appear on blank elution gradients.	Incomplete elution of the previous sample.	Wash the column.
Spikes in chromatogram	Air bubbles trapped in UV monitor flow cell.	Always use degassed buffers.
	Buffer impurities.	Use high-quality reagents.

03

Resins for hydrophobic interaction chromatography

Historically, scientists have used several distinct types of material as a base matrix to covalently attach ligands to form a HIC resin. Chapter 1 describes how matrix characteristics determine chromatographic properties like efficiency, capacity, and recovery as well as chemical, and physical stability and flow properties.

Capto™ resins are based on a rigid, high-flow agarose base matrix with an optimized pore structure that offers good pressure and flow. These resins are intended for use in process development and large-scale manufacturing, but also in research labs. Their high flow rates allow increased productivity and large-volume processing.

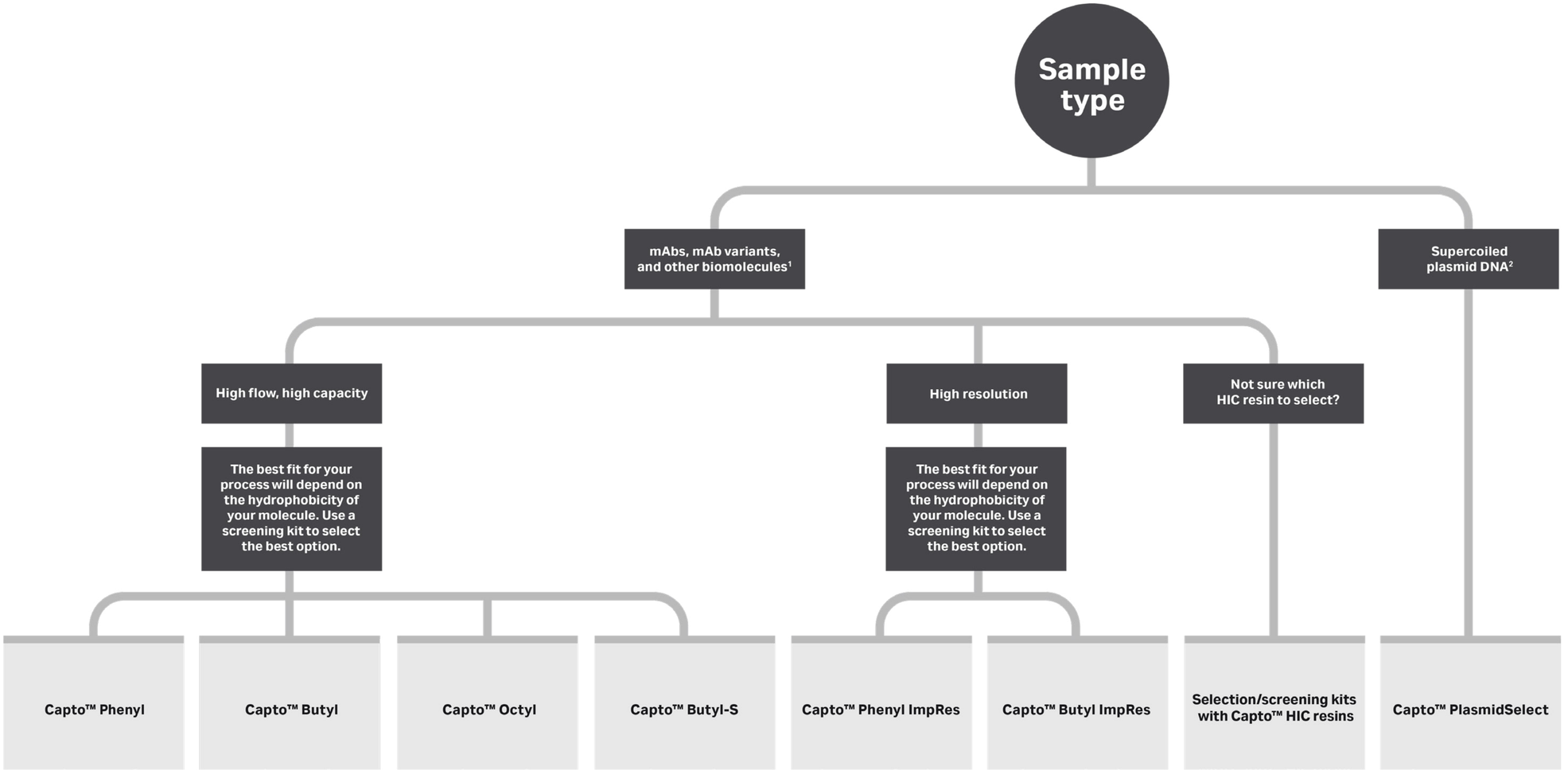
This chapter describes commercially available resins along with recommendations for use, purification examples, separation and cleaning protocols, and tips and hints for achieving excellent performance. Figure 3.1 gives a selection guide for the resins and prepacked columns currently available for HIC. This chapter guides you to faster separations and improved performance.

Capto™ resins: High-flow resins with high resolution

Capto™ HIC resins enable high-productivity purification steps, where the ligands are coupled to a chemically modified, high-flow agarose matrix. The high-flow agarose matrix provides particle rigidity without compromising the pore size. This allows for fast mass transfer resulting in high dynamic binding capacities at high flow rates, making the resin suitable for process-scale applications.

You can use Capto™ Phenyl (high sub), Capto™ Octyl, and Capto™ Butyl and Capto™ Butyl-S for capture, and intermediate purification of biomolecules. Capto™ Phenyl ImpRes and Capto™ Butyl ImpRes are high-resolution resins designed for intermediate purification and polishing.

Capto™ and Capto™ ImpRes offer improved pressure-flow properties compared to Sepharose™ Fast Flow, and Sepharose™ High Performance, respectively, due to the increased mechanical stability of the base matrix (Fig 3.2).



[1] For example, oligonucleotides, peptides, other recombinant proteins, protein complexes.
[2] Thiophilic aromatic adsorption.

Fig 3.1. Selection guide for Capto™ HIC resins.

Resin characteristics

Capto™ HIC resins are modern resins that meet the demands of large-scale biopharmaceutical manufacturing. They combine high capacity, narrow specification range, high flow rate, and low backpressure to reduce process cycle times and increase productivity.

Capto™ ImpRes HIC chromatography resins extend the well-established Capto™ platform to include high-resolution resins. The high-flow characteristics of Capto™ resin are combined with smaller particle size, to deliver good resolution with excellent pressure, and flow properties. Scientists and process developers can run them at higher flow velocities with higher bed heights, increasing flexibility in process design.

Table 3.1. Characteristics of Capto™ HIC resins

Resin	Functional group	pH stability	Particle size, d_{50v} (μm)‡
Capto™ Phenyl (high sub)	Phenyl	Operational*: 3 to 13 CIP†: 2 to 14	75
Capto™ Butyl	Butyl	Operational*: 3 to 13 CIP†: 2 to 14	75
Capto™ Butyl-S	Butyl-S	Operational*: 3 to 13 CIP†: 2 to 14	75
Capto™ Octyl	Octyl	Operational*: 3 to 13 CIP†: 2 to 14	75
Capto™ Phenyl ImpRes	Phenyl	Operational*: 3 to 13 CIP†: 2 to 14	40
Capto™ Butyl ImpRes	Butyl	Operational*: 3 to 13 CIP†: 2 to 14	40
Capto™ PlasmidSelect	2-mercaptopyridine	Operational*: 3 to 13 CIP†: 2 to 14	40

*pH range where resin can be operated without significant change in function. All ranges are estimates based on the experience and knowledge within Cytiva.

†pH range where resin can be subjected to cleaning-in-place (CIP) or sanitization-in-place without significant change in function.

‡Median particle size of the cumulative volume distribution.

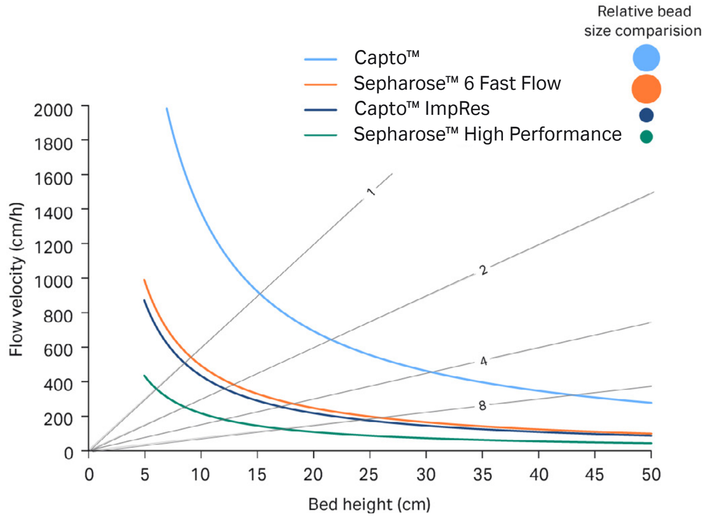


Fig 3.2. The window of operation (area below the curves) of Capto™ vs Sepharose™ Fast Flow, and Capto™ ImpRes vs Sepharose™ High Performance. The data corresponds to a process diameter column at 20°C and viscosity equivalent to water. Gray contours show the residence time in the column in minutes. Capto™ and Capto™ ImpRes HIC resins are excellent modern alternatives to Sepharose™ Fast Flow, and Sepharose™ High Performance.

Purification options

Capto™ HIC resins are available prepacked in HiTrap™, and HiScreen™ columns, and in chromatography resin lab packs. Table 3.2 shows different Capto™ resins for HIC. Some of the resins are also available in PreDictor™ filter plates, PreDictor™ RoboColumn™, and ReadyToProcess™ columns.

Use prepacked HiTrap™ columns (1 mL or 5 mL) for resin selection, group separations, and small-scale purification.

Note: The binding capacity of a HIC resin depends on the properties of the target protein, and contaminants, the selectivity of the resin, and the binding conditions. You should determine the capacity empirically during resin screening and method development.

Use the PreDictor™ Capto™ HIC Screening Kit, which consists of 4 × 96-well plates prefilled with six different Capto™ HIC resins for use in high-throughput process development. Data generated using PreDictor™ plates correlates well with data from chromatography columns, making the plates an excellent tool for the initial screening of process conditions.

Use prepacked HiScreen™ columns (4.7 mL, 10 cm bed height) for method development and optimization before scaling up.

For column packing: You can use Capto™ resins with most modern chromatography equipment from laboratory to production scale. Due to the higher rigidity of Capto™ resins, packing procedures differ slightly compared to procedures for packing Sepharose™ resins. For more details on packing laboratory-scale columns, check the instructions for the resin or column you are using.

Table 3.2. Technical properties of Capto™ HIC resins and prepacked columns

Product	Recommended operating flow[1]	Maximum operating flow[1]	Maximum operating pressure (MPa/psi) 1 MPa = 10 bar
HiTrap™ Capto™ Butyl, 1 mL	1 mL/min	4 mL/min	0.3/43
HiTrap™ Capto™ Butyl, 5 mL	5 mL/min	20 mL/min	0.3/43
HiTrap Capto™ Phenyl (high sub), 1 mL	1 mL/min	4 mL/min	0.3/43
HiTrap Capto™ Phenyl (high sub), 5 mL	5 mL/min	20 mL/min	0.3/43
HiTrap Capto™ Octyl, 1 mL	1 mL/min	4 mL/min	0.3/43
HiTrap Capto™ Octyl, 5 mL	5 mL/min	20 mL/min	0.3/43
HiTrap Capto™ Phenyl ImpRes, 1 mL	1 mL/min	4 mL/min	0.3/43
HiTrap Capto™ Phenyl ImpRes, 5 mL	5 mL/min	20 mL/min	0.3/43
HiTrap Capto™ Butyl ImpRes, 1 mL	1 mL/min	4 mL/min	0.3/43
HiTrap Capto™ Butyl ImpRes, 5 mL	5 mL/min	20 mL/min	0.3/43
HiTrap Capto™ PlasmidSelect, 1 mL	1 mL/min	4 mL/min	0.3/43
HiTrap Capto™ PlasmidSelect, 5 mL	5 mL/min	20 mL/min	0.3/43
HiScreen™ Capto™ Butyl	2.7 mL/min	4.7 mL/min	0.3/43
HiScreen™ Capto™ Phenyl (high sub)	2.7 mL/min	4.7 mL/min	0.3/43
HiScreen™ Capto™ Octyl	2.7 mL/min	4.7 mL/min	0.3/43
HiScreen™ Capto™ Butyl ImpRes	1.2 mL/min	1.7 mL/min	0.3/43
HiScreen™ Capto™ Phenyl ImpRes	1.2 mL/min	1.7 mL/min	0.3/43
HiScreen™ Capto™ PlasmidSelect	1.2 mL/min	2.3 mL/min	0.3/43

[1]At 25°C using buffers with the same viscosity as water.

Table 3.3. List of suitable empty columns at laboratory scale for packing of Capto™ resins

Column	Max. operating pressure			Inner diameter (mm)	Bed volume (mL)	Bed height (mm)*
	bar	psi	MPa			
Tricorn™ 5/20	100	1450	10	5	0.0 to 0.5	0 to 26
Tricorn ™ 5/50	100	1450	10	5	0.2 to 1.1	8 to 56
Tricorn™ 5/100	100	1450	10	5	1.2 to 2.1	58 to 106
Tricorn™ 5/150	100	1450	10	5	2.1 to 3.1	108 to 156
Tricorn™ 5/200	100	1450	10	5	3.1 to 4.1	158 to 206
Tricorn 10/20	50	725	5	10	0.0 to 2.1	0 to 26
Tricorn™ 10/50	50	725	5	10	0.0 to 4.4	0 to 56
Tricorn™ 10/100	50	725	5	10	3.6 to 8.4	46 to 106
Tricorn™ 10/150	50	725	5	10	7.6 to 12.3	96 to 156
Tricorn™ 10/200	50	725	5	10	11.5 to 16.2	146 to 206
Tricorn™ 10/300	50	725	5	10	19.4 to 24.1	246 to 306
HiScale™ 10/40	20	290	2	10	8 to 31	100 to 400
HiScale™ 16/20	20	290	2	16	0 to 40	0 to 200
HiScale™ 16/40	20	290	2	16	16 to 80	80 to 400
HiScale™ 26/20	20	290	2	26	0 to 106	0 to 200
HiScale™ 26/40	20	290	2	26	69 to 212	130 to 400
HiScale™ 50/20	20	290	2	50	0 to 393	0 to 200
HiScale™ 50/40	20	290	2	50	274 to 785	140 to 400
XK 16/40	5	72.5	0.5	16	16 to70	80 to 350
XK 26/20	5	72.5	0.5	26	0 to 66	0 to 125
XK 26/40	5	72.5	0.5	26	45 to 186	85 to 350
XK 50/20	3	43.5	0.3	50	0 to 274	0 to 140
XK 50/30	3	43.5	0.3	50	0 to 559	0 to 280

*The minimum value corresponds to a packing performed with the adapters.

Empty columns for packing Capto™ chromatography resins are shown in Table 3.3. AxiChrom™, BPG, and Chromaflow™ columns can be used for packing Capto™ resins at pilot and production scale.

Purification examples

Plasmid purification using Capto™ PlasmidSelect

Purified plasmid DNA is required in increasing quantities to meet emerging requirements for gene therapy and DNA vaccine applications.

Capto™ PlasmidSelect is a thiophilic aromatic adsorption chromatography resin with a selectivity that allows supercoiled covalently closed circular forms of plasmid DNA to be separated from open circular forms. The resin is designed for the purification of supercoiled DNA to high quality for gene therapy and DNA vaccine applications. The resin is based on a rigid base matrix, delivering excellent pressure-flow properties to plasmid production. It forms the basis of a generic process for purifying supercoiled covalently closed circular plasmid DNA suitable for bulk as well as for clinical-grade applications.

The resin can also be used to rapidly analyze the quantity and quality of plasmid DNA in complex solutions.

Figure 3.3 shows the purification of plasmid DNA from Plasmid-containing feedstream using Capto™ PlasmidSelect.

Column:	HiScreen™ PlasmidSelect
Sample:	Plasmid-containing feedstream
Sample load:	3 mg plasmid/mL resin
Equilibration:	5 CV of 100 mM of Tris, 2 M of ammonium sulfate, 10 mM EDTA, pH 7.5 at 100 cm/h
Elution:	10 CV of 100 mM Tris, 1.7 M ammonium sulfate, 10 mM EDTA, pH 7.5 + 0.3 M NaCl at 80 cm/h
Wash:	5 CV of 100 mM Tris, 2 M ammonium sulfate, 10 mM EDTA, pH 7.5 at 100 cm/h
System:	ÄKTA avant™ 150
CIP:	3 CV of 100 mM Tris, 10 mM EDTA at 100 cm/h
Detection:	254 nm

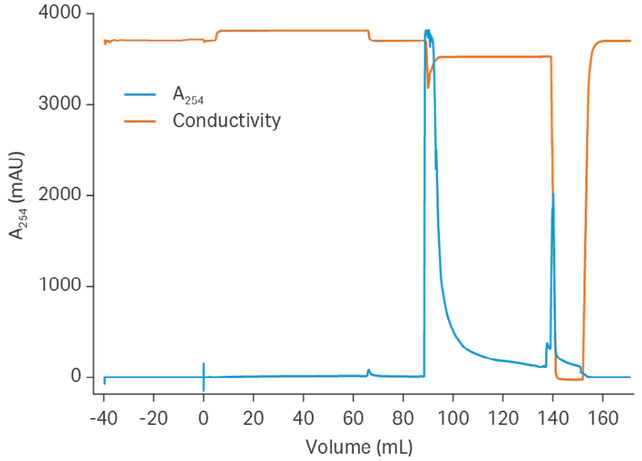


Fig 3.3. The purification of plasmid DNA using Capto™ PlasmidSelect.

Polishing of mAb

We compared aggregate removal results using Capto™ Butyl ImpRes, and its corresponding predecessor Butyl Sepharose™ High Performance resin (Fig 3.4). Capto™ Butyl ImpRes achieved a similar decrease in aggregate content with the potential to use higher bed heights and increased flow rates (Fig 3.5).

Table 3.4. Summary of aggregate removal methods

	Gradient elution	Step elution
Flow rate	0.25 mL/min (75 cm/h)	0.65 mL/min (200 cm/h)
Bed height (cm)	5	20
Buffers	A: 25 mM citrate with 1 M ammonium sulfate, pH 5.0 B: ultrapure deionized water	A: 25 mM citrate, 850 mM ammonium sulfate, pH 5.0 B: 25 mM citrate, pH 5.0
UNICORN™ method	Equilibrium, 85% A and 15% B, 2 CV Sample load, 66% of Q_{b10} [1] Column wash, 15% B, 5 CV Gradient elution, 15% B to 100% B, 10 CV Strip, 100% B, 5 CV Re-equilibration, 15% B, 5 CV	Equilibrium with the buffer, 0% B, 2 CV Sample load, 65% of Q_{b10} [1] Column wash, 0% B, 2 CV Step elution, 50% B, 5 CV Strip, 100% B, 5 CV Re-equilibration, A buffer, 3 CV
CIP[2]	0.1 M NaOH, 0.5 mL/min, 1 CV	

[1] Q_{b10} = Dynamic binding capacity at 10% breakthrough by frontal analysis (displacement chromatography)

[2] Cleaning- or sanitization-in-place without significant change in function.

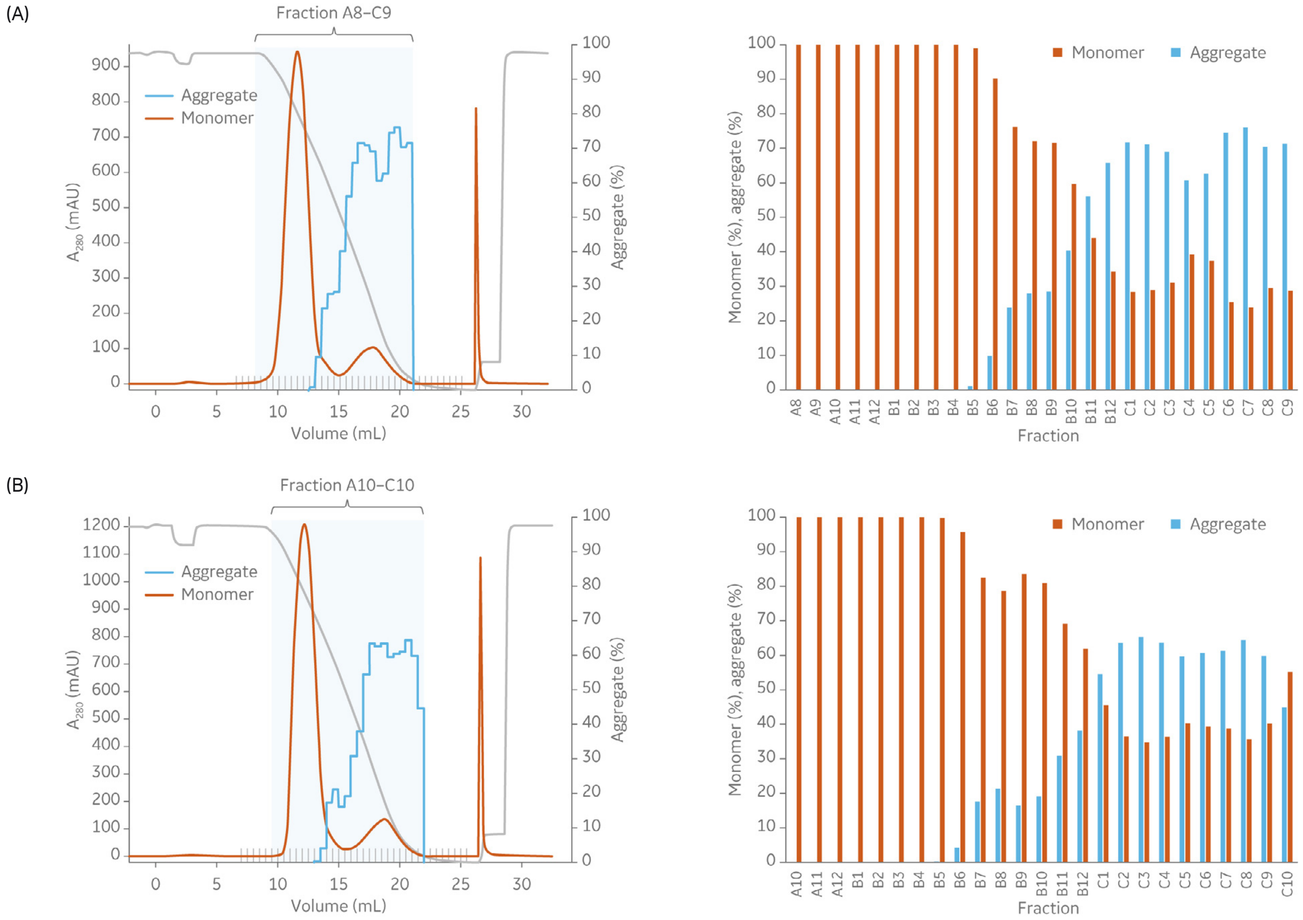


Fig 3.4. At a load of 66% of Q_{b10} in 1 mL columns (10 cm bed height) with a flow velocity of 75 cm/h, (A) Capto™ Butyl ImpRes, (B) Butyl Sepharose™ High Performance resins both effectively decrease the aggregate content and show similar elution profiles.

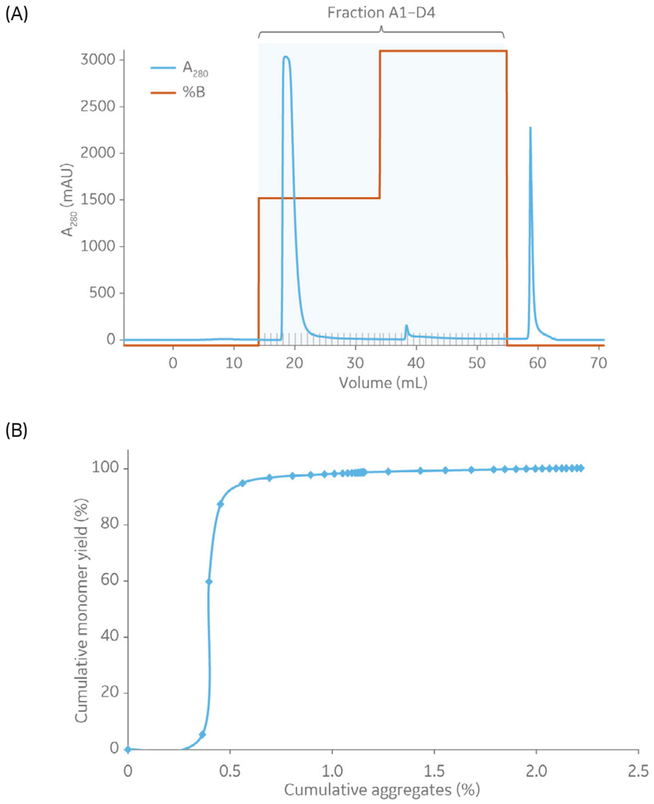


Fig 3.5. (A) Results from Capto™ Butyl Impres with a load of 65% of Q_{b10} for mAb and aggregate mixture show favorable monomer yield with increased flow velocity (200 cm/h), and bed height (20 cm). From left to right, peaks represent the monomer, aggregate, and CIP. The marked fractions were analyzed for aggregates. (B) Cumulative monomer yield versus cumulative aggregate content using Capto™ Butyl ImpRes and a mAb sample containing 10.3% aggregate at 20 cm bed height, and a flow velocity of 200 cm/h. The aggregate content is below 1% at 98% monomer yield after this HIC purification step.

Capto™ Butyl ImpRes and Butyl Sepharose™ High Performance resins showed similar capabilities in aggregate removal (Fig 3.4) when using gradient elution. Using Capto™ Butyl ImpRes instead of Butyl Sepharose™ High Performance with step elution improves productivity as higher bed heights and flow rates can be used while maintaining monomer purity and favorable yield (Fig 3.5).

Chemical stability

For daily use, Capto™ resins are stable in all common, aqueous buffers, 1 M NaOH, denaturing agents (8 M urea, 6 M guanidine hydrochloride), 70% ethanol, 1 M acetic acid, and with additives like nonionic detergents.

Storage

For column storage, wash with 5 CV of distilled water followed by 5 CV of 20% ethanol. Store at 4°C to 30°C. Do not freeze. Seal the column well to keep it from drying out.

SOURCE™ resins

Use SOURCE™ resin for polishing steps if you cannot find the right selectivity in resins with larger particle sizes.

Run SOURCE™ columns on instruments like ÄKTA™ systems, and HPLC. *See* Appendix 3 for guidance on selecting purification equipment

SOURCE™ resins are based on a hydrophilic matrix made from monodispersed, rigid, polystyrendivinyl benzenene, and substituted with hydrophobic ligands; isopropyl or phenyl (Fig 3.6). The resins 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 ensure high flow rates at low backpressure.

The high flow rates that you can use with SOURCE™ resins are more likely to be limited by the equipment rather than the physical properties of the resin.

You can easily scale up separation methods from prepacked RESOURCE™ columns to large-scale columns like FineLINE™ columns.

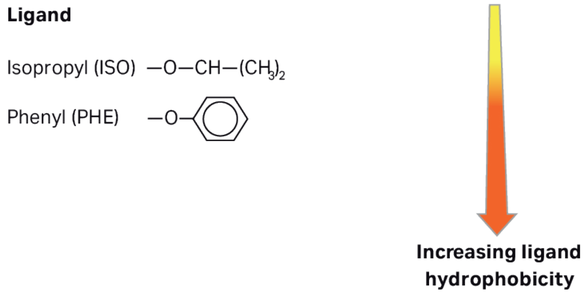


Fig 3.6. Ligands couple to monodispersed SOURCE™ resin particles through uncharged, chemically stable O-ether linkages.

Resin characteristics

The resin's composition is rigid, and monodisperse, having polystyrene, and divinylbenzene particles (15 µm) with an optimized pore size distribution. The base matrix couples to one of two hydrophobic ligands; isopropyl or phenyl.

Table 3.5. Characteristics of SOURCE™ HIC resins

Products	Matrix	pH stability	Particle size (d_{50V})‡
SOURCE™ 15ISO, SOURCE™ 15PHE	Polystyrene/divinyl particles	Operational*: 2 to 12 CIP†: 1 to 14	15 µm

*pH range where resin can be operated without significant change in function. All ranges are estimates based on the experience, and knowledge within Cytiva.

†pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.

‡Mean particle diameter, monodisperse size distribution.

Purification options

Fig 3.7. HIC resins based on a SOURCE™ matrix are available in prepacked columns and as resin packs.

SOURCE™ resins are available in resin packs and prepacked in RESOURCE™ columns (Fig 3.7 and Table 3.6). An example of screening purification of a mouse mAb with SOURCE™ resins for HIC is shown in Figure 3.8.

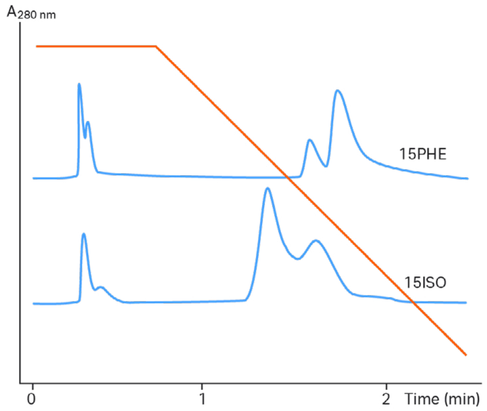
Table 3.6. SOURCE™ resins and prepacked columns

Product	Ligand*	Recommended operating flow†	Maximum operating flow rate†	Maximum operating pressure‡ (MPa/psi) 1 MPa = 10 bar
RESOURCE™ PHE, 1 mL column	phenyl	0.8 to 4.8 mL/min	10 mL/min	1.5/220
SOURCE™ 15PHE 4.6/100 PE, 1.7 mL column	phenyl	0.5 to 2.5 mL/min	5 mL/min	4/580
SOURCE™ 15PHE resin	phenyl	150 to 900 cm/h	1800 cm/h	0.5/72
RESOURCE™ ISO, 1 mL column	isopropyl	0.8 to 4.8 mL/min	10 mL/min	1.5/220
SOURCE™ 15ISO resin	isopropyl	150 to 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™ resin-based products.

†*See* Appendix 4 to convert flow velocity (cm/h) to volumetric flow rates (L/min), and *vice versa*. Note that the final working flow will depend also on factors such as column size, and bed height, sample characteristics and loading conditions, the equipment you are using, and the backpressure that the equipment can withstand.

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



Column: SOURCE™ 15ISO, and SOURCE™ 15PHE (1 mL prepacked)
Sample: Purified monoclonal mouse IgM antibodies in phosphate-buffered saline
Sample load: 20 µL
Start buffer: 2.0 M ammonium sulfate, pH 7.0, 100mM sodium phosphate, pH 7.0
Elution buffer: 100 mM sodium phosphate, pH7.0
Flow rate: 5 mL/min
Gradient: 20% to 100% elution buffer, 10 CV
Detection: 280 nm

Fig 3.8. Screening of HIC resins for an antibody purification.

Note: The binding capacity of a HIC resin depends on the properties of the target protein and contaminants, the selectivity of the resin, and the binding conditions. You should determine capacity empirically during resin screening and method development.

Use prepacked RESOURCE™ columns for fast resin selection, method scouting, group separations, or sample concentration.

Use SOURCE™ 15PHE 4.6/100 PE columns to improve resolution by increasing column length. Further optimization might be required. Use optimized conditions as the first step toward scaling up.

Packing volumes for SOURCE™ resins in Tricorn™ chromatography columns are shown in Table 3.7.

Table 3.7. Packing volumes and bed heights for SOURCE™ resins

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

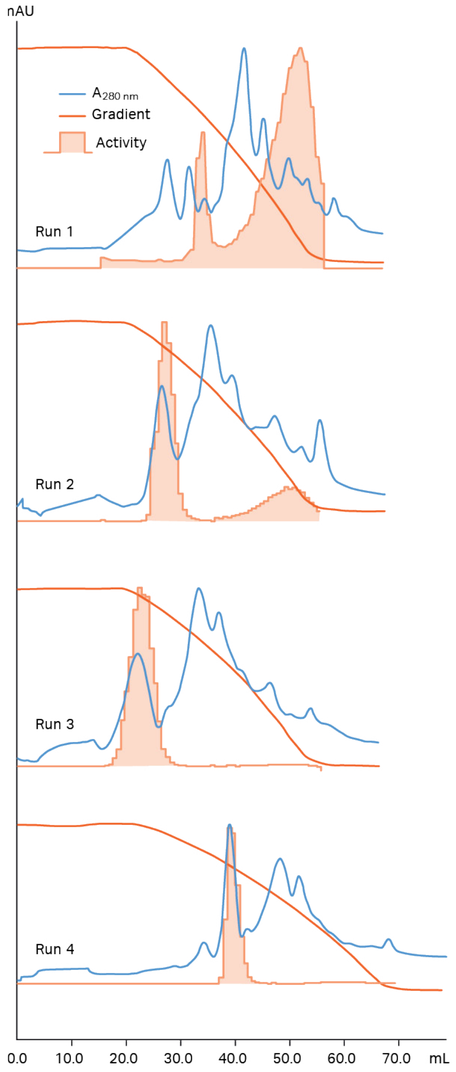
Select a production column like the FineLINE™ column for larger volumes.

Purification examples

Method optimization

Figure 3.9 shows examples of runs made while optimizing the intermediate purification step for a recombinant protein, tyrosine phosphatase. We partially purified the protein in an initial capture step, using ion exchange chromatography on Q Sepharose™ XL. We isolated the active fraction, applied it 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, we added glycerol to reduce the strong binding of tyrosine phosphatase, and to better facilitate elution (*see* additives used during HIC separations in Table 2.2 in Chapter 2).

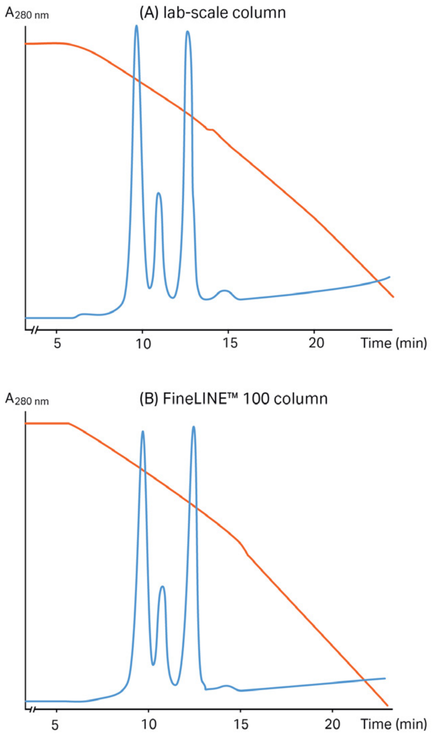


Purification protocol: CIPP
Starting material: Diluted *E. coli* supernatant
Capture: Q Sepharose™ XL resin
Intermediate purification: SOURCE™ 15PHE column
Polishing: Superdex™ 75 column

Column:	SOURCE™ 15PHE 4.6/100 PE
Sample:	Recombinant protein tyrosine phosphatase, partially purified on HiTrap™ Q XL 5 mL
Sample load:	10 mL
Start buffer (run 1):	25 mM Tris, 1 mM EDTA , 2 mM DTT, pH 7.5
Elution buffer (run 1):	1.5 M $(NH_4)_2SO_4$, 25 mM Tris, 1 mM EDTA, 2 mM DTT, pH 7.5
Start buffer (run 2):	1.5 M $(NH_4)_2SO_4$, 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 $(NH_4)_2SO_4$, 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 $(NH_4)_2SO_4$, 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 rate:	a) 1.5 mL/min (run 1) b) 1.0 mL/min (runs 2, 3, 4)
Gradient:	0% to 100% elution buffer in 20 CV (runs 1,2,3) 0% to100% elution buffer in 27 CV (run 4)
Detection:	280 nm
Activity measurement:	p-nitrophenyl phosphate (pNPP) activity assay at 405 nm.

Fig 3.9. Optimization steps for intermediate purification of a recombinant protein.

Scaling up

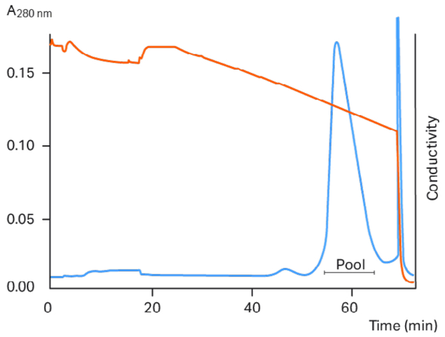


Columns: (A) SOURCE™ 15ISO 7.5 × 50 mm (2.2 mL)
(B) FineLINE™ 100, 100 × 50 mm (400 mL)
Sample: lactalbumin, chymotrypsinogen
Sample load: 0.3 mg/mL resin
Start buffer: 100 mM potassium phosphate, 2.0 M ammonium sulfate, pH 7.0
Elution buffer: 100 mM potassium phosphate, pH 7.0
Flow rate: (A) 2.2 mL/min (300 cm/h)
(B) 385 mL/min (300 cm/h)
Gradient: 20% to 100% elution buffer, 20 CV
Detection: 280 nm
System: ÄKTA™ chromatography system

Fig 3.10. 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

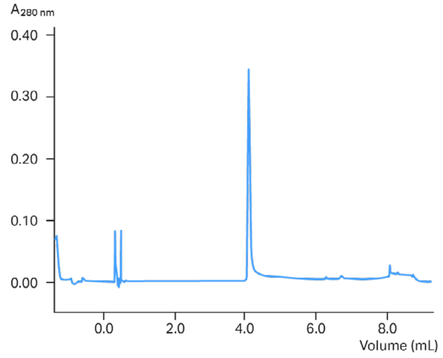
Figure 3.11 shows the use of SOURCE™ 15PHE as the final polishing step in large-scale purification of a recombinant protein, rExotoxin A (PE553D), expressed in the periplasm of *Pseudomonas aeruginosa*. We added ammonium sulfate (1.0 M) to the partially purified protein before sample application. We eluted the bound exotoxin A using a linear gradient from 1.0 to 0.55 M ammonium sulfate over 15 column volumes (CV). This step removed the remaining contaminant proteins, as shown by a single peak on reversed phase chromatography (Fig 3.12).



Purification protocol:	CIPP
Starting material:	Unclarified *E.coli* homogenate
Capture:	STREAMLINE™ DEAE resin
Intermediate purification:	Phenyl Sepharose™ 6 FF resin
Intermediate purification:	SOURCE™ 30Q resin
Polishing:	SOURCE™ 15PHE resin

Column: SOURCE™ 15PHE, 35 mm i.d. × 100 mm
Sample: 0.5 L/cycle was applied from the previous SOURCE™ 30Q step, adjusted to 1.0 M ammonium sulfate
Sample load: 0.5 L/cycle
Start buffer: 50 mM phosphate, 1.0 M ammonium sulfate, pH 7.4
Elution buffer: 50 mM phosphate, pH 7.4
Flow velocity: 200 cm/h
Gradient: 0% to 45% elution buffer, 15 CV
Detection: 280 nm

Fig 3.11. SOURCE™ 15PHE was used as the final polishing step in large-scale purification of a recombinant protein, rExotoxin A (PE553D).



Column: Analytical RPC column
Sample: Pooled fractions from SOURCE™ 15PHE
Sample load: 50 µL
Start buffer: 0.1% trifluoroacetic acid (TFA) in water
Elution buffer: 0.1% TFA in acetonitrile
Flow rate: 150 µL/min
Gradient: 25% to 75% elution buffer over 47 min
Detection: 280 nm

Fig 3.12. Chromatographic analysis demonstrates purity after polishing step on SOURCE™ 15PHE.

Performing a separation

See Chapter 2 for guidelines on selecting buffer, salt concentration, and pH, and optimizing your method.

You can use the instructions here as the basis for optimizing your separation.

To optimize separation and avoid any deterioration in column performance, it is essential to prepare your sample and buffer correctly. Your samples must dissolve fully and be free from any particles or material that can interfere with separation. *See* Chapter 2, and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers, and samples after adding all salts and additives. Use high-quality water and chemicals. Filter solutions using 0.22 µm filters. To avoid forming air bubbles in a packed column, and ensure reproducible results, keep the column and buffers at the same temperature when you are preparing for a run.

To avoid problems with precipitation, check the salt stability window of the sample components. Avoid working at concentrations near the stability limit of the target protein. For samples with unknown hydrophobic properties, try this:

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

Note: You might need to reduce flow rates due to the viscosity of your chosen start buffer, sample characteristics, loading requirements, and the equipment you are using.

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

1. Remove any ethanol. To do this, wash with 5 CV 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
2. Wash with 5 CV of start buffer using the same flow rate as the first step.
3. Perform a blank elution (i.e., perform a run without loading any sample) to check the 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.

1. Equilibrate the column with 5 to 10 CV of start buffer, or until the UV baseline, and conductivity are stable.
2. Adjust the sample to your chosen salt concentration (and pH if needed). Filter, and apply it to the column.
3. Wash with 5 to 10 CV 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 to 20 CV, increasing the proportion of elution buffer until the salt concentration reaches a minimum (i.e., salt-free buffer).
5. Wash with 2 to 5 CV of salt-free elution buffer to elute any remaining hydrophobically bound material.
6. Re-equilibrate with 5 to 10 CV 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 the column with 5 to 10 CV of start buffer, or until the baseline, eluent pH, and conductivity is stable.
2. Adjust the sample to your chosen salt concentration (and pH if needed). Filter, and apply it to the column.
3. Wash with 5 to 10 CV 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 CV of elution buffer, and salt at your chosen concentration.
5. Repeat step 4 at lower salt concentrations until you have eluted the target protein(s).
6. Wash with 2 to 5 CV of salt-free elution buffer to elute any remaining hydrophobically bound material.
7. Re-equilibrate with 5 to 10 CV of start buffer, or until conductivity reaches your required value.

You can 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 resin.

Check your column performance regularly by determining column efficiency, and peak symmetry. *See* Appendix 2 for more details.

Never leave columns or equipment in high-salt solutions.

Cleaning

Correctly preparing your samples, and buffers and applying 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 backpressure, or complete blockage are all signs that you need to clean the resin using more stringent procedures.

Whenever possible, reverse the direction of flow during cleaning so that contaminants do not pass through the entire column length. Due to the column design, we do not recommend that you reverse the direction of flow for RESOURCE™ columns. The number of CV and time required for each cleaning step can vary according to the degree of contamination. If the cleaning procedure does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Take care when changing the filter, as it can affect the column packing and interfere with performance.

To remove common contaminants like precipitated proteins, use this procedure:

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 to 2 h, for SOURCE™ packed in larger columns. Note that you might need to reduce flow rates due to the condition of the column or the viscosity of the sample, buffers, or storage solutions.

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

3a. To start a new separation, re-equilibrate with at least 3 CV of start buffer or until you have achieved the correct eluent pH.

3b. For storage, wash with at least 5 CV of storage solution. Let the UV baseline stabilize before storing the column.

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

Chemical stability

For daily use, HIC resins based on SOURCE™ resins 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 CV of distilled water followed by 5 CV of 20% ethanol. Degas the ethanol and water mixture thoroughly and apply at a low flow rate to avoid over pressuring the column. Make sure you seal the column well to keep it from drying out. Store your columns, and unused resins at 4°C to 30°C in 20% ethanol. Do not freeze.

To avoid forming air bubbles, make sure that your columns, buffers, and equipment are at the same temperature before using them.

Sepharose™ High Performance resin

Sepharose™ High Performance resin is a legacy base matrix, which is used in many applications. For developing new processes or protein purification methods, we currently recommend using one of our more recent resins based on the Capto™ ImpRes base matrix (*see* Table 3.1).

Sepharose™ High Performance resin is based on a matrix of particles (with a mean size of 34 µm) made from 6% agarose and highly cross-linked for chemical and physical stability. The small particle size supports fast binding and dissociation even at high sample loads and flow rates. Combined with the right selectivity, you can achieve high-resolution separations. The particle size and bed volumes remain stable despite changes in salt concentration, keeping separations fast at high flow rates. Ligands are coupled through a chemically stable O-ether linkage (Fig 3.13). The different selectivity characteristics of Sepharose™ based resins are shown in Figure 3.14.

You can use Sepharose™ High Performance for group separations, sample concentration, or capture steps. However, you should limit these separations to reasonably clean samples to avoid the risk of blocking the column filter — 34 µm particles require finer column filters.

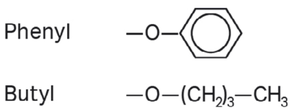


Fig 3.13. Ligands are a couple to Sepharose™ High Performance matrix with uncharged, chemically stable O-ether linkages.

Resin characteristics

Sepharose™ High Performance is based on highly cross-linked, 6% agarose formed into spherical particles (with a mean size of 34 µm) and substituted with s ligands through uncharged, chemically stable O-ether linkages (Table 3.8). Table 3.9 shows the currently available products based on Sepharose™ High Performance resin.

Table 3.8. Characteristics of Phenyl and Butyl Sepharose™ High Performance resins

Product	Matrix	pH stability	Particle size (d_{50V})‡
Phenyl Sepharose™ High Performance	6% cross-linked agarose, spherical particles	Operational*: 3 to 12 CIP†: 3 to 13	34 µm
Butyl Sepharose™ High Performance	6% cross-linked agarose, spherical particles	Operational*: 3 to 12 CIP†: 2 to 14	34 µm

*pH range where resin can be operated without significant change in function.

†pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.

‡Median particle size of the cumulative volume distribution.

Purification options

Table 3.9. HIC products based on Sepharose™ High Performance resins

Product	Recommended working flow rate (mL/min) *	Maximum operating flow rate (mL/min)	Maximum operating pressure † (MPa/psi) 1 MPa=10 bar
HiTrap™ Phenyl HP, 1 mL	up to 1 mL/min	4 mL/min	0.3/43
HiTrap™ Phenyl HP, 5 mL	up to 5 mL/min	20 mL/min	0.3/43
HiPrep™ Phenyl HP 16/10, 20 mL	up to 5 mL/min	5 mL/min	0.3/43
Phenyl Sepharose™ High Performance	30 to 150 cm/h	150 cm/h	0.5/72
HiTrap™ Butyl HP, 1 mL	up to 1 mL/min	4 mL/min	0.3/43
HiTrap™ Butyl HP, 5 mL	up to 5 mL/min	20 mL/min	0.3/43
Butyl Sepharose™ High Performance	30 to 150 cm/h	150 cm/h	0.5/72

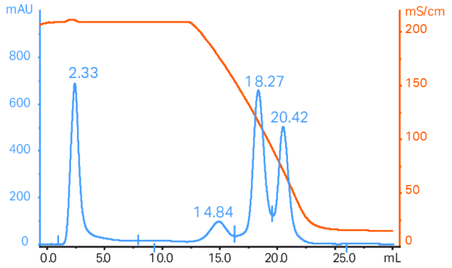
*Recommendations are for separations at room temperature in aqueous buffers. *See* Appendix 4 to convert flow velocity (cm/h) to volumetric flow rates (mL/min) and *vice versa*. Note that the final working flow also depends on factors like column size and bed height, sample characteristics, and loading conditions, the equipment you are using, and the backpressure your equipment can withstand.

†Maximum operating pressure refers to the pressure above which the resin begins to compress.

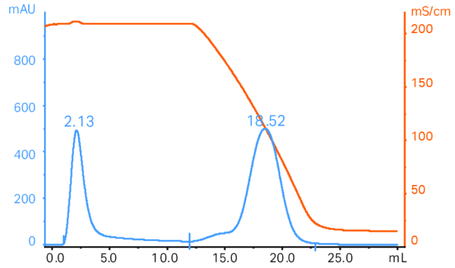
The binding capacity of a HIC resin depends highly on the properties of the target protein, and contaminants, the selectivity of the resin, and the binding conditions. You need to determine capacity empirically during resin screening and method development.

Comparison of the different selectivity characteristics of various HIC resins are shown in Figure 3.14. Select the resin that gives the optimal selectivity, resolution, and loading capacity at the lowest salt concentration.

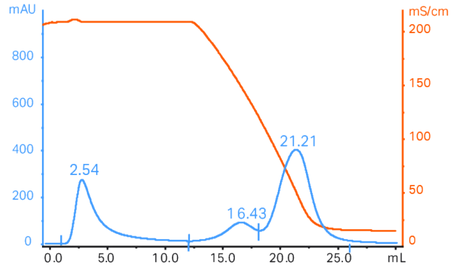
Phenyl Sepharose™ High Performance



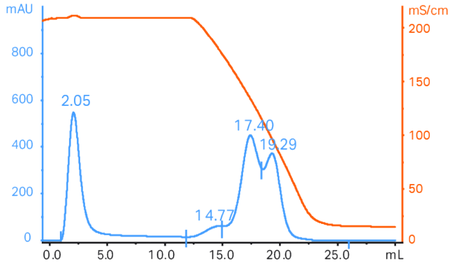
Phenyl Sepharose™ 6 Fast Flow (low sub)



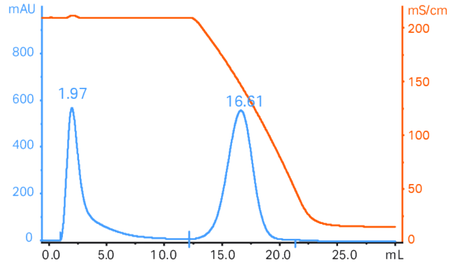
Phenyl Sepharose™ 6 Fast Flow (high sub)



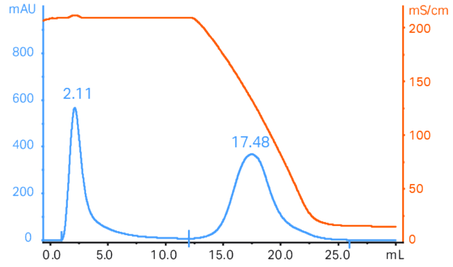
Butyl Sepharose™ 4 Fast Flow



Butyl-S Sepharose™ 6 Fast Flow



Octyl Sepharose™ 4 Fast Flow



Column: HiTrap™ HIC Selection Kit
Sample: Cytochrome C, ribonuclease A , lysozyme, α-chymotrypsinogen, 6 mg protein/mL, (1:3:1:1) in start buffer
Sample volume: 1 mL
Sample Load: 6 mg protein/mL resin
Start buffer: 1.7 M $(NH_4)_2SO_4$, 0.1 M Na_2HPO_4, pH 7.0
Elution buffer: 0.1 M Na_2HPO_4, pH 7.0
Flow rate: 1.0 mL/min, (150 cm/h)
Gradient: 0% to 100% elution buffer in 10 CV

Fig 3.14. Comparison of the different selectivity characteristics of Sepharose™ based resins.

Use prepacked HiTrap™ columns (1 mL or 5 mL) for method scouting, group separations, small-scale purification, sample concentration, or cleanup. When you need increased capacity, use prepacked columns HiPrep™ 16/10 Phenyl HP (20 mL).

Phenyl Sepharose™ High Performance resin and Capto™ Phenyl ImpRes usually exhibit similar hydrophobic properties. Capto™ Phenyl ImpRes might give a similar resolution at higher flow rates.

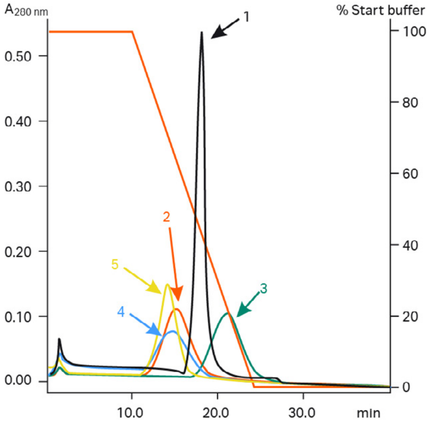
The butyl ligand in Butyl Sepharose™ High Performance resin offers an alternative selectivity to Phenyl Sepharose™ High Performance resin.

Purification examples

Resin screening: Developing a monoclonal antibody purification

Albumin and transferrin are the most common contaminants in monoclonal antibody preparations. However, since most monoclonal antibodies are more hydrophobic than these contaminants, you can use HIC to bind the antibody as the contaminants wash through the column.

Figure 3.15 shows an example of screening using columns from the HiTrap™ HIC Selection Kit to select a HIC resin that could offer the best selectivity and resolution for purifying monoclonal IgG. Phenyl Sepharose™ High Performance resin produced a well-resolved peak containing IgG. This peak is not necessarily the pure IgG, but it can represent a series of components with similar hydrophobicity to the IgG.



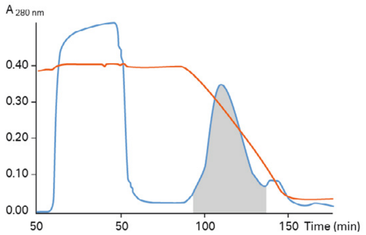
Columns: 1. HiTrap™ Phenyl HP
2. HiTrap™ Phenyl FF (low sub)
3. HiTrap™ Phenyl FF (high sub)
4. HiTrap™ Octyl FF
5. HiTrap™ Butyl FF

Samples: 0.8 mg pure mouse monoclonal IgG
Start buffer: 50 mM sodium phosphate, 1.0 M ammonium sulfate, pH 7.0
Elution buffer: 50 mM sodium phosphate, pH 7.0
Flow rate: 1 mL/min
Gradient: 0% to 100% elution buffer in 15 CV
System: ÄKTA™ chromatography system

Fig 3.15. Resin screening for monoclonal antibody purification.

Capture: Monoclonal antibody purification

Figure 3.16 shows an example of high selectivity used in a capture step. In this purification, we aimed to produce a monoclonal antibody with enough purity for *in vitro* diagnostic use. The mouse IgG_1 anti-IgE, produced in a hybridoma cell culture, bound very strongly to Phenyl Sepharose™ High Performance resin, and most fetal calf serum proteins passed through the column. The capture step yielded > 95% purity, removing the need for an intermediate step. By concentrating the sample into a small volume, it could transfer directly to a polishing step.

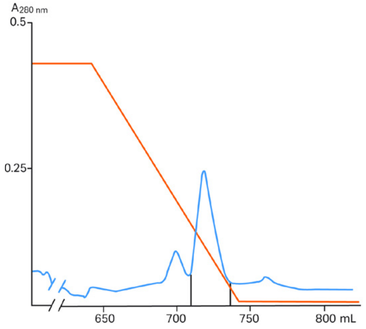


Column: Phenyl Sepharose™ HP packed in XK 16/10 column
Sample: Hybridoma cell culture supernatant, mouse IgG_1, anti-IgE. Ammonium sulfate added to 0.5 M
Start buffer: 20 mM potassium phosphate, 0.5 M ammonium sulfate, pH 7.0
Elution buffer: 20 mM potassium phosphate, pH 7.0
Flow velocity: 100 cm/h
Gradient: 0% to 100% elution buffer in 10 CV
System: ÄKTA™ chromatography system

Fig 3.16. Concentration and purification of a monoclonal antibody from hybridoma cell culture.

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 (Fig 3.17). After adding ammonium sulfate, we used HIC to concentrate, and further purify the sample using a gradient elution prior to a final polishing step using another ion exchange resin.



Column: Phenyl Sepharose™ HP in XK 16/10 column
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)
System: ÄKTA™ chromatography system

Fig 3.17. Concentration, and purification of HIV reverse transcriptase using Phenyl Sepharose™ HP in XK 16/10 column.

Performing a separation

For guidelines on selecting resin, buffer, salt, and pH conditions, as well as guidelines on optimizing your method, *see* Chapter 2.

You can use these instructions as the basis to optimize your separation:

To optimize separation, and avoid any deterioration in column performance, it is essential to prepare your sample and buffer correctly. Your samples must dissolve fully, be free from any particles or material that can interfere with separation. *See* Chapter 2, and Appendix 1 for recommendations, and advice on sample preparation.

Filter buffers, and samples after adding all salts, and additives. Use high-quality water, and chemicals. Filter solutions using 0.45 µm or 0.22 µm filters. To avoid forming air bubbles in a packed column, and ensure reproducible results, keep the column and buffers at the same temperature when you are preparing for a run.

To avoid problems with precipitation, check the salt stability window of the sample components. Avoid working at concentrations near the stability limit of the target protein. For samples with unknown hydrophobic properties, try this:

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

Note: You might need to reduce flow rates due to the viscosity of your chosen start buffer, sample characteristics, loading requirements, and the equipment you are using.

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

1. Remove any ethanol. To do this, wash with 5 CV of distilled water or elution buffer.
   Flow: 1 mL/min, HiTrap™ 1 mL
   5 mL/min, HiTrap™ 5 mL
   0.8 mL/min, HiPrep™ 16/10, 20 mL
   25 cm/h for Sepharose™ High Performance packed in larger columns

2. Wash with 5 CV of start buffer.
   Flow: 1 mL/min, HiTrap™ 1 mL
   5 mL/min, HiTrap™ 5 mL
   3 mL/min, HiPrep™ 16/10, 20 mL
   50 cm/h for Sepharose™ High Performance packed in larger columns

3. 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, HiPrep™ 16/10, 20 mL
50 to 100 cm/h for Sepharose™ High Performance packed in larger columns

Collect fractions throughout the separation.

1. Equilibrate column with 5 to 10 CV of start buffer, or until the UV baseline and conductivity are stable.
2. Adjust the sample to your chosen salt concentration (and pH if needed). Filter, and apply it to the column
3. Wash with 5 to 10 CV of start buffer or until the UV baseline and conductivity are stable. This ensures that all unbound material has washed through the column.
4. Begin elution using a gradient volume of 10 to 20 CV, increasing the proportion of elution buffer until the salt concentration reaches a minimum, that is, salt-free buffer.
5. Wash with 2 to 5 CV of salt-free elution buffer to elute the remaining hydrophobically bound material.
6. Re-equilibrate with 5 to 10 CV 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, HiPrep ™16/10, 20 mL
50 to 100 cm/h for Sepharose™ High Performance packed in larger columns

Collect fractions throughout the separation.

1. Equilibrate the column with 5 to 10 CV of start buffer, or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to your chosen salt concentration (and pH if needed). Filter, and apply it to the column.
3. Wash with 5 to 10 CV 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 CV of elution buffer, and salt at your chosen concentration.
5. Repeat step 4 at lower salt concentrations until the target protein(s) has been eluted.
6. Wash with 2 to 5 CV of salt-free elution buffer to elute any remaining hydrophobically bound material.
7. Re-equilibrate.

You can save time by using higher flow rates during the salt-free wash and re-equilibration steps, but do not exceed the maximum recommended rate for the resin.

Check your column performance regularly by determining column efficiency, and peak symmetry. *See* Appendix 2 for more details.

Never leave columns or equipment in high-salt solutions.

Cleaning

Correctly preparing your samples and buffers and applying 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 backpressure, or complete blockage are all signs that you need to clean the resin 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 CV and time required for each cleaning step can vary according to the degree of contamination. If the cleaning procedure does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Take care when changing the filter, as it can affect the column packing and interfere with performance.

To remove common contaminants like precipitated proteins, use this procedure:

Flow: 1 mL/min, HiTrap™ 1 mL
5 mL/min, HiTrap™ 5 mL
3 mL/min, HiPrep™ 16/10, 20 mL
40 cm/h, with a contact time of 1 to 2 h, for Sepharose™ High Performance packed in larger columns

Note: You might need to reduce flow rates due to the condition of your column and the viscosity of your sample, buffers, or storage solutions.

1. Wash with up to 4 CV of 1 M NaOH.
2. Wash with at least 3 CV of water, or until the eluent pH is neutral.

3a. To start a new separation: re-equilibrate with at least 3 CV of start buffer, or until the correct eluent pH is achieved.

3b. For storage, wash with at least 5 CV of storage solution. Let the UV baseline stabilize before storing the column.

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

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 CV of distilled water followed by 5 CV of 20% ethanol. Degas the ethanol and water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Seal the column well to keep it from drying out. Whenever possible, use a storage and shipping device supplied by the manufacturer. Store your columns and unused resins at 4°C to 30°C in 20% ethanol. Do not freeze.

To avoid forming air bubbles, make sure that your columns, buffers, and equipment are at the same temperature before using them.

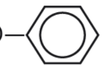
Sepharose™ Fast Flow resins

Sepharose™ Fast Flow resins are based on a legacy base matrix, which is still used in many applications.

For developing new processes or protein purification methods, we currently recommend using one of our more recent resins based on the Capto™ base matrix.

Sepharose™ Fast Flow resins are based on a matrix of particles (with a mean size of 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 through a chemically stable O-ether or S-ether linkage (Fig 3.18). Two different degrees of substitution of the phenyl ligand provide a further chance to optimize binding capacity and elution conditions. The particle size and bed volumes remain stable despite changes in salt concentration, keeping separations fast at high flow rates. You can easily scale-up methods from small HiTrap™ columns to large-scale columns like AxiChrom™ or Chromaflow™ columns. The performance of Sepharose™ Fast Flow resins is well documented, and there are many examples of the smooth transfer from the laboratory to pilot scale and on to production.

Ligand

Phenyl —O— 

Butyl-S —S—$(CH_2)_3$—CH_3

Butyl —O—$(CH_2)_3$—CH_3

Octyl —O—$(CH_2)_7$—CH_3

Fig 3.18. Ligands couple to Sepharose™ matrices through uncharged, chemically stable O-ether or S-ether linkages.

Resin characteristics

Sepharose™ 6 Fast Flow resins are based on cross-linked, 6% agarose forming spherical particles (with a d_{50V} of 90 µm) coupled to phenyl or butyl ligands through uncharged, chemically stable, O-ether or S-ether linkages. You can choose from two levels of phenyl ligand substitution.

Sepharose™ 4 Fast Flow resins are based on cross-linked 4% agarose forming spherical particles (with a d_{50V} of 90 µm) and coupled with butyl or octyl ligands through uncharged, chemically stable, O-ether linkages.

Table 3.10. Characteristics of Sepharose™ Fast Flow resins for HIC

Product	Matrix	pH stability*	Maximum operating back pressure† (MPa/psi) 1 MPa = 10 bar
Phenyl Sepharose™ 6 Fast Flow (high sub)	6% cross-linked agarose, spherical particles	Operational*: 3 to 13	90 µm
Phenyl Sepharose™ 6 Fast Flow (low sub)	6% cross-linked agarose, spherical particles	Operational*: 3 to 13	90 µm
Butyl-S Sepharose™ 6 Fast Flow	6% cross-linked agarose, spherical particles	Operational*: 3 to 13	90 µm
Butyl Sepharose™ 4 Fast Flow	4% cross-linked agarose, spherical particles	Operational*: 3 to 13	90 µm
Octyl Sepharose™ 4 Fast Flow	4% cross-linked agarose, spherical particles	Operational*: 3 to 13	90 µm

*pH range where resin can be operated without significant change in function.
All ranges are estimates based on the experience and knowledge within Cytiva.
†Maximum operating back pressure refers to the pressure above which the resin begins to compress.

Fig 3.19. Most HIC resins based on Sepharose™ 6 Fast Flow or Sepharose™ 4 Fast Flow are available prepacked in HiTrap™ or HiPrep™ columns and resin packs.

Table 3.11. HIC products based on Sepharose™ Fast Flow resin

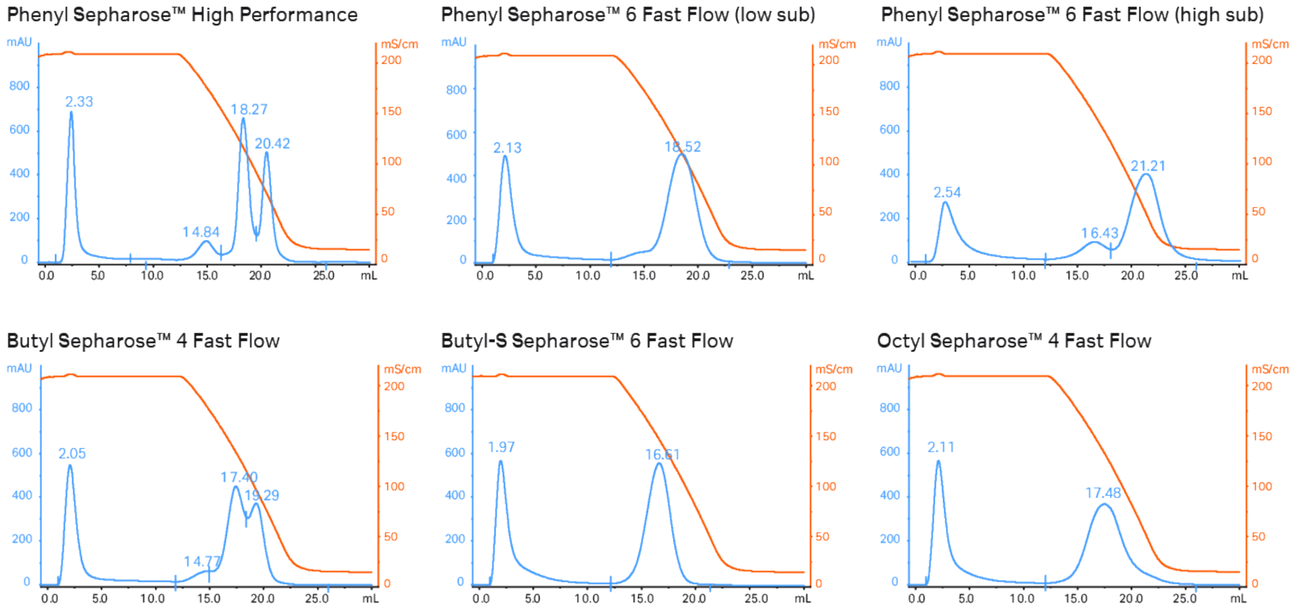
Product	Recommended working flow*	Maximum flow*	Maximum operating back pressure† (MPa/psi) 1 MPa = 10 bar
HiTrap™ Phenyl FF (high sub), 1 mL	up to 1 mL/min	4 mL/min	0.3/43
HiTrap™ Phenyl FF (high sub), 5 mL	up to 5 mL/min	20 mL/min	0.3/43
HiPrep™ 16/10 Phenyl FF (high sub), 20 mL	2 to 10 mL/min	10 mL/min	0.15/22
Phenyl Sepharose™ 6 Fast Flow (high sub)	50 to 400 cm/h	750 cm/h	0.3/43
HiTrap™ Phenyl FF (low sub), 1 mL	up to 1 mL/min	4 mL/min	0.3/43
HiTrap™ Phenyl FF (low sub), 5 mL	up to 5 mL/min	20 mL/min	0.3/43
Phenyl Sepharose™ 6 Fast Flow (low sub)	50 to 400 cm/h	750 cm/h	0.3/43
HiTrap™ Butyl FF, 1 mL	up to 1 mL/min	4 mL/min	0.3/43
HiTrap™ Butyl FF, 5 mL	up to 5 mL/min	20 mL/min	0.3/43
HiPrep™ 16/10 Butyl FF, 20 mL	2 to 10 mL/min	10 mL/min	0.15/22
HiTrap™ Butyl-S FF, 1 mL	up to 1 mL/min	4 mL/min	0.3/43
HiTrap™ Butyl-S FF, 5 mL	up to 5 mL/min	20 mL/min	0.3/43
Butyl Sepharose™ 4 Fast Flow	50 to 300 cm/h	400 cm/h	0.1/14
Butyl-S Sepharose™ 6 FF	50 to 400 cm/h	750 cm/h	0.3/43
HiTrap™ Octyl FF, 1 mL	up to 1 mL/min	4 mL/min	0.3/43
HiTrap™ Octyl FF, 5 mL	up to 5 mL/min	20 mL/min	0.3/43
HiPrep™ 16/10 Octyl FF, 20 mL	2 to 10 mL/min	10 mL/min	0.15/22
Octyl Sepharose™ 4 Fast Flow	50 to 300 cm/h	400 cm/h	0.1/14

*Recommendations are for separations at room temperature in aqueous buffers. *See* Appendix 4 to convert flow velocity (cm/h) to volumetric flow rates (mL/min) and *vice versa*. Note that the 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 backpressure that the equipment can withstand.

†Maximum operating back pressure refers to the pressure above which the resin begins to compress.

The binding capacity of a HIC resin depends highly on the properties of the target protein and contaminants, the selectivity of the resin, and the binding conditions. You need to determine capacity empirically during resin screening and method development.

Proteins of interest separate differently on the resin with various hydrophobic characteristics (Fig 3.20). Select the resin that gives the optimal selectivity, resolution, and loading capacity at the lowest salt concentration.



Sample: Cytochrome C, ribonuclease A , lysozyme, α-chymotrypsinogen, 6 mg protein/mL, (1:3:1:1) in start buffer
Sample volume: 1 mL
Sample load: 6 mg protein/mL resin
Flow: 1.0 mL/min, (150 cm/h)
Start buffer: 1.7 M $(NH_4)_2SO_4$, 0.1 M Na_2HPO_4, pH 7.0
Elution buffer: 0.1 M Na_2HPO_4, pH 7.0
Gradient: 0% to 100% elution buffer in 10 CV
System: ÄKTA™ chromatography system

Fig 3.20. Comparison of the different selectivity characteristics of various Sepharose™ based HIC resins.

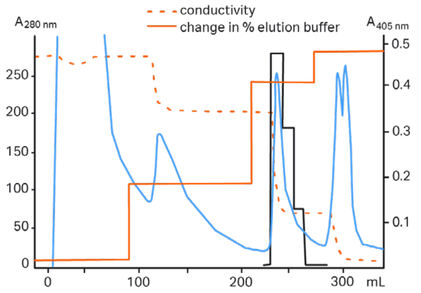
Purification examples

Resin screening

See Resin screening: Developing a monoclonal antibody purification in this chapter (Fig 3.15).

Capture: Enzyme purification

Figure 3.21 shows a capture step for partial purification of an enzyme, alkaline phosphatase, from *E. coli* homogenate. We selected conditions to ensure that the target protein binds to the column while most of the contaminants wash directly through. We concentrated the enzyme and achieved an effective purification step.



Column: HiPrep™ 16/10 Phenyl FF (high sub)
Sample: *E. coli* homogenate, centrifuged, filtered, and desalted
Sample load: 30 mL
Start buffer: 100 mM phosphate, 0.7 M ammonium sulfate, pH 7.0
Elution buffer: 100 mM phosphate, pH 7.0
Step elution: Sample application.
Wash through 37% elution buffer, 5 CV
Elution: 80% elution buffer, 3 CV
Wash: 100% elution buffer
Flow: 5 mL/min, (150 cm/h)
Detection: 280 nm
System: ÄKTA™ chromatography system

Fig 3.21. Purification of alkaline phosphatase on Phenyl Sepharose™ Fast Flow (high sub) resin. 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. World Health Organization estimates that 296 million people were living with chronic hepatitis B infection in 2019, with 1.5 million new infections each year.

For the noninfected population, scientists can produce effective vaccines at a large-scale by using recombinant technology to generate the recombinant Hepatitis B surface Antigen (r-HBsAg). Figure 3.22 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 resins. However, the mild hydrophobic properties of Butyl-S Sepharose™ Fast Flow enabled a successful purification, removing over 90% of impurities during the capture step.

Column: Butyl-S Sepharose™ 6 Fast Flow packed in XK 50/20, 130 mL
Sample: Concentrated CCS (containing approx. 12 mg of rHBsAg), 0. 6 M ammonium sulfate, pH 7.0
Sample load: 300 mL
Start buffer: 20 mM sodium phosphate, 0.6 M ammonium sulfate, pH 7.0
Elution buffer: 10 mM sodium phosphate, pH 7.0
Wash buffer: 30% isopropanol in elution buffer
Flow: 2 L/h, (100 cm/h)
System: ÄKTA™ chromatography system

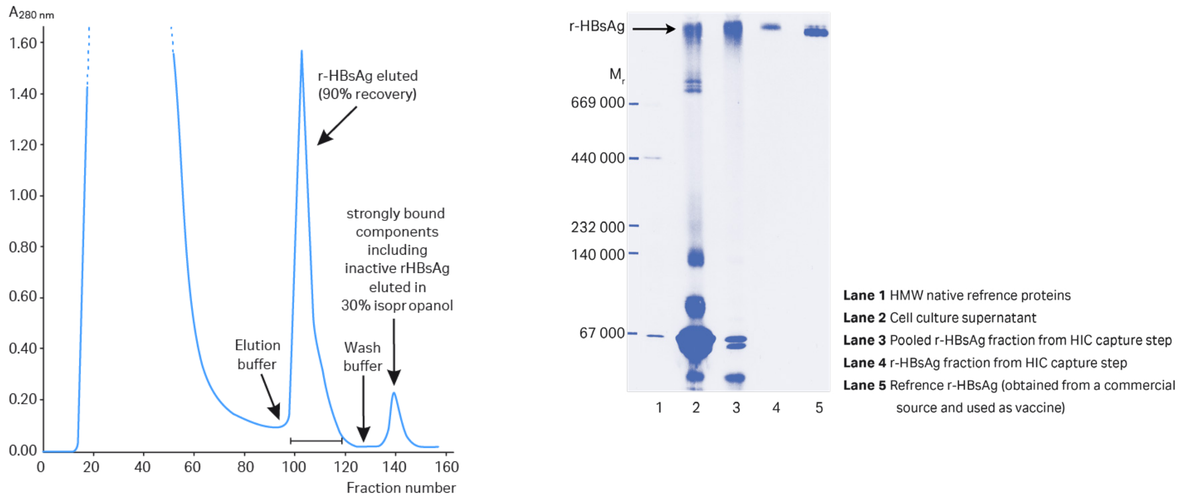


Fig 3.22. Large-scale purification of rHBsAg. Over 90% of impurities were removed by the HIC capture step (lane 3). Electrophoretic analysis (Gradient PAGE 4% to 30% under non-denaturing conditions) shows how purity increases after the HIC capture step. r-HBsAg is a particle of 22 nm, which is why the band appears at the top of the gel.

Intermediate purification: Fab fragment

Figure 3.23 shows an optimized elution scheme for intermediate purification of a Fab fragment, developed from the resin screening in Figure 2.3, Chapter 2. We optimized conditions on a 20 mL column until we could use a step elution to maximize throughput and make full use of the concentrating effect of HIC. Then we scaled up the step to a 200 mL column.

Columns:	Phenyl Sepharose™ 6 Fast Flow (high sub) in XK 16/20 (10 cm bed height), 20 mL
Sample:	Fab fraction from STREAMLINE™ SP, 80 mL
Start buffer:	1 M $(NH_4)_2SO_4$, 50 mM NaAc, pH 5.0
Elution buffer:	50 mM NaAc, pH 5.0
Flow rate:	5 mL/min
Gradient:	Step gradient to 50% elution buffer
System:	ÄKTA™ chromatography system
Detection:	280 nm

Columns:	Phenyl Sepharose™ 6 Fast Flow (high sub) in XK 50/20, 200 mL
Sample:	Fab fraction from STREAMLINE™ SP, 800 mL
Start buffer:	1 M $(NH_4)_2SO_4$, 50 mM NaAc, pH 5.0
Elution buffer:	50 mM NaAc, pH 5.0
Flow rate:	Equilibration: 100 mL/min Loading and elution: 50 mL/min
Gradient:	Step gradient to 50% elution buffer
System:	ÄKTA™ chromatography system
Detection:	280 nm

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

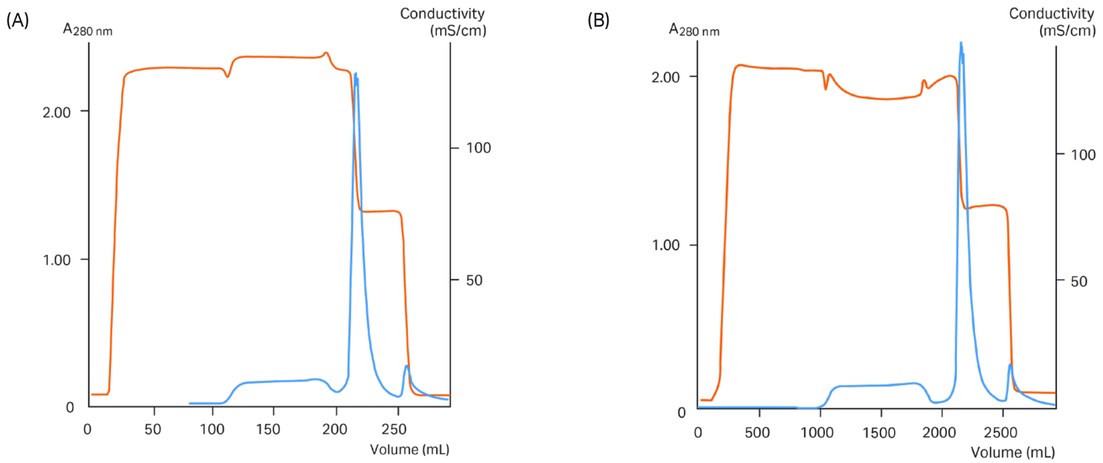


Fig 3.23. (A) Intermediate purification and (B) scale-up of a Fab fragment on Phenyl Sepharose™ 6 Fast Flow (high sub) resin.

Intermediate purification: Recombinant protein Annexin V

Figure 3.24 shows Butyl Sepharose™ 4 Fast Flow used as an intermediate step during purification of a recombinant protein, annexin V, expressed in *E. coli*.

Columns:	Butyl Sepharose™ 4 Fast Flow in XK 16/20 column
Sample:	Partially purified Annexin V expressed in *E. coli*, 5 mL
Start buffer:	20 mM sodium phosphate, 1 M ammonium sulfate, pH 7.0
Elution buffer:	20 mM sodium phosphate, pH 7.0
Flow velocity:	100 cm/h
Gradient:	0% to 50% elution buffer, 20 CV
Detection:	280 nm
System:	ÄKTA™ chromatography system

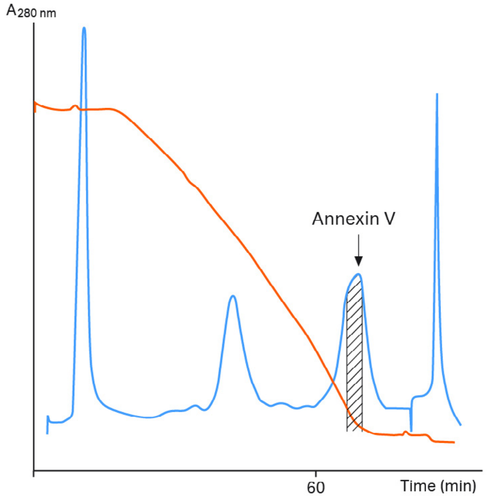


Fig 3.24. Intermediate purification of a recombinant protein.

Performing a separation

See Chapter 2 for guidelines on selecting resins, buffer, salt concentration, and pH, and optimizing your method.

You can use the instructions here as the basis for optimizing your separation.

To optimize separation and avoid any deterioration in column performance, it is essential to prepare your sample and buffer correctly. Your samples must dissolve fully and be free from any particles or material that can interfere with separation. *See* Chapter 2 and Appendix 1 for recommendations and advice on sample preparation.

Filter buffers and samples after adding all salts and additives. Use high-quality water and chemicals. Filter solutions using 1 µm filters or less. To avoid forming air bubbles in a packed column and ensure reproducible results, keep the column and buffers at the same temperature when you're preparing for a run.

To avoid problems with precipitation, check the salt stability window of the sample components. Avoid working at concentrations near the stability limit of the target protein. For samples with unknown hydrophobic properties, try this:

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

Note: You might need to reduce flow rates due to the viscosity of your chosen start buffer, sample characteristics, loading requirements, and the equipment you are using.

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

1. Remove any ethanol. To do this, wash with 5 CV 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 CV 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 the 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 the column with 5 to 10 CV of start buffer, or until the UV baseline and conductivity are stable.
2. Adjust the sample to your chosen salt concentration (and pH if needed). Filter and apply to the column.
3. Wash with 5 to 10 CV 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 to 20 CV, increasing the proportion of elution buffer until the salt concentration reaches a minimum (i.e., salt-free buffer).
5. Wash with 2 to 5 CV of salt-free elution buffer to elute any remaining hydrophobically bound material.
6. Re-equilibrate with 5 to 10 CV of start buffer, or until the conductivity reaches your 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 to 10 CV of start buffer or until the baseline, eluent pH, and conductivity are stable.
2. Adjust the sample to your chosen salt concentration (and pH if needed). Filter and apply to the column.
3. Wash with 5 to 10 CV 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 CV of elution buffer and salt at your chosen concentration.
5. Repeat step 4 at lower salt concentrations until the target protein(s) has been eluted.
6. Wash with 2 to 5 CV of salt-free elution buffer to elute any remaining hydrophobically bound material.
7. Re-equilibrate with 5 to 10 CV of start buffer, or until the conductivity reaches your required value.

You can 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 resin.

Check your column performance regularly by determining column efficiency and peak symmetry. *See* Appendix 2 for more details.

Never leave columns or equipment in high-salt solutions.

Cleaning

Correctly preparing your samples and buffers and applying 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 backpressure, or complete blockage are all signs that you need to clean the resin 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 CV and time required for each cleaning step can vary according to the degree of contamination. If the cleaning procedure does not restore column performance, change the top filter (when possible) before trying alternative cleaning methods. Take care when changing the filter, as it can affect the column packing and interfere with performance.

To remove common contaminants like precipitated proteins, use this procedure:

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 to 2 h, for Sepharose™ Fast Flow packed in larger columns

Note: You might need to reduce flow rates due to the condition of your column and the viscosity of your sample, buffers, or storage solutions.

1. Wash with up to 4 CV of 1 M NaOH.
2. Wash with at least 3 CV of water, or until eluent pH is neutral.

3a. To start a new separation, re-equilibrate with at least 3 CV of start buffer, or until you achieve the correct eluent pH.

3b. For storage, wash with at least 5 CV of storage solution. Let the UV baseline stabilize before storing the column.

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

Chemical stability

For daily use, HIC resins 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 CV of distilled water followed by 5 CV of 20% ethanol. Degas the ethanol and water mixture thoroughly and apply at a low flow rate to avoid over-pressuring the column. Seal the column well to keep it from drying out. Whenever possible, use a storage and shipping device supplied by the manufacturer. Store your columns and unused resins at 4°C to 30°C in 20% ethanol. Do not freeze.

To avoid forming air bubbles, make sure your columns, buffers, and equipment are at the same temperature before using them.

04

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 4.1.

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 a good economy. This chapter gives a brief overview of this approach, which can be recommended for any multi-step protein purification. The Strategies for Protein Purification Handbook 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 SEC.

HIC can be used with stepwise elution for a rapid capture step or with gradient elution to achieve the highest resolution in a polishing step.

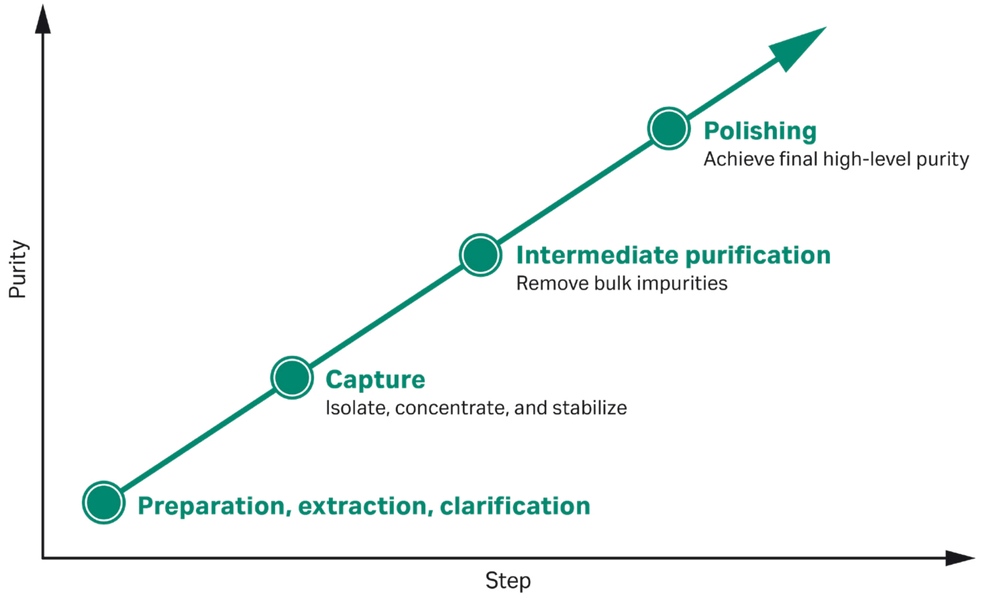


Fig 4.1. Strategy for protein purification.

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 or 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* are crucial for 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 4.1.

Table 4.1. Types of chromatographic techniques, and their properties

Technique	Property
Hydrophobicity interaction chromatography (HIC)	Hydrophobicity
Reversed phase chromatography (RPC)	Hydrophobicity
Ion exchange chromatography (IEX)	Charge
Size exclusion chromatography (SEC). Also called gel filtration	Size
Affinity chromatography (AC)	Biorecognition (ligand specificity)
Multimodal chromatography (MM)	A combination of several protein properties

In a multistep approach, factors to consider for a successful outcome are speed, recovery, resolution, or capacity. Each technique will have its characteristics and suitability for achieving any of these four performance parameters.

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 size exclusion chromatography) 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, the 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.

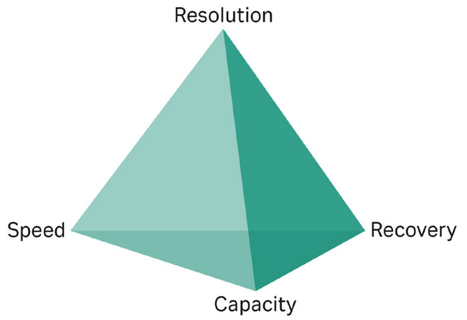


Fig 4.2. Every technique offers a balance between resolution, capacity, speed, and recovery.

A guide to the suitability of each purification technique for the stages in CIPP is shown in Table 4.2.

Table 4.2. Suitability of purification techniques for CIPP

Typical characteristics			Purification phase			Conditions	
Method	**Resolution**	**Capacity**	**Capture**	**Intermediate**	**Polishing**	**Sample start conditions**	**Sample end conditions**
AC	+++ or ++	+++ or ++	+++	++	+	Various binding conditions	Specific elution conditions
IMAC	+++	++	+++	++	+	Low concentration of imidazole, pH > 7.0	High concentration of imidazole, 500 mM NaCl, pH > 7.0
SEC	++	+	+		+++	Most conditions acceptable, limited sample volume	Buffer exchange possible, diluted sample
IEX	+++	+++	+++	+++	+++	Low ionic strength. pH depends on protein and IEX type	High ionic strength or pH changed
HIC	+++	++	++	+++	+++	High ionic strength, addition of salt required	Low ionic strength
RPC	+++	++		+	++	Ion-pair reagents and organic modifiers might be required	Organic solvents (risk for loss of biological activity)
MMC	+++	++	++	+++	+++	Various binding conditions	High ionic strength and/or pH change

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 4.2).

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 resins, 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.

Size exclusion chromatography (SEC) is a non-binding technique unaffected by buffer conditions, but with limited volume capacity. SEC 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 SEC process.

The selection of the final strategy will always depend upon specific sample properties and the required level of purification. Logical combinations of techniques for basic research are shown in Figure 4.3.

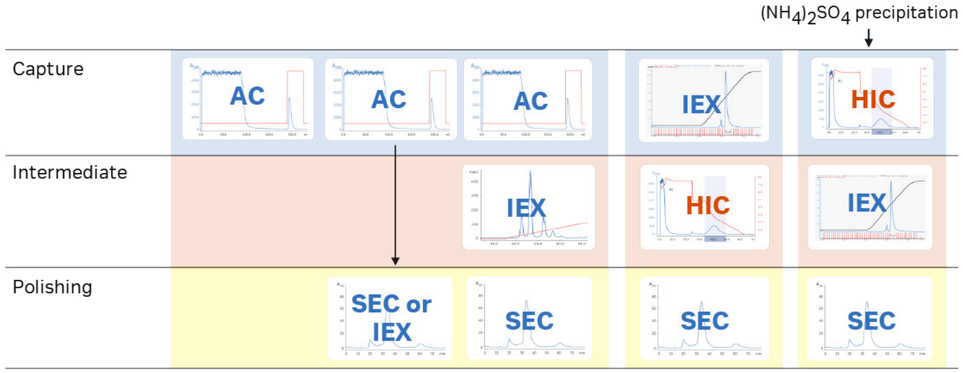


Fig 4.3. Typical combinations of chromatography techniques.

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-SEC 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 (SEC).

If nothing is known about the target protein use IEX-HIC-SEC. 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) is 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.

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

HIC resin for capture steps should offer high speed and high capacity.

1. Recommended: Capto™ chromatography resins (75 µm particle size) - excellent pressure-flow properties, and good resolution for large-scale applications using flow velocities up to 700 cm/h.
2. For established processes: Sepharose™ Fast Flow (90 µm particle size) chromatography resin— good resolution at flows up to 300 cm/h. If need higher productivity, the process can be modified to adapt to Capto™ chromatography resins.

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)

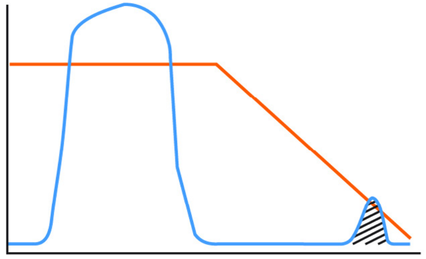
This purification represents a classical three-step purification process, using HIC-IEX-SEC, for the purification of human epidermal growth factor (h-EGF) expressed as an extracellular product by *Saccharomyces cerevisiae*.

The strategy was developed as follows: initial resin screening experiments for the capture step were performed on four different HIC resins (Fig 4.5). Phenyl Sepharose™ 6 Fast Flow (high sub) chromatography resin was selected as giving the optimal selectivity for EGF, a high binding capacity with low back pressure.

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

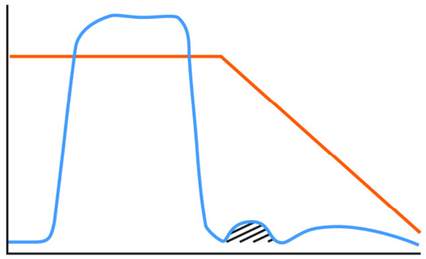
Fig 4.4. Classical HIC-IEX-SEC purification strategy.

Resin characteristics for EGF purification



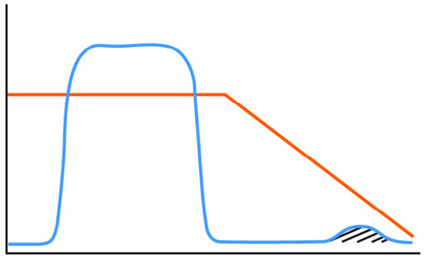
Phenyl Sepharose™ High Performance

- Very high selectivity
- Very high binding capacity
- Higher backpressure than for Fast Flow resins



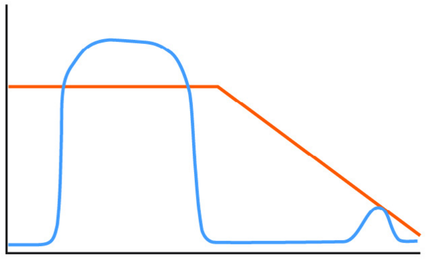
Phenyl Sepharose™ 6 Fast Flow (low sub)

- EGF comes in the wash with binding buffer
- Low selectivity for EGF



Phenyl Sepharose™ 6 Fast Flow (high sub)

- Very high selectivity
- High binding capacity
- Low back pressure



Butyl Sepharose™ 4 Fast Flow

- Was not possible to elute with low salt buffer
- Binds too tightly

Fig 4.5. Resin screening.

As Figure 4.6 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) chromatography resin and then scaled up to production scale using a BPG 300/500 column.

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

SEC on Superdex™ 75 prep grade chromatography column 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. The 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%.

Columns:	Phenyl Sepharose™ 6 Fast Flow (high sub) in XK/16/20 column, 10 cm bed height, 20 mL
Sample:	Yeast supernatant, ammonium sulfate added to 0.5 M
Sample volume:	450 mL
Sample load:	0.41 mg h-EGF/mL resin
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

Columns:	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 resin
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

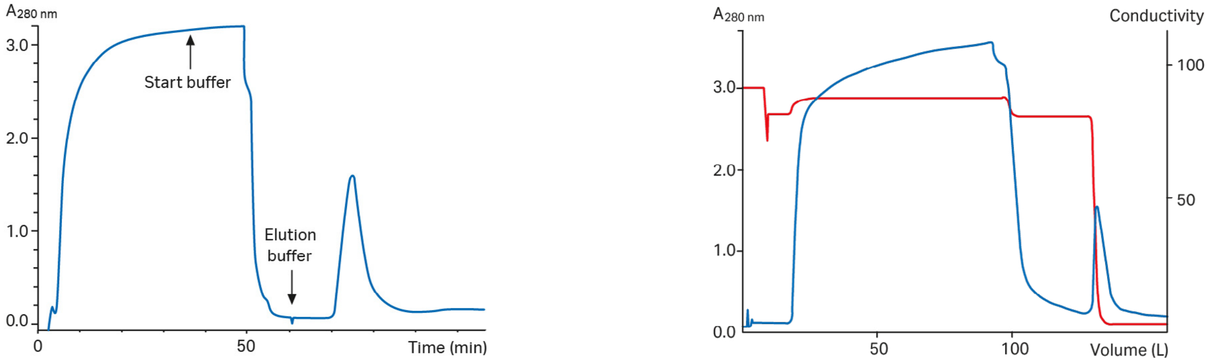


Fig 4.6. Development and scale-up of the capture step.

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 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.

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

1. Recommended for new processes: Capto™ ImpRes (40 µm particle size) chromatography resin — excellent pressure-flow properties with high resolution for large-scale applications using flow velocities up to 600 cm/h.
2. Sepharose™ High Performance (34 µm particle size) chromatography resin has been used in established processes for high resolution at flows up to 150 cm/h. If higher productivity is needed, the process can be modified to adapt to Capto™ ImpRes chromatography resins.

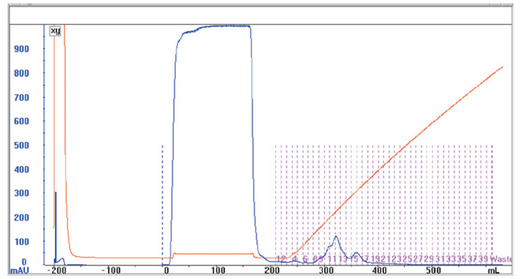
Purification of Fab fragment

Figure 4.7 shows a classic protocol, IEX-HIC-SEC, for purification of a Fab fragment against the gp 120 envelope of HIV-1. 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) ~ 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) chromatography resin were well-suited for intermediate purification, followed by a polishing step on SEC.

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

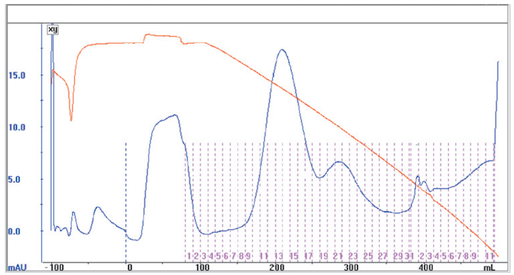
Columns: SP Sepharose™ Fast Flow packed in XK 16/10, 20 mL
Sample volume: 150 mL
Buffer: BufferPrep CIEX, pH range 3 to7.5, running pH 4.5
Gradient: 0 to 1.0 M NaCl in 20 CV
Flow rate: 150 cm/h, (5 mL/min))
System: ÄKTA™ chromatography system

Active fractions pooled: 10–14



Columns: 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% to 100% elution buffer in 20 CV
System: ÄKTA™ chromatography system

Active fractions pooled: 11–16



Columns: 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)
System: ÄKTA™ chromatography system

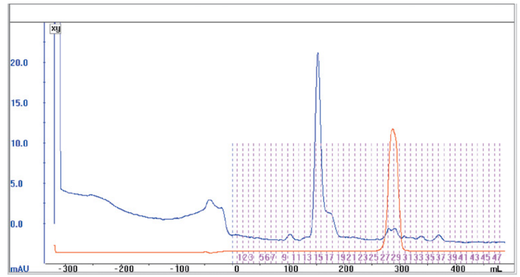


Fig 4.7. Protocol for purification of a Fab fragment against the gp 120 envelope of HIV-1.

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 mostly used, a polishing step will therefore focus on achieving the highest possible resolution.

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

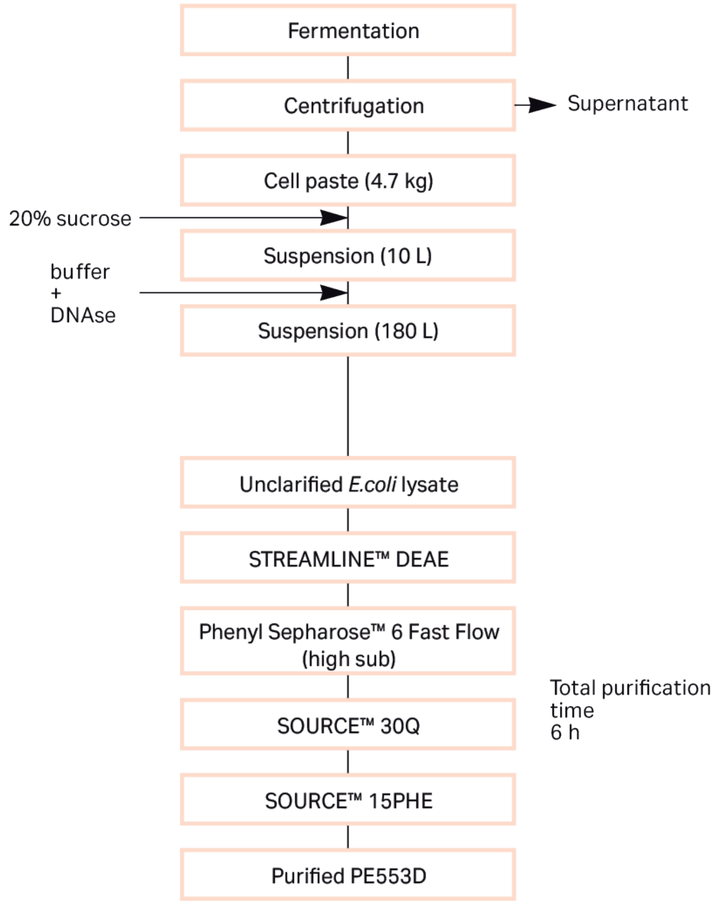
1. Capto™ ImpRes (40 µm particle size) chromatography resin — excellent pressure-flow properties for large-scale applications using flow velocities up to 600 cm/h.
2. SOURCE™ 15 (15 µm mean particle size) chromatography resin if other resins do not offer the required selectivity.

Optimize the gradient elution to maximize selectivity. Use high-efficiency resin 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 4.8 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.



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

Table 4.8. Four-step purification process.

Columns: Phenyl Sepharose™ 6 Fast Flow (high sub), in BPG 200/500 (i.d. 200 mm, 150 mm bed height), 4.7 L
Sample: 4.5 L of the pool from EBA adjusted to 0.6 M ammonium sulfate
Start buffer: 50 mM phosphate, 0.7 M ammonium sulfate, pH 7.4
Elution buffer: 20 mM phosphate, pH 7.0
Flow: 120 cm/h

Columns: SOURCE™ 30Q in FineLINE™ 100, (50 mm bed height), 375 mL
Sample: Pooled fraction from Phenyl Sepharose™ 6 Fast Flow, diluted 1 to 3 with distilled water, 1.5 L/cycle applied
Start buffer: 20 mM phosphate, pH 7.4
Elution buffer: 1.0 M sodium chloride, 20 mM phosphate, pH 7.4
Gradient: 0% to 50% elution buffer, 20 CV
Flow: 600 cm/h

Columns: SOURCE™ 15PHE, column 35 mm i.d., 100 mm bed height
Sample: Pooled SOURCE™ 30Q fraction, adjusted to 1.0 M ammonium sulfate, 0.5 L/cycle applied
Start buffer: 1.0 M ammonium sulfate, 50 mM phosphate, pH 7.4
Elution buffer: 50 mM phosphate, pH 7.4
Gradient: 0% to 45% elution buffer, 15 CV

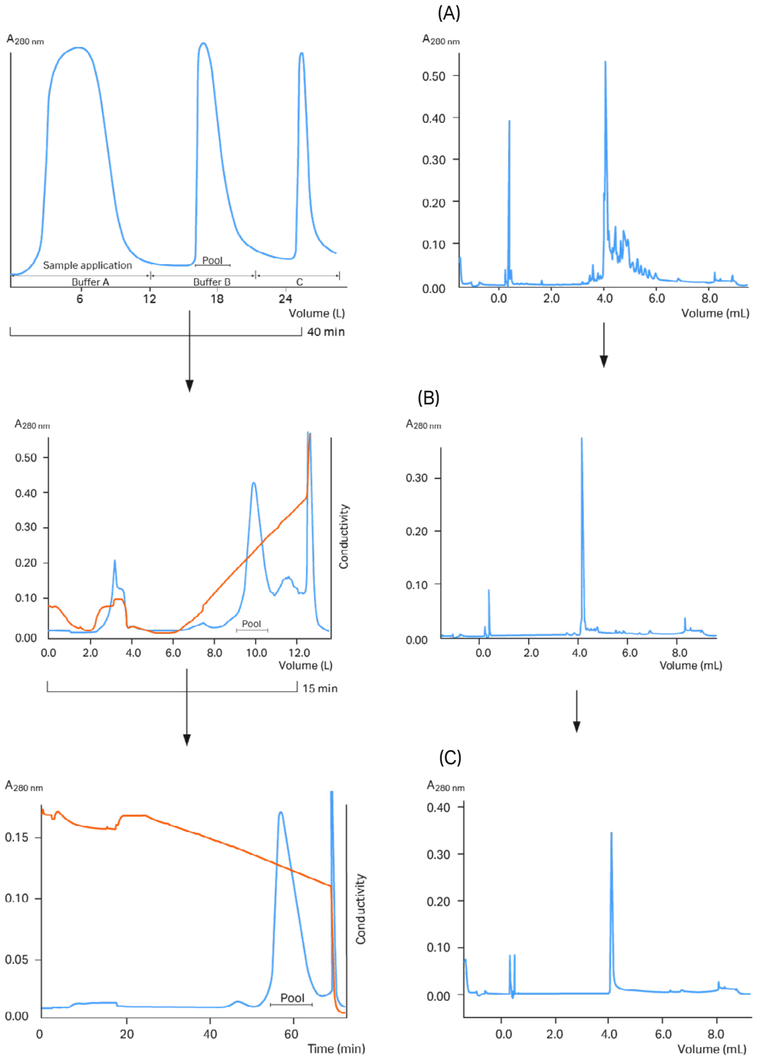


Fig 4.9. Purification of Exotoxin A.

Exotoxin A was captured directly from unclarified *E. coli* homogenate by an ion exchange column. The collected fraction was transferred directly to an intermediate purification step using Phenyl Sepharose™ 6 Fast Flow (high sub) chromatography resin to remove a substantial part of the UV absorbing material that could interfere with the following steps (Fig 4.9A). 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 chromatography resin, to remove most of the remaining contaminants, based on differences in their net surface charge (Fig 4.9B).

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 chromatography resin (Fig 4.9C). The process resulted in a pure protein, according to SDS-PAGE and RPC analysis, and the overall recovery was 51%.

Columns: Analytical RPC column
Sample: (A) Pool from Phenyl Sepharose™ 6 Fast Flow (high sub)
(B) Pool from SOURCE™ 30Q
(C) Pool from SOURCE™ 15PHE
Sample load: 50 µL
Start buffer: 0.1% trifluoroacetic acid (TFA) in water
Elution buffer: 0.1% TFA in acetonitrile
Gradient: 25% to 75% B over 47 min
Flow: 150 µL/min

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 SEC is an excellent choice 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 4.10.

SEC 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 SEC after techniques that reduce sample volume so that smaller columns can be used. Resins for polishing steps should offer the highest possible resolution.

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 based on hydrophobicity. RPC is a high-selectivity (high-resolution) technique, usually requiring the use of organic solvents. The RPC technique is used for purity check analyses when recovery of activity and tertiary structure is not essential. Since many proteins are denatured by organic solvents, RPC is not generally recommended for protein purification. However, in the polishing phase, when most 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 biomolecules, 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.

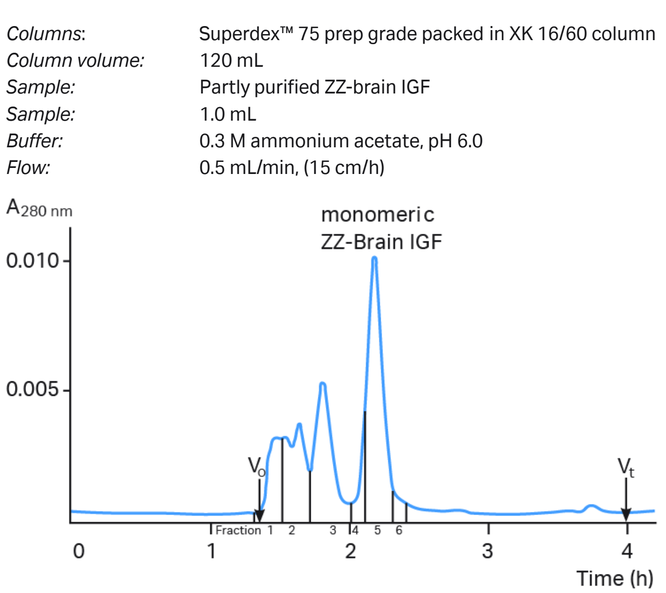


Fig 4.10. Final polishing step: separation of monomers, dimers, and multimers on Superdex 75™ prep grade chromatography column.

05

Large-scale purification

Our BioProcess™ chromatography resin family includes resins widely used by biopharmaceutical manufacturers. The support for these products comprise validated manufacturing methods, secure long-term chromatography resin supply, safe handling, and regulatory support files (RSF) to assist process validation and submissions to regulatory authorities. In addition, the Fast Trak™ Training and Education team provides you with the high-level hands-on training for all key aspects of bioprocess development and manufacturing. BioProcess™ chromatography resins have high chemical stability to allow efficient cleaning/sanitization procedures and validated packing methods established for a wide range of large-scale chromatography columns.

BioProcess™ chromatography resins for HIC

BioProcess™ resins for HIC are designed for large-scale purification and use in industrial processes. Chromatography resins for HIC purification for new methods or processes are mentioned in Table 5.1; while our legacy resins used in established processes or methods are listed in Table 5.2 for your reference. Figure 3.1 in Chapter 3 also guides you on how to select the resins.

Most of the resins are available in HiTrap™ and HiScreen™ formats for the development of efficient and robust purification parameters before the scaling-up process. Some of the resins are also available in high-throughput process development (HTPD) formats, such as PreDictor™ 96-well plates for fast and easy parallel screening of running conditions. PreDictor™ RoboColumn™ miniaturized columns (which you can use with a robotic station) are available for testing of a wide range of parameters such as DBC with small sample consumption. These small-scale formats in the early stages of process development, will save you time, buffer and reduce sample consumption.

Previously, HIC resins based on Sepharose™ Fast Flow and Sepharose™ High Performance were used in purification processes. Today, Capto™ and Capto™ ImpRes resins are more suitable choices. These consist of beads (particles) based on a very rigid, high-flow agarose base matrix. The beads with an optimized pore structure offer outstanding pressure or flow properties. Their high flow rates allow you to increase productivity and large-volume processing.

Table 5.1. BioProcess™ HIC chromatography resins for a new method or process development

Resin	Particle size, d_{50v} (µm)*	Recommended flow rate	Use
Capto™ Phenyl (high sub)	75	High	Capture
Capto™ Butyl	75	High	Capture
Capto™ Butyl-S	75	High	Capture
Capto™ Octyl	75	High	Capture
Capto™ Phenyl ImpRes	40	Medium	Polishing
Capto™ Butyl ImpRes	40	Medium	Polishing
Capto™ PlasmidSelect	40	Medium	Polishing
SOURCE™ 15PHE	15†	High	Polishing

* Median particle size of the cumulative volume distribution.
† Mean particle diameter (monodisperse size distribution).

Table 5.2. Legacy BioProcess™ HIC chromatography resins used in established processes or methods

Resin	Particle size, d_{50v} (µm)[1]	Recommended flow rate	Main use
Phenyl Sepharose™ 6 Fast Flow (low sub)	90	Medium	Capture
Phenyl Sepharose™ 6 Fast Flow (high sub)	90	Medium	Capture
Butyl Sepharose™ 4 Fast Flow	90	Medium	Capture
Butyl-S Sepharose™ 6 Fast Flow	90	Medium	Capture
Octyl Sepharose™ 4 Fast Flow	90	Medium	Capture
Phenyl Sepharose™ High Performance	34	Low	Polishing
Butyl Sepharose™ High Performance	34	Low	Polishing

[1] Median particle size of the cumulative volume distribution.

Capture purification

General capture purification is performed using Capto™ or Sepharose™ Fast Flow. When HIC is used as a capture step, the objective is to quickly adsorb the protein of interest from the crude sample and remove critical contaminants such as proteases. Figure 5.1 shows a comparison of properties of Capto™ with Sepharose™ chromatography resins. Sepharose™ Fast Flow resins are available with a wide range of hydrophobic interaction ligands and are still widely used in biopharmaceutical processes. However, for modern industrial applications, Capto™ resins are usually the preferred option having benefits such as high rigidity and ability to run at high flow rates, which gives you improved throughput without compromising performance. Capto™ HIC resins have been developed in collaboration with biopharmaceutical manufacturers specifically to improve productivity when processing recombinant proteins.

Figure 5.1 compares the pressure-flow performance of a Capto™ resin with Sepharose™ 6 Fast Flow in a representative large-scale situation. The pressure-flow properties of Capto™ are significantly better than Sepharose™ 6 Fast Flow due to the exceptional mechanical stability of the high-flow agarose base matrix.

Capto™ chromatography resins provide you with a good DBC over a wide range of residence times due to excellent mass transfer properties. When you increase flow velocities, the DBC decreases. However, a three-fold increase in loading flow velocity, from 200 to 600 cm/h, results only in a capacity decrease of 25% as shown in Figure 5.2.

When you optimize upstream processes to yield high titers, the need for better downstream productivity increases. In general, decreasing process times in large-scale chromatographic purifications with Capto™ HIC resins increases productivity and improves the final process economy.

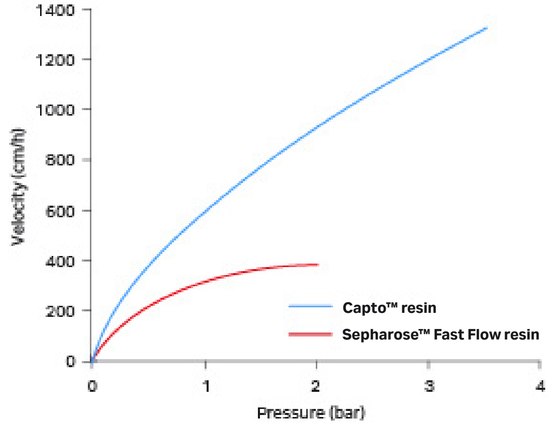


Fig 5.1. Pressure-flow properties of Capto™ vs Sepharose 6 Fast Flow chromatography resin. Running conditions: BPG 300 column (30 cm i.d), open bed at settled bed height = 20 cm with water at 20°C.

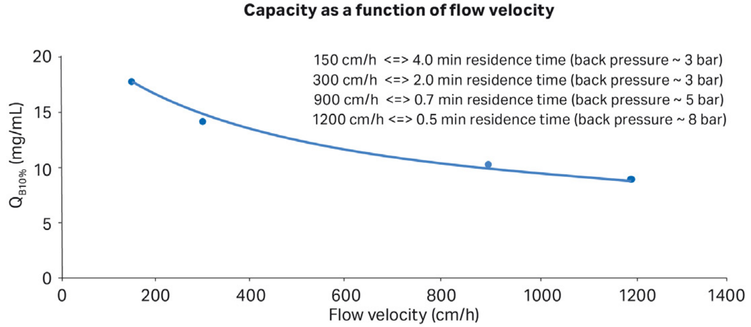


Fig 5.2. DBC of Capto™ Phenyl chromatography resin as a function of flow velocity with an increase in the loading flow velocity that decreased its capacity and improved productivity. Capto™ Phenyl packed in a Tricorn™ 5/100 GL column. Binding buffer: 1.2 M ammonium sulfate, 100 mM sodium dihydrogen phosphate. Elution buffer: 100 mM sodium dihydrogen phosphate, 10% ethylene glycol.

Polishing purification

The polishing purification is performed using chromatography resins with smaller average bead sizes such as Capto™ ImpRes resins. In contrast to capture purification where a fast, high-capacity step elution is commonly used, a polishing purification will focus on achieving high resolution (resulting in high purity).

Capto™ Phenyl ImpRes and Capto™ Butyl ImpRes are resins developed for the intermediate and polishing steps in a downstream protein purification process. These resins extend the well-established Capto™ platform to include high-resolution resin. These resins combine the high-flow characteristics of Capto™ resin with smaller particle sizes, they deliver both excellent pressure flow properties and resolution. The ability to run at higher flow velocities and higher bed heights increases flexibility in process design and might help to increase your productivity.

Figure 5.3 shows the pressure-flow properties of a high-resolution Capto™ based resin compared with a legacy Sepharose™ based resin. Although the bead sizes of the resins are similar, the pressure-flow properties of Capto™ ImpRes are significantly improved because of the greater mechanical stability of its high-flow base matrix.

We optimized the Capto™ ImpRes resin to bring similar retention times using Capto™ Phenyl Impres and Phenyl Sepharose™ High Performance for lysozyme. Likewise, Capto™ Butyl ImpRes was optimized to have a similar retention time to Butyl Sepharose™ High Performance for α-chymotrypsinogen. The overlay chromatograms shown in Figure 5.4 show the similarities in retention times for the harmonized model proteins, whereas the differences in retention times for the other two proteins indicate a slight difference in selectivity.

Capto™ ImpRes resins provide you with improved performance over Sepharose™ High Performance resins when scaling up due to their good pressure-flow properties, allowing high flow rates. You can use SOURCE™ 15 if the required selectivity is not available in a resin of larger particle size.

Prepacked, disposable solutions speed up the downstream process

In addition to a wide range of industrial-scale columns such as AxiChrom™ columns and bulk resins for purification of proteins, Cytiva offers you the large-scale, disposable ReadyToProcess™ columns: prepacked, prequalified, and presanitized process chromatography columns available with a range of BioProcess™ resins — including Capto™, Capto™ ImpRes, Sepharose™ Fast Flow, and Sepharose™ High-Performance product families.

ReadyToProcess™ columns are available in several different sizes (Fig 5.5) and are designed for the purification of biopharmaceuticals (e.g., proteins and antibodies, vaccines, plasmids, and viruses) for phase I and II clinical studies. Depending on the scale of operations, you can use the columns for manufacturing, as well as preclinical studies. ReadyToProcess™ columns make column packing, qualification, and sanitization redundant in the purification process, which provides you with substantial time saving.

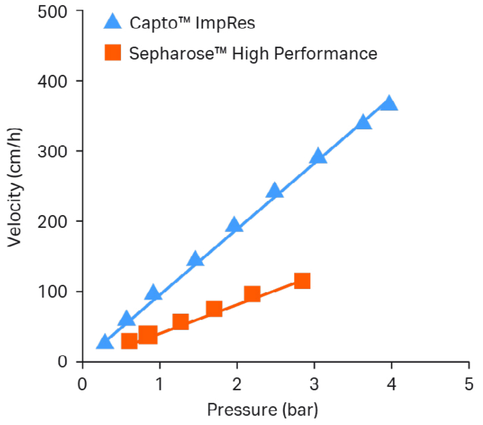


Fig 5.3. Pressure-flow properties of Capto™ ImpRes compared to Sepharose™ High Performance resin. Running conditions: AxiChrom™ 300 column, bed height with water at 20°C: 20 cm.

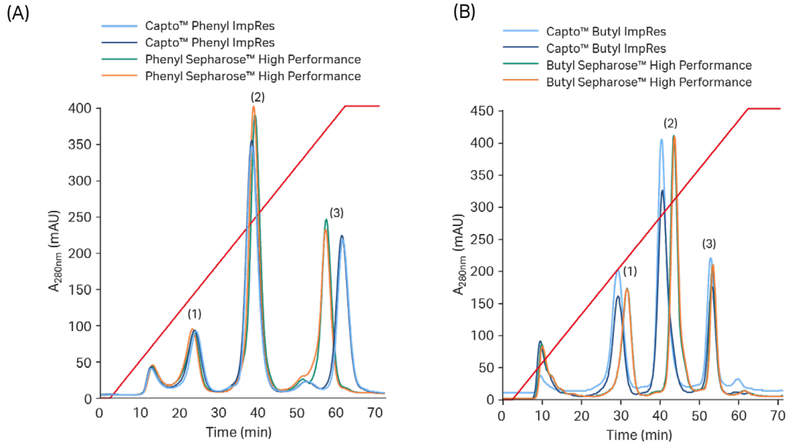


Fig 5.4. Selectivity test using three model proteins: ribonuclease A (1), lysozyme (2), and α-chymotrypsinogen (3). (A) Comparison of Capto™ Phenyl ImpRes vs Phenyl Sepharose™ High Performance (B) Comparison of Capto™ Butyl Impres vs Butyl Sepharose™ High Performance.

Process Characterization Kit

With the increased molecular diversity and drive for higher productivity, the challenge of getting a deeper understanding of sources of variability and set mitigation strategies grows. A Process Characterization Kit (Fig 5.6) is a tool that provides you with a deeper process understanding. It is available for multiple Capto™ and Capto™ ImpRes HIC resins. The kit consists of three bottles of the same chromatography resin. Each bottle has a defined ligand density level (high, average, low), which enables you to study the potential impact that the critical material attribute (CMA) may have on the process outcome.

Custom Designed Media (CDM)

The CDM group customize resins based on your specific industrial process separations when suitable resins are not available from the standard range. The CDM group works in close collaboration with your team to design, manufacture, test, and deliver resins for your specific purification requirements.

Mechanistic chromatography modeling enabling smarter and faster process development

Mechanistic models use computer simulations to decrease the number of experiments needed during process development. The simulations are based on known physiochemical phenomena involved in chromatography.

Mechanistic modeling is a complement to other process development approaches, such as high-throughput process development (HTPD) and multivariate data analysis (MVDA). Unlike in MVDA, physiochemical effects are also considered in mechanistic modeling and allow interpretation of the model parameter values for increased process understanding.

With mechanistic models, process developers can get a better understanding of both the entire process and the parameters that can influence the process with a smaller number of experiments. That way, process development can become faster, more scientific, and more reliable.

Learn more about mechanistic modeling at cytiva.com/modeling

Fig 5.5. ReadyToProcess™ columns are easily connected to the system and can be disposed of after completed production.

Fig 5.6. The Process Characterization Kit provides deeper process understanding.

06

Reversed phase chromatography: principles and methods

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 a good choice for applications such as peptide mapping or purity checking and is often used for the 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 resin. However, in practice, the techniques are very different. The surface of an RPC resin is usually more hydrophobic than that of a HIC resin. This leads to stronger interactions that for successful elution must be reversed using nonpolar, organic solvents such as acetonitrile or methanol. HIC resins 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 often, *gradient elution* is used to minimize the run time.

Oligonucleotides are often labeled, for example, with fluorescein, Dye667, or Dye550. For purity checking, an RPC resin with different selectivity would be used under high-resolution separation conditions as shown in Figure 6.1.

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 (MS).

RPC is often combined with other chromatography techniques to achieve identification and characterization unknown proteins and peptides. *Multidimensional liquid chromatography* (MDLC) followed by MS 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 nonpolar solvents, complex enzymes and multicomponent 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 state that may interact differently with an RPC resin. 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.

Columns:	RESOURCE™ RPC 1 mL
Sample:	5′-Cy™5-labeled 20-mer from a 0.2 mmol synthesis
Sample load:	Approx. 0.5 mg
Buffer A:	0.1 M triethyl ammonium acetate (TEAA), 5% acetonitrile
Buffer B:	0.1 M TEAA, 34% acetonitrile
Gradient:	10% to 65% eluent B, 10 CV
Flow rate:	1 mL/min

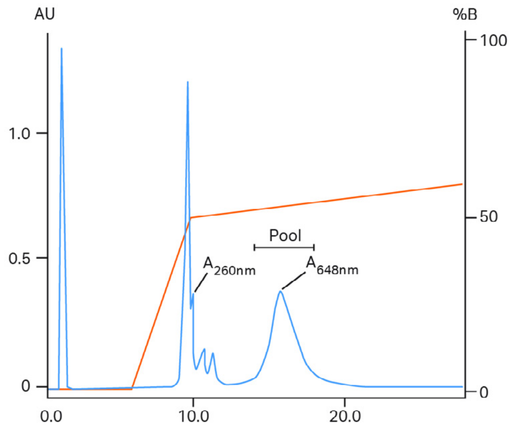


Fig 6.1. RPC can be used to separate the hydrophobic, labeled oligonucleotides from the free oligonucleotides.

Terminology

Certain terminology occasionally associated with RPC reflects the developmental history of the technique. The term "reversed phase" derives from "normal phase" chromatography — a "normal phase" technique utilizes a *hydrophilic stationary phase* together with hydrophobic *mobile phases* (using organic solvents such as hexane or methylene chloride). In RPC the *stationary phase* is hydrophobic, hence, water or organic solvent is used in the mobile phase, that is, the stationary phase is more hydrophobic than the mobile phase. RPC resins may be referred to as adsorbents while buffer 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 buffer and the resin. Initial conditions are primarily aqueous, favoring a high degree of organized water structure surrounding the sample molecule. Frequently, a small percentage of organic modifier (3% to 5%) of acetonitrile is present to achieve a "wetted" surface. As sample binds to the resin, the hydrophobic area exposed to the buffer is minimized buffer.

Separation relies on sample molecules existing in an equilibrium between the buffer and the surface of the resin. The distribution of the sample depends on the properties of the resin, the hydrophobicity of the sample, and the composition of the buffer (mobile phase), as illustrated in Figure 6.2. 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 resin has the nature of a multipoint attachment.

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*.

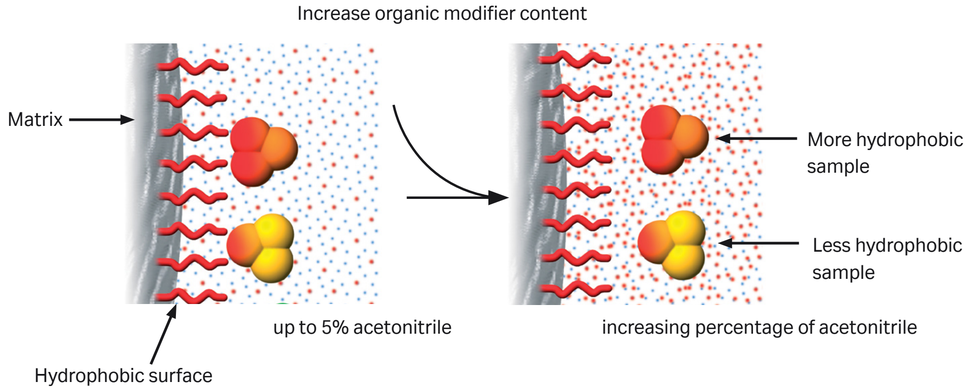


Fig 6.2. Proteins and peptides bind to an RPC resin under aqueous conditions and elute as the buffer becomes more hydrophobic.

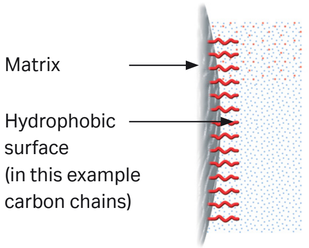
Steps in an RPC separation

There are two main types of RPC resin, 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 other 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, you must pack RPC resin into a column to form a *packed bed*. The bed is then equilibrated with buffer 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.

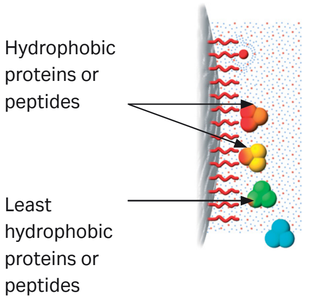
A typical biological sample contains a complex mixture of molecules with a correspondingly diverse range of hydrophobicity. Most biomolecules are sufficiently hydrophobic to bind strongly to RPC resins under aqueous conditions, in the presence of a low concentration of organic modifier, and to elute 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 6.3.

The 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* Typical eluent protocols for separation of proteins and peptides in this chapter) 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 to elute the bound sample. Elution begins by increasing the concentration of organic modifiers, such as acetonitrile. Molecules with the lowest hydrophobicity will elute first. By controlling the increase in organic modifiers, 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.



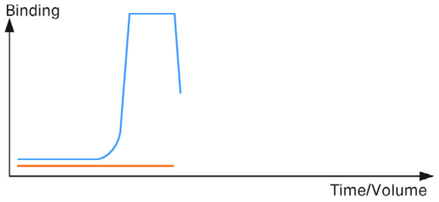
Equilibration

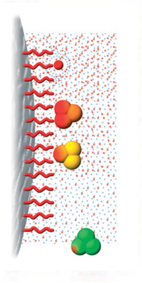
RPC resin equilibrated with start buffer.



Sample application

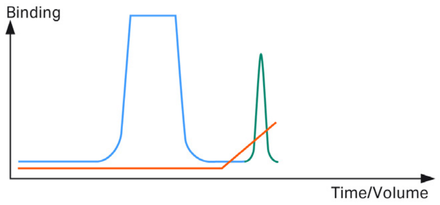
Hydrophobic molecules bind to hydrophobic resin, becoming concentrated on the column. Proteins or peptides with insufficient hydrophobicity elute during or just after sample application.

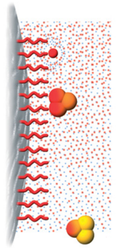




Elution 1

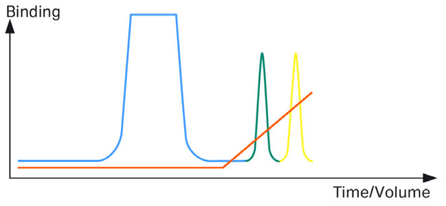
Gradient elution begins when UV signal returns to baseline. As % organic modifier increases the least hydrophobic molecules begin to elute first.

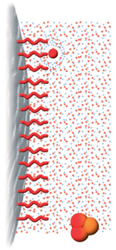




Elution 2

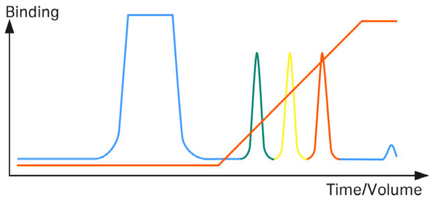
Samples elute in order of increasing hydrophobicity.

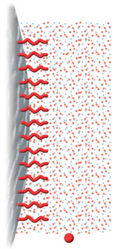




Elution 3

Molecules with the highest degree of hydrophobicity will be most strongly retained and eluted last.





Wash

Final wash in a high concentration of organic modifier removes any very hydrophobic contaminants before re-equilibration.

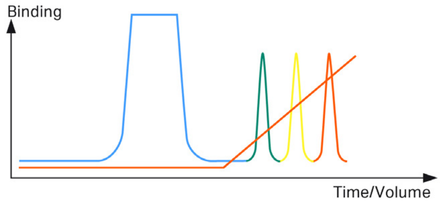


Fig 6.3. Steps involved in an RPC separation using gradient elution.

Components of an RPC resin

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 polymer matrix

Synthetic organic polymers, such as beaded polystyrene, provide excellent chemical stability, particularly under strongly acidic or basic conditions (from pH 1.0 to pH 12.0). 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 ion-pairing agents in this chapter); greater chemical stability facilitates any cleaning that may be required after working with biological samples (Fig 6.4). 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.

Matrices and ligands used in RPC resins from Cytiva are shown in Table 6.1.

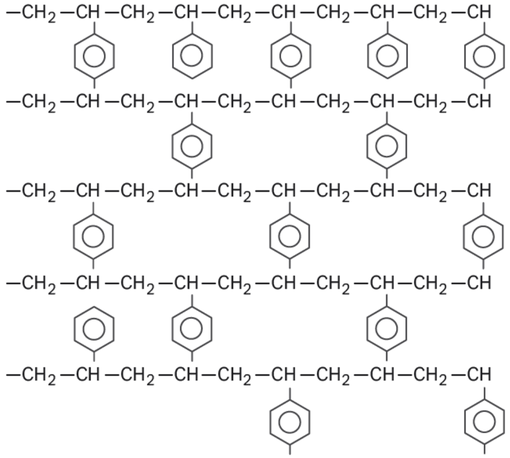


Fig 6.4. Partial structure of a polystyrene-based RPC resin.

Table 6.1. Matrices used for RPC resins available from Cytiva

Product	Matrix	Ligand	Mean particle diameter[1]	pH stability, operational[2]
SOURCE™ 15RPC	Nonpolar (hydrophobic) polystyrene/divinyl benzene, highly spherical, monodispersed	Matrix surface	15 µm	1–12
SOURCE™ 30RPC	As above	Matrix surface	30 µm	1–12

[1]Monodisperse size distribution.

[2]pH range where resin can be operated without significant change in function.

Use 15 µm resin for intermediate purification or polishing of laboratory-scale separations.

Use 15 µm or 30 µm resins for large-scale preparative and process separation, for example, SOURCE™ 15RPC or SOURCE™ 30RPC. These resins 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 good for the polishing stage of industrial processes.

Eluents

pH

Optimum pH is one of the most important parameters to establish. Figure 6.5 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.

Polystyrene-based resin separations can be performed over a broad pH range from 1.0 to 12.0. 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 to12.0. 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 8.0. In addition, a wider working pH range facilitates method optimization.

The 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.

Columns:	RESOURCE™ RPC 3 mL (i.d. 6.4 mm, length 100 mm)
Sample:	Angiotensin II and Angiotensin III
Sample load:	(0.25 mg/mL)
Start buffer:	(A) 0.1% TFA in water, pH 2.0
	(B) 10 mM NaOH in water
Elution buffer:	(A) 0.1% TFA, 60% acetonitrile in water
	(B) 10 mM NaOH, 60% acetonitrile in water
Flow rate:	2 mL/min
Gradient:	10% to 65% B in 10 min
System:	ÄKTA™ chromatography system
Detection:	214 nm

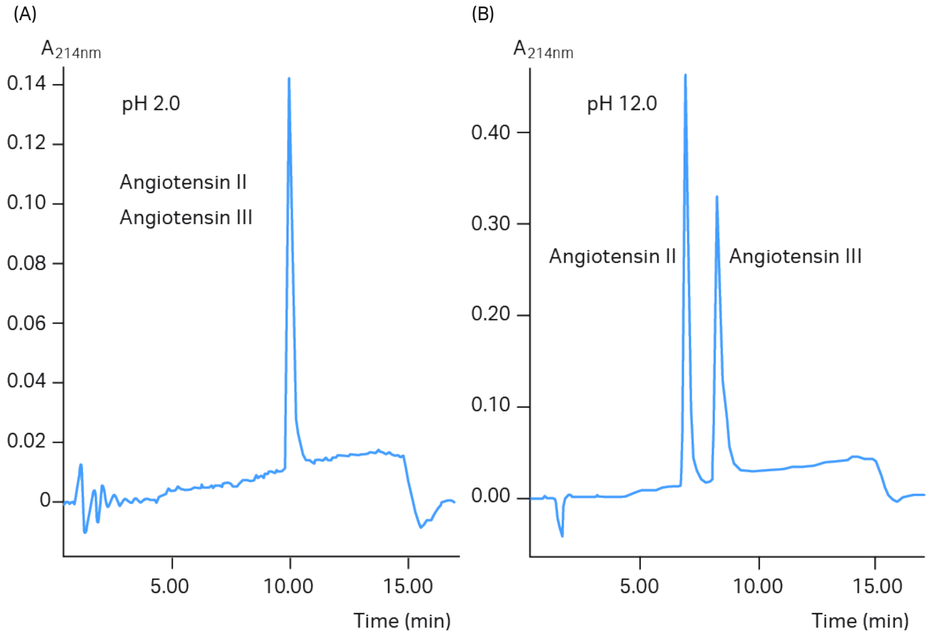


Fig 6.5. Selectivity altered by a change in pH from (A) pH 2.0 to (B) pH 12.0.

Examples of compounds used to maintain the required pH are given in Table 6.2.

Table 6.2. 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	12.0	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)	2.0 to 3.0	Pairing ion: CF_3COO^-	(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	6.0 to 10.0 and 4.3 to 5.3	Pairing ion: CH_3COO^-	10 to 100 mM	Volatile.
Phosphoric acid	2.0 to 3.0	Pairing ion: $H_2PO_4^-$, HPO_4^{2-}, PO_4^{3-}	10 to 100 mM	Less hydrophobic than TFA . Weak ion-pairing properties. Nonvolatile. Adjust to chosen pH with NaOH.
Eluent components that also act as ion-pairing agents for negatively charged molecules, e.g., oligonucleotides				
Triethylamine (TEA)	4.0 to 8.0	Pairing ion: $NH^+(C_2H_5)_3$	10 to 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	4.0 to 12.0	Pairing ion: $^+N(CH_3)_4$	5 to 100 mM	
Tetrabutylammonium chloride	4.0 to 12.0	Pairing ion: $^+N(C_4H_9)_4$	5 to 100 mM	

Ion-pairing agents

A common way to increase the hydrophobicity of charged components, enhance binding to the resin, and so alter retention time, is to add ion-pairing agents to the buffer. These agents bind *via* ionic interactions with charged groups and thereby suppress their influence on overall hydrophobicity (Figure 6.6). 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 resin. For example, an ion-pairing agent such as TFA is essential to ensure the binding of hydrophilic peptides. Examples of compounds that act as ion-pairing agents are given in Table 6.2.

The type and concentration of an ion-pairing agent can also affect retention behavior and subsequent selectivity, as shown in Figure 6.7.

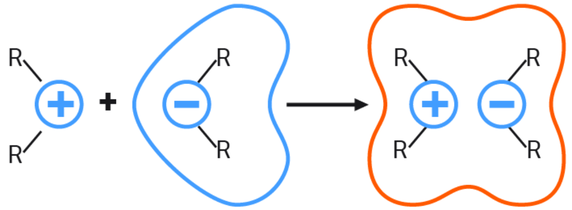


Fig 6.6. Ion-pairing agents alter net hydrophobic properties.

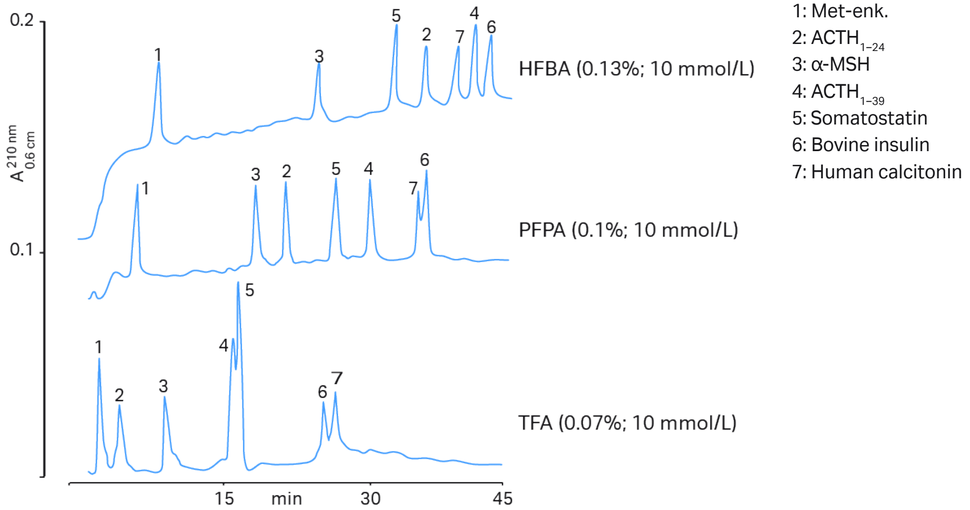


Fig 6.7. Ion-pairing agents significantly affect selectivity and even elution order.
*Figure taken from Bennett, H. P. J. *Manipulation of pH and ion-pairing reagents to maximize the performance of reversed-phase columns, in High-Performance Liquid Chromatography of Peptides and Proteins: Separation 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.

Elution

Organic modifiers

To bring about elution, an organic modifier is added to the eluent 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 6.3 reviews the commonly used modifiers in terms of their suitability for protein and peptide separations. Figure 6.8 shows how organic modifiers differ in their elution strength.

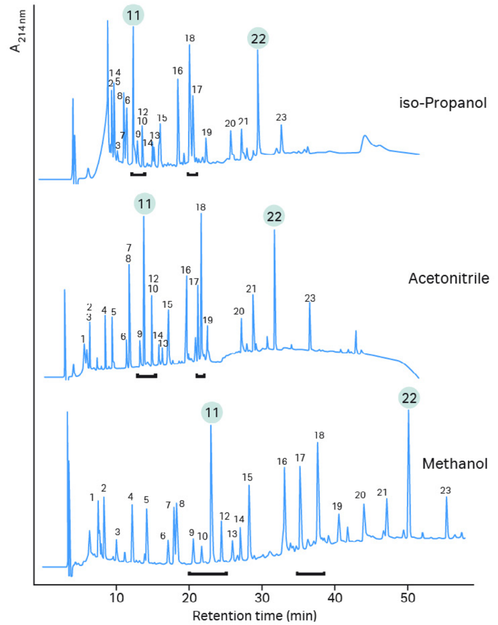


Fig 6.8. Organic modifiers differ in their elution strength rather than in their influence on selectivity when separating proteins and peptides. Figure taken from Aguilar, M.-I. and Hearn, M. T. W. High-resolution reversed-phase high-performance liquid chromatography of peptides and proteins. *Meth. Enzymol.* **270**, 3–26 (1996). Used with permission. © 1996 Academic Press, Inc.

Table 6.3. Commonly used organic modifiers. Acetonitrile is the preferred organic modifier used for protein and peptide separations

Organic modifier	Suitability	Boiling point (°C)	UV cut-off (nm)	Viscosity (cP at 20°C)	Comment
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	82	190	Low: 0.36	Most effect on protein structure.
	Proteins Peptides				More powerful denaturant than alcohols. Toxic.

Types of elution

Gradient elution is used most frequently for preparative and analytical, high-resolution separations of proteins and peptides, to minimize separation times. The UV absorbance and theoretical gradient traces shown in Figure 6.9 represents 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 4) and time (min), expressing eluent volumes as *CV* 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.

For *high-resolution analysis* a broad gradient is used 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 to separate a target molecule from all contaminants.

Step elution is used most typically for desalting (buffer exchange). Here you can use a low-resolution separation 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.

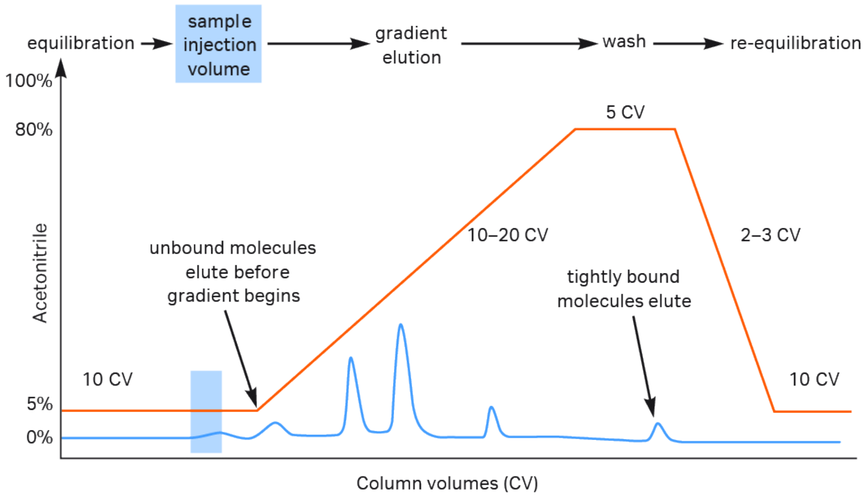


Fig 6.9. Typical high-resolution, RPC separation using gradient elution.

Isocratic elution is also used for 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 MS. Large volumes of sample can be applied (limited only by the binding capacity of the resin). 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. Size exclusion chromatography (SEC) is also used for desalting, separating low molecular weight contaminants and salts from higher molecular weight biomolecules (*see* Appendix 1). However, although SEC 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 6.10 shows a theoretical comparison between SEC and RPC used for desalting.

Binding capacity

The *available binding capacity* of an RPC resin 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 resin 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 resin, is available for binding of sample components, but high molecular weight components may be excluded from resin 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 resin) 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 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 resin later 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. Our advice focuses primarily on separation of biomolecules such as proteins and peptides. The steps for a typical RPC separation are summarized in Figure 6.11.

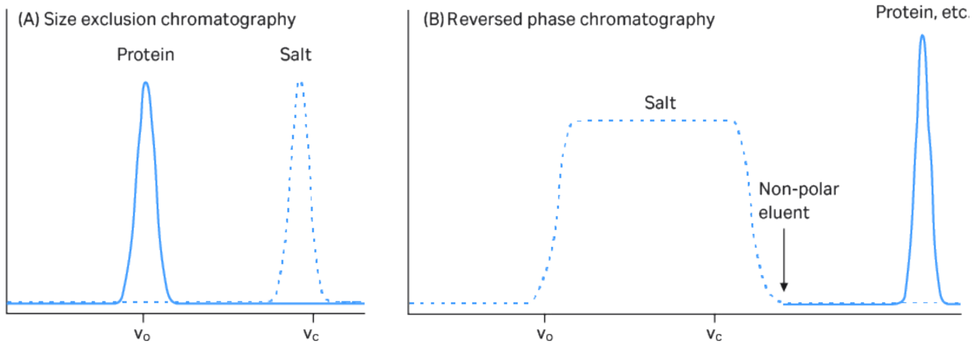


Fig 6.10. Desalting on RPC.

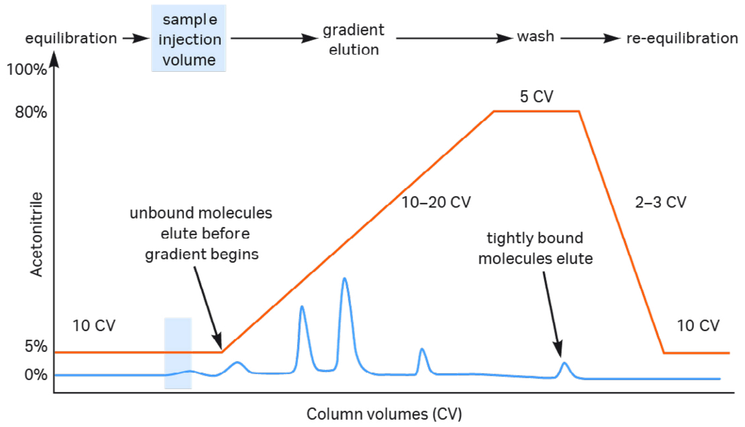


Fig 6.11. Typical RPC separation using gradient elution.

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 resins and conditions for an RPC separation of biomolecules are given here in order of priority:

1. Select a resin that provides best resolution under the simplest starting conditions, for example, TFA and acetonitrile in the eluent.
2. Scout for the pH that provides good resolution.
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.

Resin and column selection

Choosing the correct RPC resin 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 resin 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 α-helices and β-pleated sheets. The situation for proteins is further complicated by their tertiary structure.

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

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 might be traded off against speed and capacity. However, if used in the final polishing step, the resolution will be crucial.

Resolution in reversed phase chromatography depends on the efficiency of the column and the selectivity.

Scale of separation

Use 15 µm or 30 µm resins for large-scale preparative and process separation, for example, SOURCE™ 15RPC or SOURCE™ 30RPC. These resins 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 good for the polishing stage of industrial processes.

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

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 when- ever 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 to 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 6.2 and 6.3 have been chosen based on 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 6.2 shows the most used acids and bases for setting the pH of elution buffer 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. Elution buffer pH is therefore an important influence on elution order and final selectivity.

For samples with unknown properties, start with the most 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 6.2, to enhance binding of hydrophobic components to the resin. Note that other ion-pairing agents are not combined with TFA.

For samples with known specific properties, refer to Table 6.2.

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 Baseline drift: balancing eluents in this chapter). 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 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* pH and ion-pairing agents above in this chapter).

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 6.3, 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. To remove common contaminants like precipitated proteins, use this procedure:

Polymer-based resin

Since polymer-based resin 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

Buffer A: 0.065% TFA in 2% acetonitrile
Buffer B: 0.050% TFA in 80% acetonitrile

For samples known to require basic conditions

Buffer A: 0.125% ammonium solution pH 10 in 2% acetonitrile
Buffer B: 80% acetonitrile in buffer A

Other systems

Buffer 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 12.0 10 mM NaOH, 2% acetonitrile in buffer
Buffer B: 70% acetonitrile in buffer 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 µm 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 you must store eluents, 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 to 3 d 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 resin preparation

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

1. Wash the column with approximately 3 CV of buffer B at a low to moderate flow rate appropriate for the column.
2. Run a 2 to 3 CV linear gradient from 100% buffer B to 100% buffer A at the same flow rate as in step 1.
3. Equilibrate the column with 10 CV of buffer 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% buffer A and equilibrate to a stable baseline prior to sample injection.

Sample preparation

Sample preparation is critical especially for high-resolution separations. 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 buffer or a solution with a lower content of organic modifier.

Samples must be clear and free from particulate matter. Desalt very crude samples using SEC (Appendix 1) or an RPC column packed with larger particles to remove contaminants that may foul a high-resolution RPC column.

1. Dissolve the sample in buffer A.
2. Centrifuge samples at 10 000 × g for 10 min or filter through a 0.22 or 0.45 µm sterile filter. Use a solvent-resistant filter if there is an organic modifier in buffer 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 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 buffer A. A low percentage of organic modifier in buffer 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 buffer 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 buffer 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 resin and the degree of resolution required.

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% to 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 DBC and a fast separation especially when applying large sample volumes.

Sample volume

As a binding technique, RPC is independent of sample volume. You can apply large volumes of diluted sample to concentrate and separate the sample.

Temperature

Maintain sample, eluents, columns, and chromatography equipment at the same, constant temperature throughout a separation to ensure reproducible results. Temperature will affect sample and buffer viscosity and may influence resolution. 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, you can use a stepwise or continuous gradient to elute components. The gradient shape and volume must be empirically determined for each separation. Gradient slopes are described as changes in percent buffer B per unit time (Buffer B [%]/min) or per unit volume (B [%]/mL).

In any gradient elution, the concentration of organic modifier is lower in buffer A than in buffer 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 molecule(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 to detect any baseline disturbances coming from the column or impurities/components in the buffer. Run from 5% buffer B to 80% B over 10 to 20 CV.
3. Adjust buffer conditions if the baseline drift is too great (*see* Baseline drift: balancing eluents in this chapter).

Check buffer components and cleanliness of column if there are signs of contamination (*see* Ghosting in the Troubleshooting section at the end of this chapter).

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 6.4.

Table 6.4. 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 elution buffer.
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 peaks are resolved.

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

Step gradients (i.e., a series of isocratic elution at different percent buffer 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 if 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 resin. When programming a chromatography system in time mode, remember that changes in flow rate will affect gradient slope and, therefore, resolution.

Reduce flow rate 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 DBC (*see* Binding capacity in this chapter).

The optimal flow rate for sample application must be determined empirically.

Wash and re-equilibration

1. Wash the column with at least 5 CV of 100% buffer B to remove any bound molecules.
2. Apply a decreasing gradient over 2 to 3 CV from 100% buffer B to 0% buffer A to avoid damaging the column by a sudden change in composition.
3. Re-equilibrate the column using at least 10 CV of buffer 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 buffer components can cause a phenomenon referred to as “ghosting”. Trace levels of organic impurities bind to the resin, 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 buffer.

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 a linear fashion as the proportion of buffer 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 buffer components is corrected for during column equilibration. As the proportion of organic part 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 buffer A and B and thereby balancing the “concentrations” with respect to UV-absorption properties to give a straight baseline. Because of batch-to-batch variations in the absorption properties of buffer 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% to 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 buffer A and 0.05% TFA in buffer B. Table 6.5 provides troubleshooting under various conditions in using RPC.

Table 6.5. Troubleshooting conditions in handling RPC

Situation	Possible cause	Remedy
Reduced or no flow through column.	Outlet closed or pumps not working.	Open outlet. Check pumps for signs of the leakage.
	Blocked filter, end-piece, tubing or precolumn.	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.
Sample does not elute during gradient elution.	pH-caused precipitation.	Adjust pH to avoid precipitation.
	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 resin or change organic modifier.
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 resin.
	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.
	Column not equilibrated properly.	Re-equilibrate column.
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 cutoffs of buffer 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 (see Appendix 2). Repack if necessary. Replace column if necessary.

Situation	Possible cause	Remedy
Resolution less than expected.	Large mixing spaces at top of or after column.	Top up surface of resin if possible. Reduce all post-CV.
	Suboptimal 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 elution buffer to maintain solubility.
	Column aging.	Adjust elution buffer to improve ion suppression. Replace column if necessary.
	Sample not filtered properly.	Clean the column, filter the sample and repeat.
	Poor selectivity.	Add or adjust ion-pairing agent. Change to another resin.
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.
	Incorrect buffer conditions (possibly due to evaporation).	Check conditions required. Prepare fresh solutions.
Resin/beads appears in buffer.	Column operated at too high pressure.	Do not exceed recommended operating pressure for resin or column.
Low recovery of activity, but normal recovery of mass.	Sample may be denatured or inactive in the buffer.	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 resin.
	Enzyme separated from co-factor or similar.	Test by pooling aliquots from the fractions and repeating the assay.
Sample yield lower than expected.	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 precipitated.	Decrease sample load or change buffer conditions.
	Basic sample components bound to resin 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.
	Removal of inhibitors during separation.	

Situation	Possible cause	Remedy
Air bubbles in the bed.	Eluents not properly degassed.	Degas eluents 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 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 buffer.
Unexpected peaks or spikes in chromatogram. Peaks appear on blank gradients.	Impurities in the buffer.	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 flow 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.	Buffer A and B absorb differently at the same wavelength.	Balance elution buffer components or use a different wavelength.
	Impurities in the elution buffer.	Use high-quality HPLC grade reagents.
Excessive baseline noise.	UV absorption by components in elution buffer.	Monitor at different wavelength or reduce concentration of UV absorbing component (usually the ion-pairing agent) or change organic modifier if this is absorbing.

SOURCE™ chromatography resins: rapid separation with high resolution and easy scale-up

- Use SOURCE™ RPC resins 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 8.0 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).

SOURCE™ resins are based on a matrix made from monodispersed, rigid, polystyrene/divinyl benzene (Fig 6.11). The resins demonstrate extreme chemical and physical stability and, unlike silica-based resins, can be used at extremes of pH. A range of particle sizes (30 or 15 µm) enables the use of SOURCE™ RPC from large-scale purification through to high-resolution analysis. The uniformity and stability of SOURCE™ particles ensure high flow rates at low backpressure. Such high flow rates are useful for speeding up cleaning and re-equilibration steps. Flow rates are more likely to be limited based on the equipment available and the eluents used rather than the physical properties of the resin.

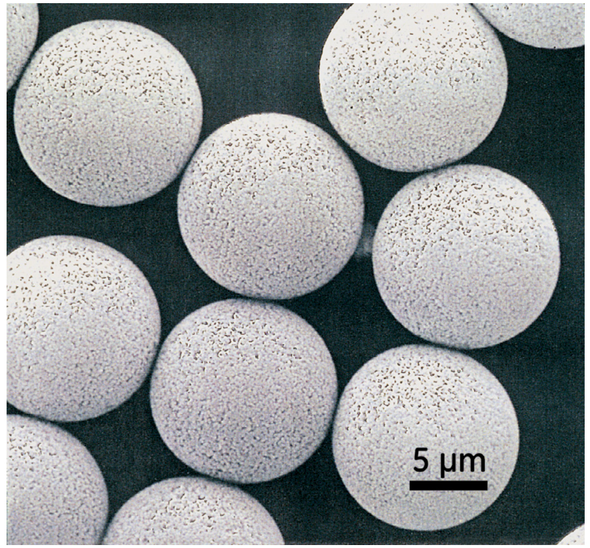


Fig 6.11. Scanning electron micrograph of SOURCE™ 15RPC particles shows the uniform size distribution.

Resin characteristics

Table 6.6. Resin characteristics for SOURCE™ RPC resins

Product	Temperature stability for regular use	pH stability	Mean particle diameter[3]
SOURCE™ 15RPC	4°C to 40°C	Operational[1]: 1 to 12 CIP[2]: 1 to 14	15 µm
SOURCE™ 30RPC	4°C to 40°C	Operational[1]: 1 to 12 CIP[2]: 1 to 14	30 µm

[1] pH range where resin can be operated without significant change in function.
[2] pH range where resin can be subjected to cleaning- or sanitization-in-place. without significant change in function.
[3] Mean particle diameter (monodisperse size distribution).
All ranges are estimates based on the experience and knowledge within Cytiva.

Separation methods can be easily scaled up from prepacked columns such as RESOURCE™ and SOURCE™ 15RPC ST 4.6/100 (Table 6.6) through to large-scale columns such as FineLINE™.

Purification options

Table 6.7. RPC resins based on SOURCE™ matrices are available in prepacked columns and as resin lab packs

Product	DBC/mL resin*	Recommended operating flow†	Maximum operating flow†	Maximum operating back pressure‡ (MPa/psi) 1 MPa = 10 bar
SOURCE™ 15RPC ST 4.6/100, 1.7 mL	~ 18 mg BSA ~ 14 mg Bacitracin ~ 45 mg insulin	0.5 to 2.5 mL/min	5 mL/min	4/580
RESOURCE™ RPC, 1 mL	~18 mg BSA ~14 mg Bacitracin ~45 mg insulin	1 to 5 mL/min	10 mL/min	4/580
RESOURCE™ RPC, 3 mL	~ 18 mg BSA ~ 14 mg Bacitracin ~ 45 mg insulin	1 to 5 mL/min	10 mL/min	4/580
SOURCE™ 15RPC	~ 18 mg BSA ~ 14 mg Bacitracin ~ 45 mg insulin	150 to 900 cm/h	1800 cm/h	
SOURCE™ 30RPC	~ 14 mg BSA ~ 23 mg bacitracin ~ 72 mg insulin	100 to 1000 cm/h	2000 cm/h	

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

† *See* Appendix 4 to convert flow velocity (cm/h) 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.

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

In RPC, many parameters, such as properties of the protein, flow rates, and selectivity of the resin 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 to RESOURCE™ RPC 3 mL column for higher resolution and method development on a 10 cm bed height. You can pack SOURCE™ RPC resins in Tricorn™ columns according to the conditions shown in Table 6.8.

Table 6.8. Packing volumes and bed heights for SOURCE™ resins 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

Purification at high pH

Figure 6.12 shows the successful purification of β-lipotropin fragment 1 to 10 (M_r 950) achieved by using high pH conditions (pH 12.0), possible only on polymer-based resin. At pH 2.0, the contaminants are eluted in the β-lipotropin peak.

Column:	RESOURCE™ RPC, 3 mL
Sample:	β-lipotropin (fragment 1–10), 0.5 mg in 300 µL water
Buffer A:	(A) 0.1% TFA pH 2.0 or (B) 10 mM NaOH (pH 12.0) in water
Buffer B:	60% acetonitrile in 0.1% TFA (pH 2.0) or 10 mM NaOH (pH 12.0)
Flow:	2 mL/min, (360 cm/h)
Gradient:	0% to 30% B in approx. 7 CV (10 min)

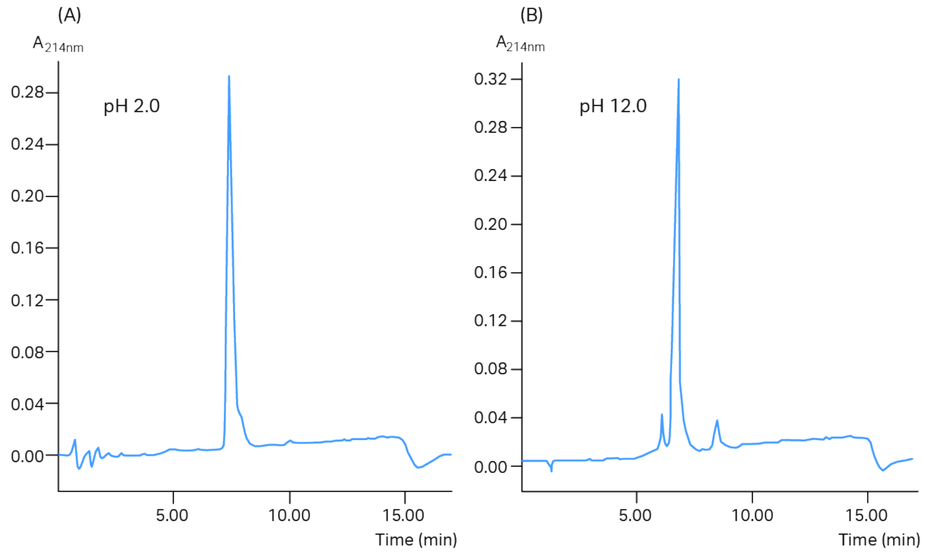


Fig 6.12. Purification of lipotropin fragment at (A) pH 2.0 and (B) pH 12.0.

Polishing step and scale-up

Figure 6.13 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
Sample load:	2 mL
Buffer A:	0.05% TFA, 5% acetonitrile
Buffer B:	0.05% TFA, 80% acetonitrile
Flow:	1.6 mL/min, (300 cm/h)
Gradient:	0% to 100% B in 40 CV
Detection:	280 nm

Column:	SOURCE™ 15RPC, 35 × 100 mm
Sample:	62.5 mL EGF pool after Q Sepharose™ High Performance
Sample load:	0.1 mg/mL resin, 10 mg total load
Buffer A:	0.05% TFA, 5% acetonitrile
Buffer B:	0.05% TFA, 80% acetonitrile
Flow:	50 mL/min, (300 cm/h)
Gradient:	0% to 100% B in 40 CV
Detection:	280 nm

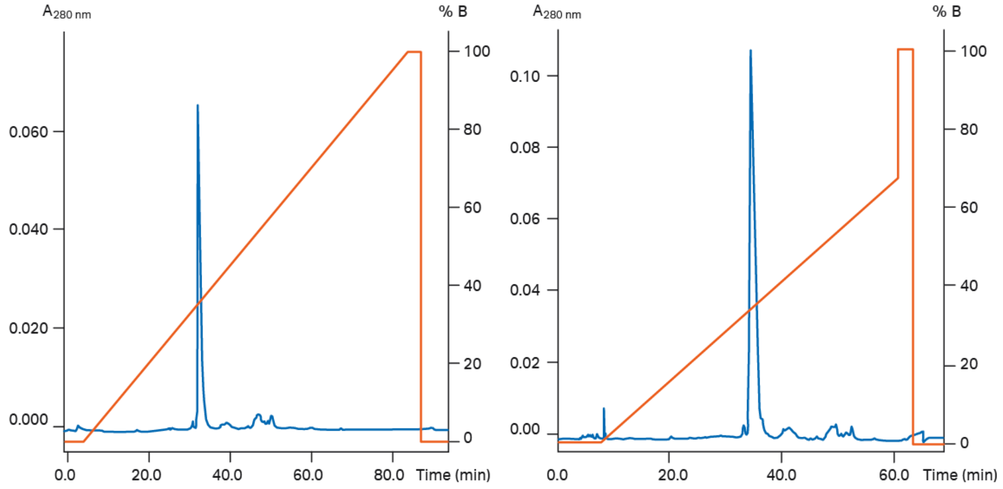


Fig 6.13. Preparative separation of a recombinant protein.

Scaling up

Figure 6.14 shows the excellent scalability of SOURCE™ 30RPC. The resin 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 L FineLINE™ column.

Column: SOURCE™ 30RPC, 10 × 300 mm column i.d. (24 mL); 200 × 300 mm column i.d. (10 L)
Sample: angiotensin II, ribonuclease A and insulin
Sample load: 0.064 mg/mL resin, total load
Buffer A: 0.1% TFA, 0.05 M NaCl
Buffer B: 0.1% TFA, 60% isopropanol
Flow velocity: 150 cm/h
Gradient: 20% to 70% B, 5 CV
Detection: 280 nm

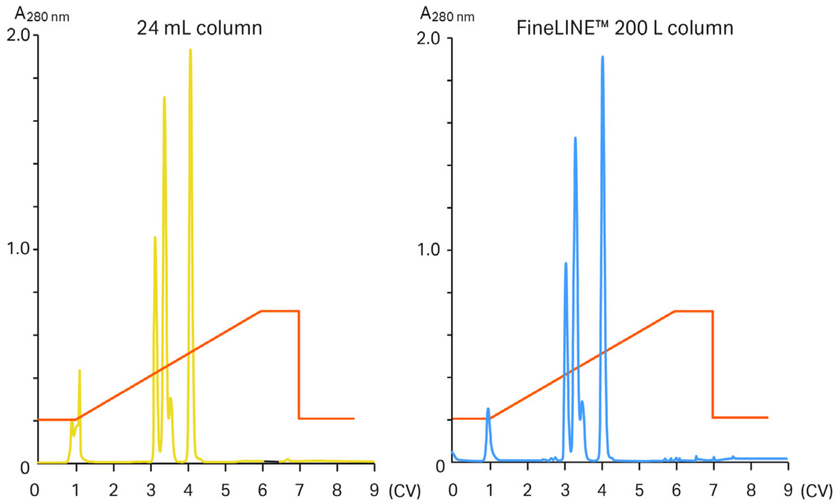


Fig 6.14. Scalability of SOURCE™ 30RPC resin from 24 mL to 10 L.

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
Buffer A: 0.1% TFA in 2% acetonitrile
Buffer B: 0.1% TFA in 80% acetonitrile

For samples known to require basic conditions
Buffer A: 0.125% ammonium solution, pH 10.0 in 2% acetonitrile
Buffer B: 80% acetonitrile in buffer A

First-time use or after long-term storage

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

Separation by gradient elution

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

Flow: 2 mL/min, (SOURCE™ 15RPC ST 4.6/100)
1 to 5 mL/min, (RESOURCE™ 1 mL)
1 to 5 mL/min, (RESOURCE™ 3 mL) or, for larger columns, 200 cm/h (SOURCE™ 15RPC) and
100 to 1000 cm/h, (SOURCE™ 30RPC)
Collect fractions throughout the separation.

1. Equilibrate the column with at least 10 CV buffer A until the UV signal is stable.
2. Dissolve the sample in a small volume of buffer 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 to 20 CV from 0% to 100% buffer B.
4. Wash the column with at least 5 CV of 100% buffer B (or until UV signal is stable) to elute any remaining material.
5. Wash with a gradient of 2 to 3 CV from 100% to 0% buffer B.
6. Re-equilibrate with 10 CV of buffer A or until UV signal is stable.

Do not exceed the maximum recommended flow for the resin.

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% buffer B, should keep most columns in good condition. However, reduced performance, reduced flow, increasing backpressure or complete blockage are all indications that the resin needs to be cleaned using more stringent procedures to remove tightly bound, precipitated, or denatured substances.

Reverse the direction of flow during cleaning when possible so that contaminants do not need to pass through the entire column length. Due to the design of the column, we do not recommend that you reverse the direction of flow for RESOURCE™ columns. The number of CV 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:

Buffer A: 0.1% TFA

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

Change to 0.1% TFA in 2-propanol for buffer 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
Buffer A: 90% acetic acid
Buffer B: 80% acetonitrile or 50% 2-propanol

Removal of alkali-soluble contaminants
Buffer A: 0.5 M NaOH
Buffer 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 to 10 CV of 6 M guanidine hydrochloride.

SOURCE™ RPC resin 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 CV of 0.5 to 1 M NaOH for cleaning. The ability to use such a strong cleaning agent is a major advantage of using SOURCE™ resins for large-scale separations. At the production scale, other cleaning protocols may be applied to fulfill regulatory requirements.

Chemical stability

For daily use, SOURCE™ RPC resins 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 or 40% 2-propanol, 6 M guanidine hydrochloride, 1-propanol, 20% ethanol, acetone.
- Aqueous solutions pH 1.0 to 12.0 including trifluoroacetic acid (up to 0.3%), pentafluoro propionic 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 to 50 mM), phosphoric acid (10 to 50 mM).
- Water-miscible organic solvents including methanol, ethanol, acetonitrile, 1-propanol, 2-propanol.
- Ion-pairing agents such as trifluoroacetic acid, tributyl phosphate, triethylammonium phosphate, tetrabutylammonium salts, hexyl sulfate.

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

Avoid as, oxidizing agents and solutions < pH 1.0 and > pH 12.0.

Storage

SOURCE™ 15RPC and SOURCE™ 30RPC columns:
wash with at least 10 CV distilled water, equilibrate with at least 10 CV of 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 resin at 4°C to 8°C in 20% ethanol. Do not freeze.

RPC and CIPP

RPC can give extremely high resolution of complex mixtures for analytical purposes and, when full recovery of activity and tertiary structure is 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 most suited as a polishing step when high resolution of similar components is required, and most contaminants have been removed. *See* Chapter 4 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 75 µm 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 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 6.13.

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 resin.

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 *Strategies for Protein Purification Handbook* and more specifically according to the target molecule in the *Affinity Chromatography Handbook, Vol.1: Antibodies* and *Affinity Chromatography Handbook, Vol.2: Tagged proteins*, available from Cytiva.

Sample stability

In most 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 nonspecific 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 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 by hydrogen bonding. Hence, peptides tolerate a wider range of conditions than proteins. This basic difference in native structures is also reflected in that proteins are not easily refolded, while peptides often refold spontaneously.

You can 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 2.0 and pH 9.0.
- Test salt stability with 0 to 2 M NaCl and 0 to 2 M $(NH_4)_2SO_4$, in steps of 0.5 M.
- Test the stability toward acetonitrile and methanol by increasing from 0% to 50% with 10% increase each time.
- 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.

We recommend that you 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 to 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. Whatman™ syringe filters, which give the least amount of nonspecific binding of proteins, are composed of cellulose acetate (CA), regenerated cellulose (RC), or polyvinylidene fluoride (PVDF) (Table A1.1).

Table A1.1. Whatman™ syringe filters for filtration of samples

Filter pore size (µm)	Up to sample volume (mL)	Whatman™ syringe filter*	Membrane
0.8	100	Puradisc™ FP	CA
0.45	1	Puradisc™ 4	PVDF
0.45	10	Puradisc™ 13	PVDF
0.45	100	Puradisc™ 25	PVDF
0.45	10	Protein Prep for ÄKTA™ systems 13/0.45 RC	RC
0.45	10	SPARTAN™ 13	RC
0.45	100	Protein Prep for ÄKTA™ systems 30/0.45 RC	RC
0.45	100	SPARTAN™ 30	RC
0.45	100	Puradisc™ FP	CA
0.2	1	Puradisc™ 4	PVDF
0.2	10	Puradisc™ 13	PVDF
0.2	100	Puradisc™ 25	PVDF
0.2	10	Protein Prep for ÄKTA™ systems 13/0.2 RC	RC
0.2	10	SPARTAN™ 13	RC
0.2	100	Protein Prep for ÄKTA™ systems 30/0.2 RC	RC
0.2	100	SPARTAN™ 30	RC
0.2	100	Puradisc™ FP 30	CA

*The number indicates the diameter (mm) of the syringe filter

For sample preparation before chromatography, you can select a filter pore size in relation to the bead size of the chromatographic resin (Table A1.2).

Table A1.2. Filter pore size selection based on bead size

Nominal pore size of filter (µm)	Particle size of the chromatographic resin (µm)
1.0	75 and upward
0.45	40
0.22	below 15 or when extra clean samples or sterile filtration is required

Check the recovery of the target protein in a test run. Some proteins might adsorb nonspecifically 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. For detailed procedures, *see* Buffer exchange and desalting later in this appendix.

At laboratory scale, when samples are reasonably clean after filtration or centrifugation, the buffer exchange and desalting step can be avoided. In HIC, you can adjust the salt concentration and pH of the sample.

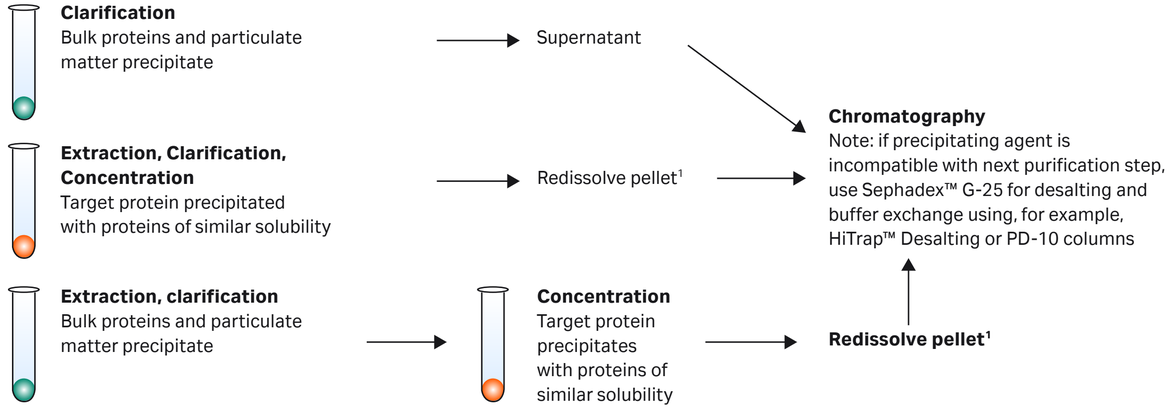
Specific sample preparation steps

Specific sample preparation steps might be required if the crude sample contain contaminants such as lipids, lipoproteins, or phenol red that might 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 A1.1.



[1] Remember: not all proteins are easy to redissolve, yield can be reduced

Fig A1.1. Three ways to use precipitation.

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

Examples of precipitation agents are reviewed in Table A1.3. The most common precipitation method using ammonium sulfate is described in more detail.

Table A1.3. Examples of precipitation agents

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.
Dextran sulfate	Add 0.04 mL of 10% dextran sulfate and 1 mL of 1 M $CaCl_2$/mL of sample, mix 15 min, centrifuge at 10 000 × g, discard pellet.	Samples with high levels of lipoprotein (e.g., ascites).	Precipitates lipoprotein.
Polyvinyl	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.
Polyethylene (PEG, M_r > 4000)	Up to 20% w/v	Plasma proteins.	No denaturation, supernatant goes directly to IEX or AC. Complete removal might be difficult. Stabilizes proteins.
Acetone (cold)	Up to 80% v/v at 0°C. Collect pellet after centrifugation at full speed in a microcentrifuge		Can 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, Protein Purification, Principles, High Resolution Methods and Applications, 3rd ed. Wiley Inc, (2011) Personal communications.*

Ammonium sulfate precipitation

Some proteins can be damaged by ammonium sulfate. You should take care when adding crystalline ammonium sulfate: high local concentrations can cause contamination of the precipitate with unwanted proteins.

For routine, reproducible purification, you should avoid precipitation with ammonium sulfate in favor of chromatography.

Precipitation is ineffective 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 h.
4. Centrifuge for 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* Buffer exchange and desalting later in this appendix).

*The percentage 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 A1.4 shows the quantities required at 20°C.

Table A1.4. Quantities of ammonium sulfate are required to reach given degrees of saturation at 20°C

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

Resolubilization of protein precipitates

Many proteins are easily resolubilized in a small amount of the buffer to be used in the next chromatographic step. However, a denaturing agent may be required for less soluble proteins. Specific conditions will depend upon the specific protein. These agents must always be removed to allow complete refolding of the protein and to maximize recovery of mass and activity. A chromatographic step often removes a denaturant during purification. Table A1.5 gives examples of common denaturing agents.

Table A1.5. Examples of denaturing agents

Denaturing agent	Typical conditions for use	Removal/comment
Urea	2 M to 8 M	Remove using Sephadex™ G-25
Guanidine hydrochloride	3 M to 6 M	Remove using Sephadex™ G-25

Reprinted with permission 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 due to proteolytic breakdown or non-specific binding to the dialysis membranes, you have a risk losing the protein sample. A simple and fast 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 of up to 30% of the total volume of the desalting column can be processed. Sample concentration does not influence the separation if the concentration of proteins does not exceed 70 mg/mL using normal aqueous buffers. The sample should be fully dissolved. Centrifuge or filter to remove particulate material.

For small sample volumes, we can dilute the sample with the start buffer for chromatographic purification, but cell debris and particulate matter must still be removed.

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 A1.2 shows a typical buffer exchange and desalting separation. The process can be monitored by following changes in UV absorption and conductivity.

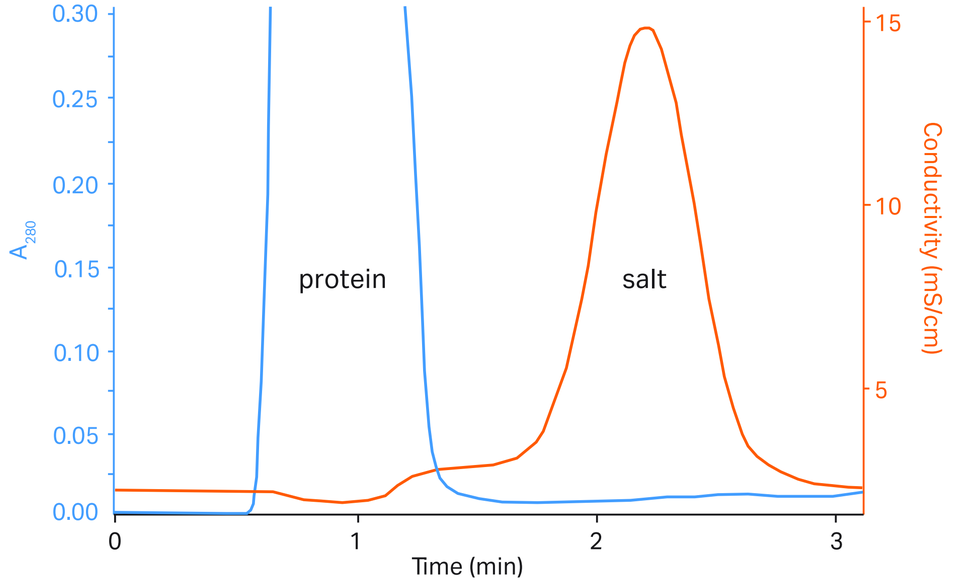


Fig A1.2. Buffer exchange of mouse plasma (10 mL) on HiPrep™ 26/10 Desalting.

For laboratory-scale operations, Table A1.6 shows a selection guide for prepacked, ready-to-use desalting and buffer exchange columns.

Table A1.6. Selection guide for desalting and buffer exchange columns

Column	Sample volume (mL)	Sample elution volume (mL)
PD™ MiniTrap™ G-25	0.2 to 0.5	0.1 to 0.5
PD™-10 (gravity column)	1.5 to 2.5 mL	2.5 to 3.5
HiTrap™ Desalting 5 mL	0.25 to 1.5	1.0 to 2.0
HiPrep™ 26/10 Desalting	2.5 to 15	7.5 to 20

To desalt larger sample volumes:

- You can connect up to five HiTrap™ Desalting 5 mL columns in series to increase the sample volume capacity, for example, two columns: sample volume 3 mL, five columns: sample volume 7.5 mL.
- You can connect up to four HiPrep™ 26/10 Desalting columns in series to increase the sample volume capacity, for example, two columns: sample volume 30 mL, four columns: sample volume 60 mL. Even with four 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.

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 syringe (2 to 5 mL) at a flow rate between 1 to 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 A1.7.

   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 A1.7 for the effect of reducing the sample volume applied to the column.

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

Table A1.7. Recommended sample and elution volumes using a syringe

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

Simple desalting with ÄKTA start™ protein purification system

ÄKTA start™ protein purification system (Fig A1.3) makes it easy to purify proteins, with predefined quick start and editable method templates, real-time run control and visualization, and single-click operations with a compact and portable size suitable for cold-room operations.

Follow the instructions in “Desalting using ÄKTA™ card” training cue card from Cytiva.

Removal of lipoproteins

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

- Centrifuge samples to avoid the risk of nonspecific 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 resin and should be removed as early as possible to avoid the risk of contamination. Phenol red is known to bind to anion exchange resin at pH greater than 7.0.

- 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, *see* Buffer exchange and desalting earlier in this appendix.

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 (*see* Buffer exchange and desalting earlier in this appendix).

Fig A1.3. ÄKTA start™ is an easy-to-use chromatography system and is an excellent choice for automated desalting of proteins.

Appendix 2
Column packing and preparation

Prepacked columns from Cytiva will provide reproducible results and good performance.

Use small, prepacked columns for resin screening and method optimization to increase efficiency in method development, for example, HiTrap™ Capto™ HIC Selection Kit, PreDictor™ Capto™ HIC Screening Kit.

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, you can follow these guidelines, which are applicable to all scales of operation:

- When using a binding technique, you can use short wide columns (typically 5 to 15 cm bed height) for rapid purification, even with low linear flow.
- The amount of resin required will depend on the binding capacity of the resin and the amount of sample. The binding capacity of a resin is influenced by the hydrophobic nature of the sample as well as the resin itself and must be determined empirically.
- Once separation parameters have been determined, scale up a purification by increasing the diameter of the column to increase column volume. You can avoid increasing the length of the column as this will alter separation conditions.

HIC resin can be packed in Tricorn™, XK, or HiScale™ columns available from Cytiva. SOURCE™ RPC resins can be packed in Tricorn™ or HR columns also available from Cytiva. Packing instruction videos for Tricorn™ and XK columns are available online. Figure.A2.1 shows some of the steps involved in packing a columns.

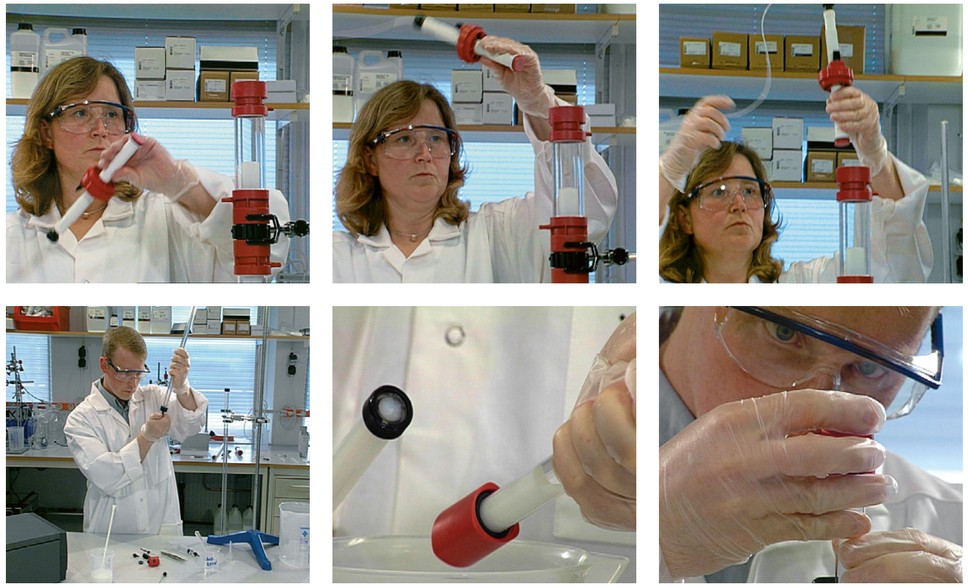


Fig A2.1. Column packing in progress.

General packing procedures

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 to 2 cm of buffer in the column.
3. Gently resuspend the resin.

Note that HIC resin from Cytiva is 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 resin) required based on the recommendations supplied.
5. You can 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, you can use the maximum flow rate the pump can deliver.

Do not exceed the maximum operating pressure of the resin or column.

9. Maintain the packing flow rate for at least three 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, you can fill the rest of the column with buffer to form an upward meniscus at the top.
11. Insert the adapter into the column at an angle, ensuring that no air is trapped under the net.
12. Slide the adapter slowly down the column (the outlet of the adapter should be open) until the mark is reached. Lock the adapter in position.
13. Connect the column to the pump and begin equilibration. Reposition the adapter if necessary.

You must wash the resins thoroughly to remove the storage solution, usually 20% ethanol. Residual ethanol may interfere with subsequent procedures.

Many resins equilibrated with sterile phosphate-buffered saline containing an antimicrobial agent may be stored at 4°C for up to 1 month, but follow the specific storage instructions supplied with the product.

Column selection

Tricorn™, XK, and HiScale™ columns are fully compatible with the high flow rates achievable with modern resins and a broad range of column dimensions are available. In most cases the binding capacity of the resin and the amount of sample to be purified will determine the column size required.

Column packing and efficiency

Column efficiency 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:

$N = 5.54 \times (V_R / W_h)^2$

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 resins) and measuring the eluted peak as shown in Figure A2.2.

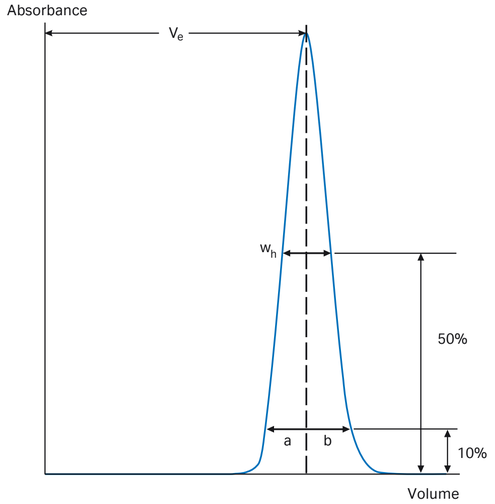


Fig A2.2. Measurement of efficiency of column performance.

As a 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 cm to 0.027 cm.

The asymmetry factor (A_s) is expressed as:

$$A_s = \frac{b}{a}$$

where

a = First half peak width at 10% of peak height

b = Second 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 to 1.80.

An extensive leading edge is usually a sign that the resin is packed too tightly, and extensive tailing is usually a sign that the resin is packed too loosely.

Run at least two column volumes of buffer through a newly packed column to ensure that the resin is equilibrated with start buffer. Use pH monitoring to check the pH of the eluent buffer.

Appendix 3
Selection of lab-scale purification equipment

Simple HIC, such as elution by a step-gradient, can be performed using a syringe or peristaltic pump with prepacked HiTrap™ columns. A chromatography system is required when reproducible results are important and when manual purification becomes time consuming and inefficient. This happens when large sample volumes are handled, or many different samples purified. The progress of the purification can be monitored automatically and high-resolution separations are accurately controlled with linear gradient elution methods.

Table A3.1 lists the standard system configurations for currently available systems. *See* ÄKTA™ Laboratory-scale chromatography Systems: Instrument Management Handbook.

Table A3.1. The AKTA™ chromatography system types and their configurations

ÄKTA™ lab-scale protein purification systems are designed for purification of biomolecules, providing speed and flexibility in research and process development. ÄKTA™ systems use intelligent UNICORN™ system control software to combine simplicity with power in protein purification from your lab bench to process development and full-scale bioprocess production. Scientists all over the world rely on ÄKTA™ systems, columns, and resins for their protein purification needs.

	ÄKTA start™	ÄKTA go™	ÄKTA pure™	ÄKTA avant™
Applications	Transition from manual to automated protein purification/education in protein purification	Achieve desired purity with ease in routine purifications—make the most of valuable bench/cold-room space	Flexibility in research—match most current and future purification challenges	Productivity in process development—fast and secure development of purification processes
Automated and reproducible protein purification including support for gradient elution	•	•	•	•
Support for affinity chromatography, ion exchange chromatography, and multimodal (mixed mode) chromatography	•	•	•	•
Support for size exclusion chromatography	Limited capabilities	•	•	•
Support for hydrophobic interaction chromatography		Limited capabilities	•	•
Software compatible with regulatory requirements, e.g., GLP, GMP		•	•	•
Microscale purification			•	
Automated buffer preparation including pH scouting				•
Scale-up, process development			Optional	•
Method development and optimization using design of experiments (DoE)			Optional	•
Automated resin or column scouting			Optional	•
Automated multistep purification			Optional	Optional
Advanced automation including external equipment			Optional	Optional
Recommended flow rate (mL/min)	0.5–5.0	0.01–25.0	0.001–25/0.01–150	0.001–25/0.01–150
Max. operating pressure (MPa)	0.5	5	20/5	20/5

Appendix 4

Converting from flow velocity to volumetric flow rates

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

From flow velocity (cm/h) to volumetric flow rate (mL/min)

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

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

where

Y = flow velocity 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 flow velocity is 150 cm/h?

Y = flow velocity = 150 cm/h
d = inner diameter of the column = 1.6 cm

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

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

From volumetric flow rate (mL/min) to flow velocity (cm/h)

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

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

where

Z = volumetric flow rate in mL/min
d = column inner diameter in cm

Example:

What is the linear flow in 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

$$\text{Flow velocity} = 1 \times 60 \times \frac{4}{\pi \times 0.5 \times 0.5} \text{ cm/h} = 305.6 \text{ cm/h}$$

From volumetric flow rate (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 5

Conversion data: proteins, column pressures

Table A5.1. Conversion data: proteins

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

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.

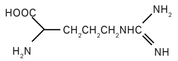
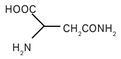
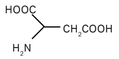
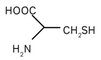
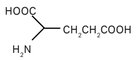
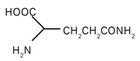
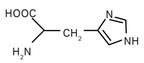
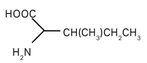
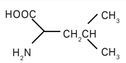
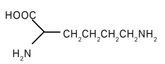
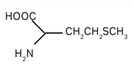
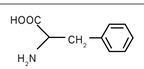
Column pressures

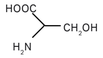
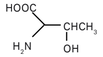
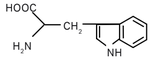
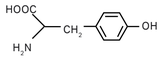
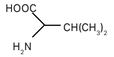
The maximum pressure drop over the packed bed refers to the pressure above which the column contents might begin to compress.

Pressure units may be expressed in megaPascal (MPa), bar, or pounds per square inch (psi) and can be converted as follows: 1 MPa = 10 bar = 145 psi

Appendix 6
Table of amino acids

Table A6.1. List of amino acids

Amino acid	Three-letter code	Single-letter code	Structure	Formula	M_r	Middle unit residue ($-H_2O$) Formula	Middle unit residue ($-H_2O$) M_r	Charge at pH 6.0 to 7.0	Hydrophobic (nonpolar)	Uncharged (polar)	Hydrophilic (polar)
Alanine	Ala	A		$C_3H_7NO_2$	89.1	C_3H_5NO	71.1	Neutral	•		
Arginine	Arg	R		$C_6H_{14}N_4O_2$	174.2	$C_6H_{12}N_4O$	156.2	Basic (+ve)			•
Asparagine	Asn	N		$C_4H_8N_2O_3$	132.1	$C_4H_6N_2O_2$	114.1	Neutral		•	
Aspartic acid	Asp	D		$C_4H_7NO_4$	133.1	$C_4H_5NO_3$	115.1	Acidic(-ve)			•
Cysteine	Cys	C		$C_3H_7NO_2S$	121.2	C_3H_5NOS	103.2	Neutral		•	
Glutamic acid	Glu	E		$C_5H_9NO_4$	147.1	$C_5H_7NO_3$	129.1	Acidic (-ve)			•
Glutamine	Gln	Q		$C_5H_{10}N_2O_3$	146.1	$C_5H_8N_2O_2$	128.1	Neutral		•	
Glycine	Gly	G		$C_2H_5NO_2$	75.1	C_2H_3NO	57.1	Neutral		•	
Histidine	His	H		$C_6H_9N_3O_2$	155.2	$C_6H_7N_3O$	137.2	Basic (+ve)			•
Isoleucine	Ile	I		$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	•		
Leucine	Leu	L		$C_6H_{13}NO_2$	131.2	$C_6H_{11}NO$	113.2	Neutral	•		
Lysine	Lys	K		$C_6H_{14}N_2O_2$	146.2	$C_6H_{12}N_2O$	128.2	Basic (+ve)			•
Methionine	Met	M		$C_5H_{11}NO_2S$	149.2	C_5H_9NOS	131.2	Neutral	•		
Phenylalanine	Phe	F		$C_9H_{11}NO_2$	165.2	C_9H_9NO	147.2	Neutral	•		

Amino acid	Three-letter code	Single-letter code	Structure	Formula	M_r	Middle unit residue ($-H_2O$)		Charge at pH 6.0 to 7.0	Hydrophobic (nonpolar)	Uncharged (polar)	Hydrophilic (polar)
						Formula	M_r				
Proline	Pro	P		$C_5H_9NO_2$	115.1	C_5H_7NO	97.1	Neutral	•		
Serine	Ser	S		$C_3H_7NO_3$	105.1	$C_3H_5NO_2$	87.1	Neutral		•	
Threonine	Thr	T		$C_4H_9NO_3$	119.1	$C_4H_7NO_2$	101.1	Neutral		•	
Tryptophan	Trp	W		$C_{11}H_{12}N_2O_2$	204.2	$C_{11}H_{10}N_2O$	186.2	Neutral	•		
Tyrosine	Tyr	Y		$C_9H_{11}NO_3$	181.2	$C_9H_9NO_2$	163.2	Neutral		•	
Valine	Val	V		$C_5H_{11}NO_2$	117.1	C_5H_9NO	99.1	Neutral	•		

Appendix 7
Analytical assays

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 standard methods to determine the total protein content. The Bradford assay is particularly suited to samples with high lipid content that may interfere with the Lowry assay.

SDS-PAGE analysis

The general steps in SDS-PAGE analysis are summarized below:

1. Prepare samples by mixing with equal volumes of 2× SDS loading buffer.
2. Vortex briefly and heat for 5 min at 90°C to 100°C.
3. Load the samples and, optionally, a MW marker onto an SDS-polyacrylamide gel.
4. Run the gel.
5. Stain the gel with Coomassie™ Blue or silver stain.

Purity determination

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

The percentage of acrylamide in the SDS-gel should be selected according to the expected molecular weight of the protein of interest (*see* Table A7.1).

Table A7.1. Separation ranges of polyacrylamide gels

Acrylamide in resolving gel (%)		Separation size range (M_r)
Single percentage:	5%	36 000 to 200 000
	7.5%	24 000 to 200 000
	10%	14 000 to 200 000
	12.5%	14 000 to 100 000
	15%	14 000 to 60 000*
Gradient:	5% to 15%	14 000 to 200 000
	5% to 20%	10 000 to 200 000
	10% to 20%	10 000 to 150 000

* The larger proteins do not move significantly into the gel.

For information and advice on electrophoresis techniques, *see* *2-D Electrophoresis Principles and Methods Handbook* from Cytiva.

The gel is usually stained after electrophoresis to make the protein bands visible by, for example, Coomassie™ Blue or silver staining. Another way of making protein visible is by prelabeling the proteins by fluorescent dye (Amersham™ Cy™5 dye reagent) before loading the sample in the gel. By doing in this way the gel image can be acquired directly after finished electrophoresis by laser scanner or CCD camera and the result is obtained much faster. This workflow is outlined below.

Protein prelabeling with CyDye™ fluorescent dyes

1. Prepare samples by prelabeling with Amersham™ Western blotting Cy™5 dye reagent.
2. Vortex briefly and heat for 5 min at 90°C to 100°C.
3. Load the samples and, optionally, a MW marker onto an SDS-polyacrylamide gel.
4. Run the gel and proceed directly to image capture.

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, depending on the choice of detection reagents. Protran™ (NC) or Hybond™ P (PVDF) membranes are recommended for chemiluminescent detection using ECL™ start, ECL™, ECL Prime, or ECL Select™ Western blotting detection reagents. Protran™ Premium (NC) or Hybond™ LFP (PVDF) membranes are recommended for fluorescent detection with ECL Plex™ Western blotting detection system.
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 *2-D Electrophoresis Principles and Methods Handbook* from Cytiva.

- ELISAs are used as activity assays.
- Functional assays using the phenomenon of surface plasmon resonance to detect immunospecific interactions. Biacore™ surface plasmon resonance systems enable the label-free 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 of GST-tagged proteins. Further details on the detection and quantitation of GST- and His tagged proteins, *see* *Affinity Chromatography, Vol. 2: Tagged proteins* and the *GST Gene Fusion System Handbook*.

Appendix 8
Storage

Storage of biological samples

Overall recommendations are provided for your convenience. You should consider the properties of the specific sample and its intended use before following any of these recommendations.

Recommendations

- Add stabilizing agents, if essential. Stabilizing agents are often required for storage of purified proteins.
- Serum, culture supernatants and ascitic fluid should be kept frozen at -20° C or -70° C, in small aliquots.
- Avoid repeated freeze/thawing or freeze drying/redissolving that may reduce biological activity.
- Avoid conditions close to stability limits, for example, pH or salt concentrations, reducing or chelating agents.
- Keep refrigerated at 4° C in a closed vessel to minimize bacterial growth and protease activity.

Sodium azide can interfere with many coupling methods and some biological assays and can be a health hazard. You can remove using a desalting column (*see* Desalting and exchange section in Appendix 1).

Overall 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% to 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 might need to be removed at a later stage.

• Avoid repeated freeze/thawing or freeze drying/redissolving that may reduce biological activity.

Sodium azide can interfere with many coupling methods and some biological assays. You can remove it with a desalting column (*see* Desalting and exchange section in Appendix 1).

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

Appendix 9
Column cleaning for HIC resins

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 resins need to be cleaned using more stringent procedures 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. You should take care changing a filter as this affects the column packing and interfere with your performance.

Removal of common contaminants

The following procedure should be good 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 four column volumes of 1 M NaOH at the recommended flow rate.
2. Wash with at least 3 CV of water or until eluent pH is neutral.

3a. To start a new separation: reequilbrate with at least 3 CV of start buffer or until the correct eluent pH is achieved at the recommended flow rate.

3b. For storage: wash with at least 3 CV of storage solution. Allow UV baseline to stabilize before storing the column.

Removal of lipids, lipoproteins, and 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 resin or column.

Before using organic solvents, wash the resins with at least 4 CV 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 CV of water at the recommended flow rate.
2. Wash with at least 4 CV of 70% ethanol or 30% isopropanol at the recommended flow rate.
3. Wash with at least 4 CV of distilled water, same flow as step 1.

4a. To start a new separation: re-equilibrate with at least 3 CV of start buffer, or until the correct eluent pH is achieved at the recommended flow rate.

4b. For storage: wash with at least 5 CV of storage solution. Allow UV baseline to stabilize before storing the column.

Alternative 2

1. Wash with up to two column volumes of 0.05% nonionic detergent (e.g., Tween™-20) in 1 M acetic acid at the recommended flow rate.
2. Wash with at least 5 CV of 70% ethanol (to remove detergent) at the recommended flow rate.
3. Wash with at least 4 CV of distilled water (same flow as step 1).

4a. To start a new separation: re-equilibrate with at least 3 CV of start buffer, or until the correct eluent pH is achieved at the recommended flow rate.

4b. For storage: wash with at least 5 CV of storage solution. Allow UV baseline to stabilize before storing the column.

Use detergents with care as they may bind to the resins thereby reducing binding capacity in subsequent runs.

Handbooks from Cytiva

Handbooks	Code number
Affinity Chromatography - Vol. 1: Antibodies	CY13981
Affinity Chromatography - Vol. 2: Tagged Proteins	CY13980
Affinity Chromatography - Vol. 3: Specific Groups of Biomolecules	CY13979
Ion Exchange Chromatography Principles and Methods	CY13983
Multimodal Chromatography Principles and Methods	CY14738
Size Exclusion Chromatography Principles and Methods	CY12707
ÄKTA™ Laboratory-Scale Chromatography Systems	CY13989
Cross Flow Filtration Method Handbook	CY14739
Design of Experiments in Protein Production and Purification	CY14782
GST Gene Fusion System	CY14588
High Throughput Process Development (HTPD) with PreDictor Plates	CY16051
Purifying Challenging Proteins	CY14791
Strategies for Protein Purification	CY13986
Molecular Imaging	CY14828
Protein sample preparation	CY14735
2-D Electrophoresis	CY14825

These handbooks can be downloaded from cytivalifesciences.com/handbooks

Ordering information

Hydrophobic interaction chromatography products

Capto™, Capto™ ImpRes, SOURCE™, Sepharose™ High Performance, and Sepharose™ Fast Flow resins are also all available as BioProcess™ resins for large-scale production, *see* cytiva.com for details.

Capto™ HIC products

Screening kits

Format	Quantity	Product code
HiTrap™ Capto™ HIC Selection Kit Kit includes the following Capto™ HIC resins: Capto™ Phenyl (high sub), Capto™ Phenyl ImpRes, Capto™ Butyl, Capto™ Butyl ImpRes, and Capto™ Octyl. Prepacked, ready-to-use 1 mL HiTrap™ columns	5 × 1 mL	29321087
PreDictor™ Capto™ HIC Screening Kit Kit includes the following Capto™ HIC resins: Capto™ Phenyl (high sub), Capto™ Phenyl ImpRes, Capto™ Butyl, Capto™ Butyl ImpRes, Capto™ Octyl, and Capto™ Butyl-S	6 µL, 4 × 96 well plates	29711438
	20 µL, 4 × 96 well plates	29711439

Capto™ Phenyl (high sub) resin

Format	Quantity	Product code
Bulk	25 mL	17545101
	100 mL	17545102
HiTrap™ column	5 × 1 mL	17545108
	5 × 5 mL	17545109
HiScreen™ column	1 × 4.7 mL	28992472
PreDictor™ plate	6 µL, 4 × 96-well plates	17545116
	20 µL, 4 × 96-well plates	29716136
	50 µL 4 × 96-well plates	17545117
PreDictor™ RoboColumn™ unit	200 µL, 1 × 8 columns	28986088
	600 µL, 1 × 8 columns	28986182

Capto™ Butyl resin

Format	Quantity	Product code
Bulk	25 mL	17545901
	100 mL	17545902
HiTrap™ column	5 × 1 mL	17545908
	5 × 5 mL	17545909
HiScreen™ column	1 × 4.7 mL	28992473
PreDictor™ plate	6 µL, 4 × 96-well plates	17545916
	20 µL, 4 × 96-well plates	29716137
	50 µL, 4 × 96-well plates	17545917
PreDictor™ RoboColumn™ unit	200 µL, 1 × 8 columns	28986097
	600 µL, 1 × 8 columns	28986183

Capto™ Octyl resin

Format	Quantity	Product code
Bulk	25 mL	17546501
	100 mL	17546502
HiTrap™ column	5 × 1 mL	17546508
	5 × 5 mL	17546512
HiScreen™ column	1 × 4.7 mL	17546510
PreDictor™ plate	6 µL, 4 × 96-well plates	17546516
	20 µL, 4 × 96-well plates	29716138
	50 µL 4 × 96-well plates	17546517
PreDictor™ RoboColumn™ unit	200 µL, 1 × 8 columns	29275286
	600 µL, 1 × 8 columns	29275287

Capto™ Butyl-S resin

Format	Quantity	Product code
Bulk	25 mL	17549701
	100 mL	17549702

Capto™ PlasmidSelect resin

Format	Quantity	Product code
Bulk	25 mL	17549901
	100 mL	17549902
HiTrap™ column	5 × 1 mL	17549911
	5 × 5 mL	17549912
HiScreen™ column	1 × 4.7 mL	29201790

Capto™ Phenyl ImpRes resin

Format	Quantity	Product code
Bulk	25 mL	17548401
	100 mL	17548402
HiTrap™ column	5 × 1 mL	17548411
	5 × 5 mL	17548412
HiScreen™ column	1 × 4.7 mL	17548410
PreDictor™ plate	6 µL, 4 × 96-well plates	29711440
	20 µL, 4 × 96-well plates	29711441
PreDictor™ RoboColumn™ unit	200 µL, 1 × 8 columns	29701638
	600 µL, 1 × 8 columns	17548441

Capto™ Butyl ImpRes

Format	Quantity	Product code
Bulk	25 mL	17371901
	100 mL	17371902
HiTrap™ column	5 × 1 mL	17371911
	5 × 5 mL	17371912
HiScreen™ column	1 × 4.7 mL	17371910
PreDictor™ Plate	6 µL, 4 × 96-well plates	29711442
	20 µL, 4 × 96-well plates	29711443
PreDictor™ RoboColumn™ unit	200 µL, 1 x 8 columns	29701637
	600 µL, 1 x 8 columns	17371941

SOURCE™ HIC products

SOURCE™ 15PHE

Format	Quantity	Product code
Bulk	200 mL	17014702
RESOURCE™ column	1 × 1 mL	17118601
4.6/100 PE column	1 × 1.7 mL	17518601

SOURCE™ 15ISO

Format	Quantity	Product code
Bulk	200 mL	17014802
RESOURCE™ column	1 × 1 mL	17118501

Sepharose™ HIC products

Screening kits

Format	Quantity	Product code
HiTrap™ HIC selection kit Kit includes the following resins: Phenyl Sepharose™ High Performance, Phenyl Sepharose™ 6 Fast Flow (low sub), Phenyl Sepharose™ 6 Fast Flow (high sub), Butyl Sepharose™ High Performance, Butyl-S Sepharose™ 6 Fast Flow, Butyl Sepharose™ 4 Fast Flow, Octyl Sepharose™ 4 Fast Flow	7 × 1 mL	28411007

Phenyl Sepharose™ 6 Fast Flow (high sub) resin

Format	Quantity	Product code
Bulk	25 mL	17097310
	200 mL	17097305
HiTrap™ column	5 × 1 mL	17135501
	5 × 5 mL	17519301
HiScreen™ column	1 × 4.7 mL	28926988

Phenyl Sepharose™ 6 Fast Flow (low sub) resin

Format	Quantity	Product code
Bulk	25 mL	17096510
	200 mL	17096505
HiTrap™ column	5 × 1 mL	17135301
	5 × 5 mL	17519401
HiScreen™ column	1 × 4.7 mL	28926989

Butyl Sepharose™ 4 Fast Flow resin

Format	Quantity	Product code
Bulk	25 mL	17098010
	200 mL	17098001
HiTrap™ column	5 × 1 mL	17135701
	5 × 5 mL	17519701
HiScreen™ column	1 × 4.7 mL	28926984

Octyl Sepharose™ 4 Fast Flow resin

Format	Quantity	Product code
Bulk	200 mL	17094602
HiTrap™ column	5 × 1 mL	17135901
	5 × 5 mL	17519601
HiScreen™ column	1 × 4.7 mL	28926986

Butyl-S Sepharose™ 6 Fast Flow resin

Format	Quantity	Product code
Bulk	200 mL	17097802
HiTrap™ column	5 × 1 mL	17097813
HiScreen™ column	1 × 4.7 mL	28926985

Phenyl Sepharose™ High Performance resin

Format	Quantity	Product code
Bulk	75 mL	17108201
HiTrap™ column	5 × 1 mL	17135101
	5 × 5 mL	17519501
HiScreen™ column	1 × 4.7 mL	28950516

Butyl Sepharose™ High Performance resin

Format	Quantity	Product code
Bulk	200 mL	17543202
HiTrap™ column	5 × 1 mL	28411001
	5 × 5 mL	28411005
HiScreen™ column	1 × 4.7 mL	28978242

Reversed phase chromatography products

SOURCE™ 15RPC and SOURCE™ 30RPC resins are also available as BioProcess™ resins for large-scale production, *see* cytiva.com for details

SOURCE™ 15RPC resin

Format	Quantity	Product code
Bulk	10 mL	17072720
	200 mL	17072702
	500 mL	17072703
RESOURCE™ column	1 × 1 mL	17118101
	1 × 3 mL	17118201
4.6/100 ST column	1 × 1.7 mL	17506801

SOURCE™ 30RPC resin

Format	Quantity	Product code
Bulk	10 mL	17512020
	200 mL	17512002
	500 mL	17512003

Other columns and accessories

Desalting columns

Product name	Quantity	Product code
HiTrap™ Desalting	5 × 5 mL	17140801
HiPrep™ 26/10 Desalting	1 × 53 mL	17508701
HiPrep™ 26/10 Desalting	5 × 53 mL	17508702
Disposable PD-10 Desalting Column	30	17085101

Empty columns

Complete information on the range of Tricorn™ columns is available on Cytiva's website

Product name	Quantity	Product code
Tricorn™ 5/100 column	1	28406410
Tricorn™ 10/100 column	1	28406415
HiScale™ 26/20 column	1	28964514
HiScale™ 26/40 column	1	28964513
HiScale™ 50/20 column	1	28964445
HiScale™ 50/40 column	1	28964444

Accessories and spare parts

For a complete listing, refer to Cytiva's website

Product name	Quantity	Product code
Tricorn™ Packing Equipment 5/50	1	18115324
Tricorn™ Packing Equipment 5/50 for Tricorn™ 5 mm columns includes: Tricorn™ Packing Connector 5-5, Tricorn™ Glass Tube 5/50, bottom unit, and stop plug.		
Tricorn™ Packing Equipment 10/100	1	18115325
Tricorn™ Packing Equipment 10/100 for Tricorn 10 mm columns includes: Tricorn™ Packing Connector 10-10, Tricorn™ Glass Tube 10/100, bottom unit, and stop plug.		
Tricorn™ Packing Connector 5-5	1	18115321
Tricorn™ Packing Connector 5-10	1	18115322
Tricorn™ Packing Connector 10-10	1	18115323
Packing tube 20, complete for HiScale™ 26	1	28980383
Packing tube 20, complete for HiScale™ 50	1	28980251

cytiva.com/HIC

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