instructions

HiTrap

HiTrap Phenyl FF (high sub), 1 ml and 5 ml
HiTrap Phenyl FF (low sub), 1 ml and 5 ml
HiTrap Phenyl HP, 1 ml and 5 ml
HiTrap Butyl FF, 1 ml and 5 ml
HiTrap Octyl FF, 1 ml and 5 ml

HiTrap hydrophobic interaction columns

HiTrap™ Phenyl FF (high sub), HiTrap Phenyl FF (low sub), HiTrap Phenyl HP, HiTrap Butyl FF and HiTrap Octyl FF are prepacked 1 ml and 5 ml, ready to use hydrophobic interaction chromatography columns. The special design of the column, together with modern chromatography media, provides fast, reproducible and easy separation in a convenient format. Separations are easily performed with a syringe, a pump or a chromatography system such as ÄKTAdesign™ or FPLC™ System.

ⓘ 71-5018-24 Edition AE

Amersham Biosciences

- p2

Code No.	Designation	No. supplied
17-1355-01	HiTrap Phenyl FF (high sub)	5 x 1 ml
17-5193-01	HiTrap Phenyl FF (high sub)	5 x 5 ml
17-1353-01	HiTrap Phenyl FF (low sub)	5 x 1 ml
17-5194-01	HiTrap Phenyl FF (low sub)	5 x 5 ml
17-1351-01	HiTrap Phenyl HP	5 x 1 ml
17-5195-01	HiTrap Phenyl HP	5 x 5 ml
17-1357-01	HiTrap Butyl FF	5 x 1 ml
17-5197-01	HiTrap Butyl FF	5 x 5 ml
17-1359-01	HiTrap Octyl FF	5 x 1 ml
17-5196-01	HiTrap Octyl FF	5 x 5 ml
	Connectors	
	1/16" male/luer female	1
	Union luerlock female/M6 female	1
	Union 1/16" female/M6 male	1
	Tubing connector flangeless/M6 male	1
	Tubing connector flangeless/M6 female	1
	Union M6 female/1/16" male	1
	Stop plug female, 1/16"	2, 5 or 7
	Instructions	1

Descriptions

Media properties

The HIC media are based on the highly cross-linked beaded agarose matrices, Sepharose™ Fast Flow and Sepharose High Performance.

The media have excellent flow properties with high physical and chemical stabilities. All Sepharose matrices show virtually no non-specific adsorption and are resistant to microbial degradation due to the presence of the unusual sugar, 3,6-anhydro-L-galactose. The hydrophobic ligands are coupled to the monosaccharide units via glycidylethers. The resulting ether bonds are both stable and uncharged. Characteristics of

the different HiTrap HIC media are listed in Table 1 and their chemical stability is shown in Table 3.

Phenyl Sepharose High Performance is based on a 34 µm matrix and is ideal for laboratory and intermediate process scale separations and for final step purifications where high resolution is needed. The degree of substitution gives Phenyl Sepharose High Performance a selectivity similar to that of Phenyl Sepharose 6 Fast Flow (low sub).

Phenyl Sepharose 6 Fast Flow (low sub) and Phenyl Sepharose 6 Fast Flow (high sub) are based on a 90 µm matrix. They are ideal for initial and intermediate step purifications requiring a matrix with low to medium hydrophobicity. The availability of two degrees of substitution grades increases the possibility of finding the best selectivity and capacity for a given application.

Butyl Sepharose 4 Fast Flow is based on a 90 µm matrix. It is intended for initial and intermediate step purifications requiring a matrix with low to medium hydrophobicity. Butyl Sepharose 4 Fast Flow often works efficiently with rather low salt concentrations. The mechanism of adsorption and desorption onto the butyl ligand is different from that onto the phenyl ligand, giving a different selectivity.

Octyl Sepharose 4 Fast Flow is based on a 90 µm matrix. It has a different hydrophobic character from the phenyl and butyl ligands and is an important complement to the other hydrophobic matrices.

Table 1. HiTrap HIC media characteristics

	Phenyl Sepharose High Performance	**Phenyl Sepharose 6 Fast Flow (low sub)**	**Phenyl Sepharose 6 Fast Flow (high sub)**	**Butyl Sepharose 4 Fast Flow**	**Octyl Sepharose 4 Fast Flow**
Hydrophobic ligand	Phenyl	Phenyl	Phenyl	*n*-Butyl	*n*-Octyl
Ligand density	25 µmol/ml medium	25 µmol/ml medium	40 µmol/ml medium	40 µmol/ml medium	5 µmol/ml medium
Mean bead size	34 µm	90 µm	90 µm	90 µm	90 µm
Bead size range	24–44 µm	45–165 µm	45–165 µm	45–165 µm	45–165 µm
Bead structure	6% cross-linked agarose, spherical	6% cross-linked agarose, spherical	6% cross-linked agarose, spherical	4% cross-linked agarose, spherical	4% cross-linked agarose, spherical
pH stability*					
Short term	2–14	2–14	2–14	2–14	2–14
Working	3–13	3–13	3–13	3–13	3–13
Long term	3–13	3–13	3–13	3–13	3–13
Storage	0.01 M NaOH or 20% ethanol	0.01 M NaOH or 20% ethanol	0.01 M NaOH or 20% ethanol	0.01 M NaOH or 20% ethanol	0.01 M NaOH or 20% ethanol

* The ranges given are estimates based on our knowledge and experience. Please note the following:
pH stability, long term refers to the pH interval where the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance.
pH stability, short term refers to the pH intervalfor regeneration, cleaning-in-place and sanitization procedures.

Table 2. Characteristics of HiTrap column

Column volumes	1 ml or 5 ml
Column dimensions	0.7 x 2.5 cm (1 ml) or 1.6 x 2.5 cm (5 ml)
Maximum flow rates*	HiTrap 1 ml: 4 ml/min, HiTrap 5 ml: 20 ml/min
Recommended flow rates	HiTrap 1 ml: 1 ml/min, HiTrap 5 ml: 5 ml/min
Maximum back pressure	0.3 MPa, 3 bar

* Room temperature, aqueous buffers. Stay below maximum pressure.

Column

The column is made of polypropylene, which is biocompatible and non-interactive with biomolecules. The top and bottom frits

are manufactured from porous polyethylene. It is delivered with a stopper on the inlet and a snap-off end on the outlet. Characteristics of HiTrap column are listed in Table 2.

Table 3. Chemical stability

	Phenyl Sepharose High Performance	Phenyl Sepharose 6 Fast Flow (low sub)	Phenyl Sepharose 6 Fast Flow (high sub)	Butyl Sepharose 4 Fast Flow	Octyl Sepharose 4 Fast Flow
1 M NaOH	X	X	X	X	X
1 M acetic acid	X	n.d.	n.d.	n.d.	n.d.
1 mM HCl	n.d.	n.d.	n.d.	X	X
3 M $(NH_4)_2SO_4$	n.d.	X	X	n.d.	n.d.
70 % ethanol	X	X	X	X	X
30 % isopropanol	X	X	X	X	X
6 M Gua-HCl	X	X	X	X	X
8 M Urea	X	X	X	n.d.	n.d.

X = Functionally stable for 7 days at + 40°C
n.d. = not determined

The separation can be easily achieved using a syringe together with the supplied luer adaptor, a peristaltic pump, or in a chromatography system such as ÄKTA™ or FPLC.

Note: To prevent leakage it is essential to ensure that the adaptor is tight.

The column cannot be opened or refilled.

Hydrophobic Interaction Chromatography

Substances are separated on the basis of their varying strengths of hydrophobic interactions with hydrophobic ligands immobilized to an uncharged matrix. This technique is usually performed with moderately high concentrations of salts in the

start buffer (salt promoted adsorption). Elution is achieved by a linear or stepwise decrease in salt concentration.

Factor affecting HIC

The type of ligand, the degree of substitution, the pH and the type and concentration of salt used during the adsorption stage have a profound effect on the overall performance (e.g. selectivity and capacity) of a HIC matrix. Other factors that affect HIC are temperature, detergents, polarity of solvents, type of matrix and ligand coupling chemistry.
The type of immobilized ligand determines primarily the selectivity of the HIC adsorbent. In general, HIC media fall into two groups, depending on their interactions with sample components. Straight alkyl chains (butyl, octyl) show a ”pure” hydrophobic character, while aryl ligands (phenyl) show a mixed mode behaviour, where both aromatic and hydrophobic interactions as well as lack of charge play simultaneous roles. The choice of ligand must be determined empirically through screening experiments for each individual separation problem.
The protein binding capacity of HIC adsorbents increases with increased degree of substitution up to a certain level and then levels off. Simultaneously, the strength of the interaction increases, which may lead to difficulties in the elution of bound compounds. This potential problem has been addressed by the development of matrices with different levels of ligand density.
The solvent is one of the most important parameters which influences capacity and selectivity in HIC. In general, the adsorption process is more selective than the desorption process. It is therefore important to optimize the start buffer with respect

to pH, type of solvent, type of salt and concentration of salt. The addition of various "salting-out" salts to the sample promotes ligand-protein interactions in HIC. As the concentration of salt is increased, the amount of bound protein increases up to the precipitation point for the protein. Each type of salt differs in its ability to promote hydrophobic interactions and it may be worthwhile testing several salts. The most commonly used salts are $(NH_4)_2SO_4$, Na_2SO_4, NaCl, KCl and CH_3COONH_4. At a given concentration, ammonium sulphate often gives the best resolution of a mixture of standard proteins compared to other salts. If sodium chloride is used, a concentration of up to 3-4 M is usually needed. Due to instability, ammonium sulphate is not suitable when working at pH values above 8.0. Sodium sulphate is also a very good salting-out agent but protein solubility problems may exclude its use at high concentrations.

The effect of pH in HIC is not straightforward. In general, an increase in pH weakens hydrophobic interactions. Retention of proteins changes more drastically at pH values above 8.5 or below 5.0 than in the range 5.0-8.5. These findings suggest that pH is an important separation parameter and it is advisable to check the applicability to the particular problem at hand.

Increasing the temperature enhances hydrophobic interactions in most cases. One should thus be aware that a process developed at room temperature might not be reproducible in the cold room and vice versa.

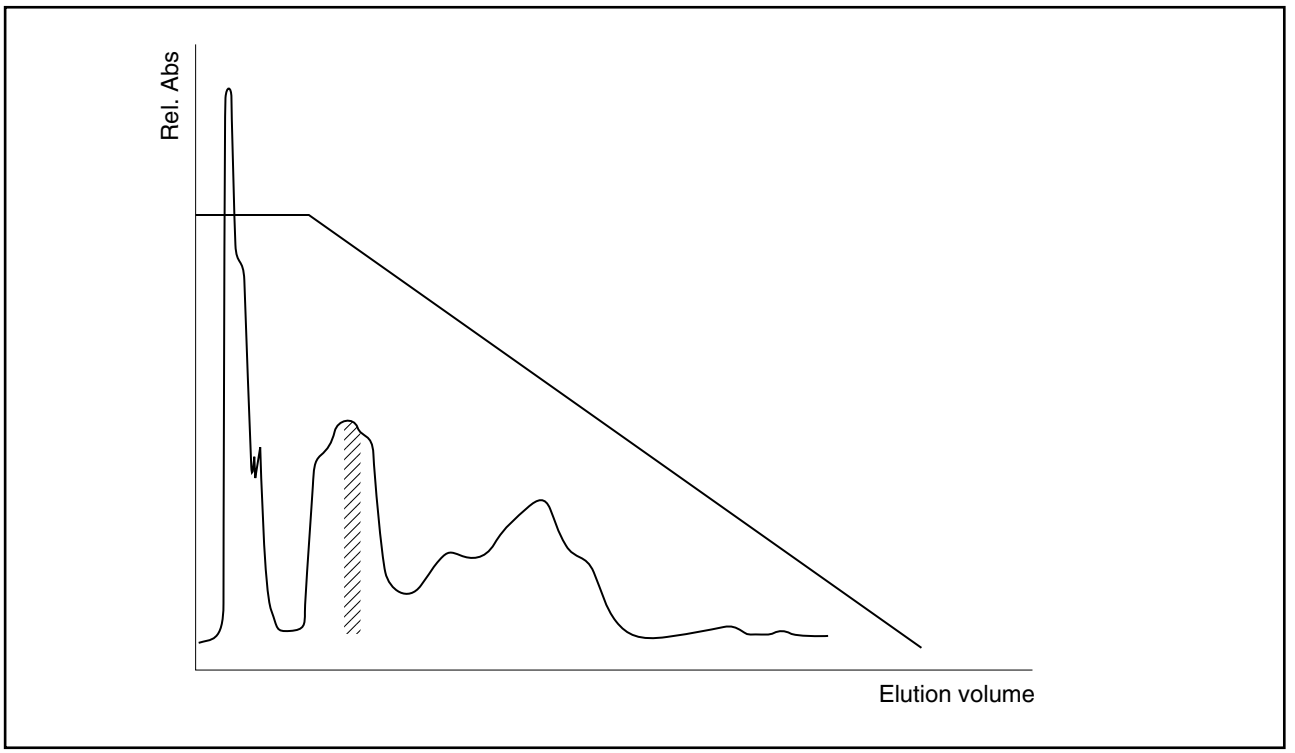
Sometimes it is necessary to weaken the protein-ligand interactions by including different additives. Commonly used are water-miscible alcohols (propanol, ethylene glycol), detergents

(SDS) and solutions of chaotrophic salts (lithium perchlorate, urea, guanidine hydrochloride).
Further information about hydrophobic interaction chromatography can be found in the Handbook "HIC & RPC, Principles and Methods", see Ordering Information or other relevant textbooks.

Screening experiments

Protein binding to HIC adsorbents is promoted by moderate to high concentrations of "salting-out" salts, which also have a stabilizing influence on protein structure. Elution is achieved by a linear or step-wise decrease in concentration of the salt.
The HIC medium should bind the protein of interest at a reasonably low concentration of salt. As mentioned before, binding conditions are dependent on the salt chosen. The salt concentration should be below that which causes precipitation of proteins in the sample. We recommend 1 M ammonium sulphate as a good starting buffer for screening experiments. If the substance does not bind, a more hydrophobic medium should be chosen. If the substance binds so strongly that non-polar additives are required for elution, a less hydrophobic medium should be tried. The bound protein should be eluted from the column with high recovery.
Figures 1 to 6 show some typical elution profiles from screening experiments and recommendations for further experimental work. The shaded area shows the elution position of the protein of interest.

Fig. 1

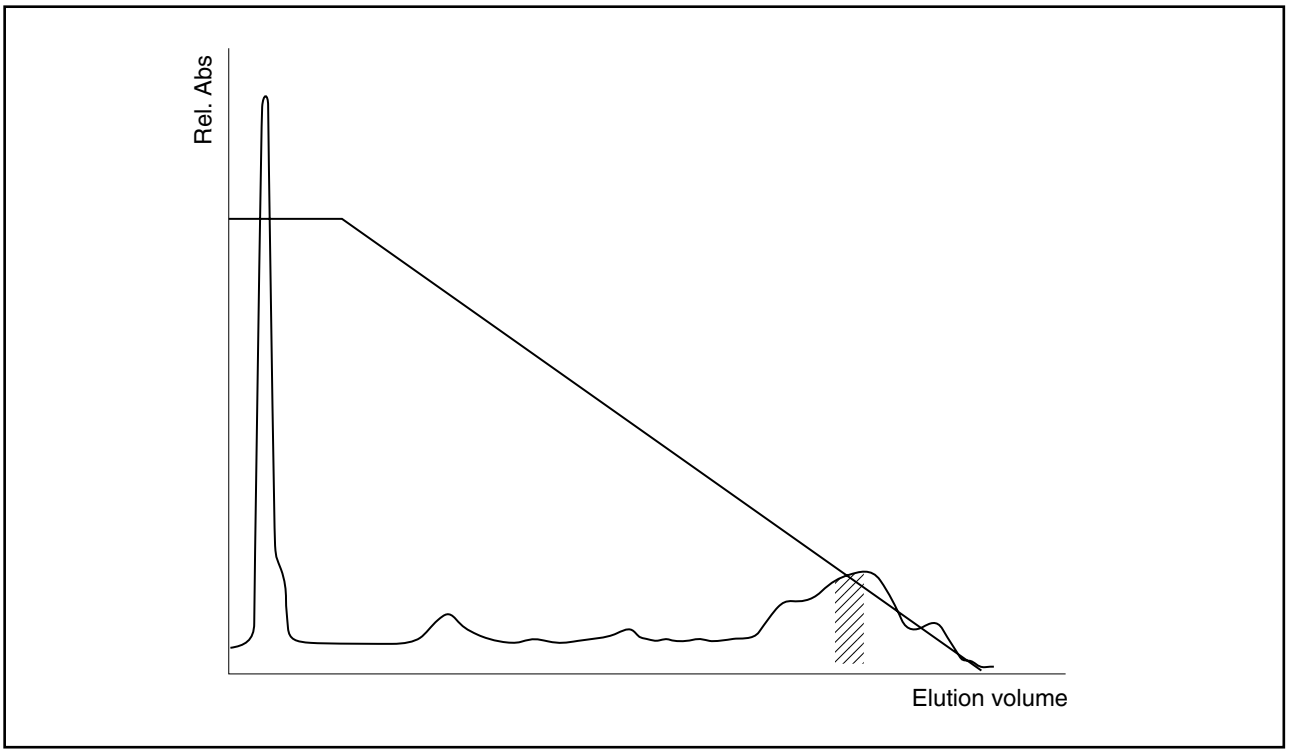


Result: The protein is eluted early in the gradient. Resolution is not satisfactory.

Discussion: Not much can be gained by changing the salt concentration. A decrease in the salt concentration of the start buffer will decrease binding capacity and might even lead to co-elution with the unbound fraction. An increase in the salt concentration might lead to the co-adsorption of unwanted impurities leading to a decrease in selectivity. Change of pH in the start buffer might result in stronger binding and higher selectivity. Trying another salt may also improve performance.

Next step: Repeat the experiment at a lower or higher pH or with a salt of higher salting-out strength. If no improvement in selectivity is obtained, try a medium with a different ligand or with a higher degree of ligand substitution.

Fig. 2

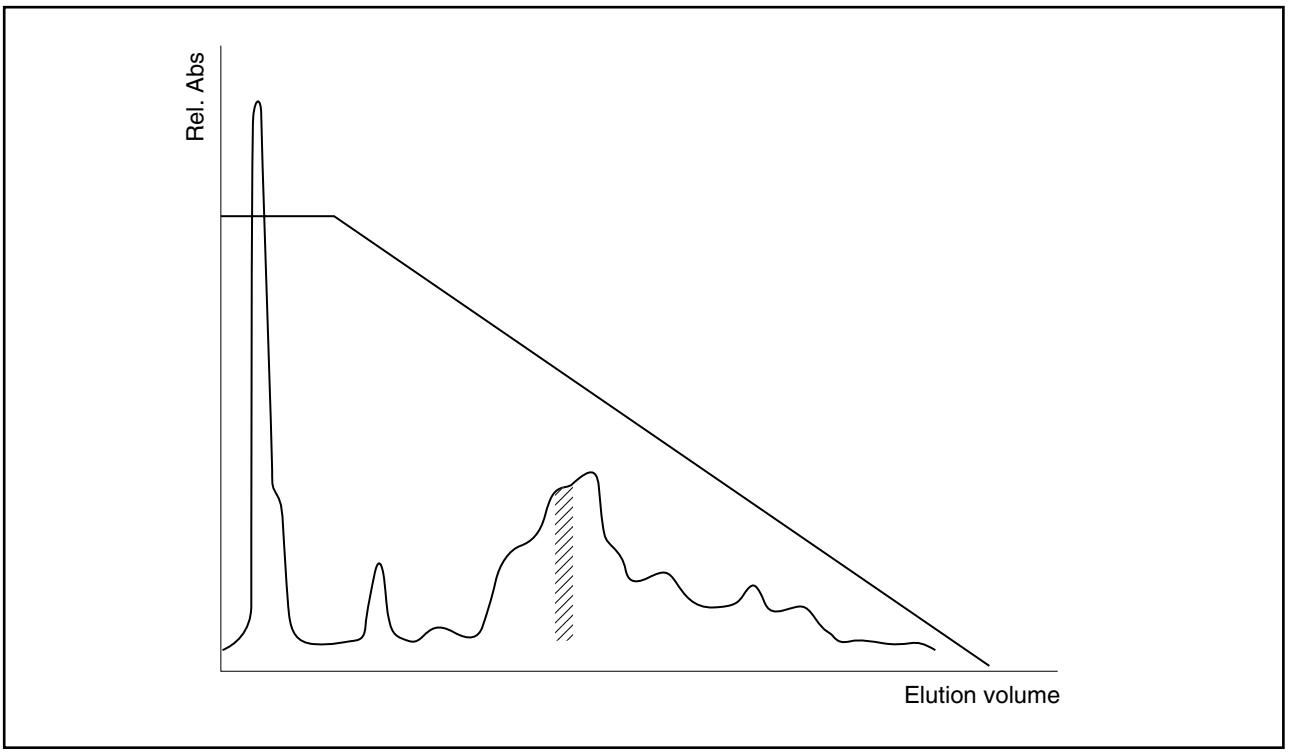


Result: The protein is eluted near the end of the gradient. Resolution is not satisfactory.

Discussion: A decrease in the initial salt concentration will weaken the binding, resulting in earlier elution of the protein. It may, however, not have a positive effect on selectivity, since the contaminants are eluted very close to, both before and after, the protein of interest. Changing the pH of the start buffer or changing to another salt may have more impact on resolution and should be tried.

Next step: Repeat the experiment at a lower or higher pH value or with a salt with lower salting-out strength. If no improvement in selectivity is obtained, try a medium with a different ligand or with a lower degree of ligand substitution.

Fig. 3.

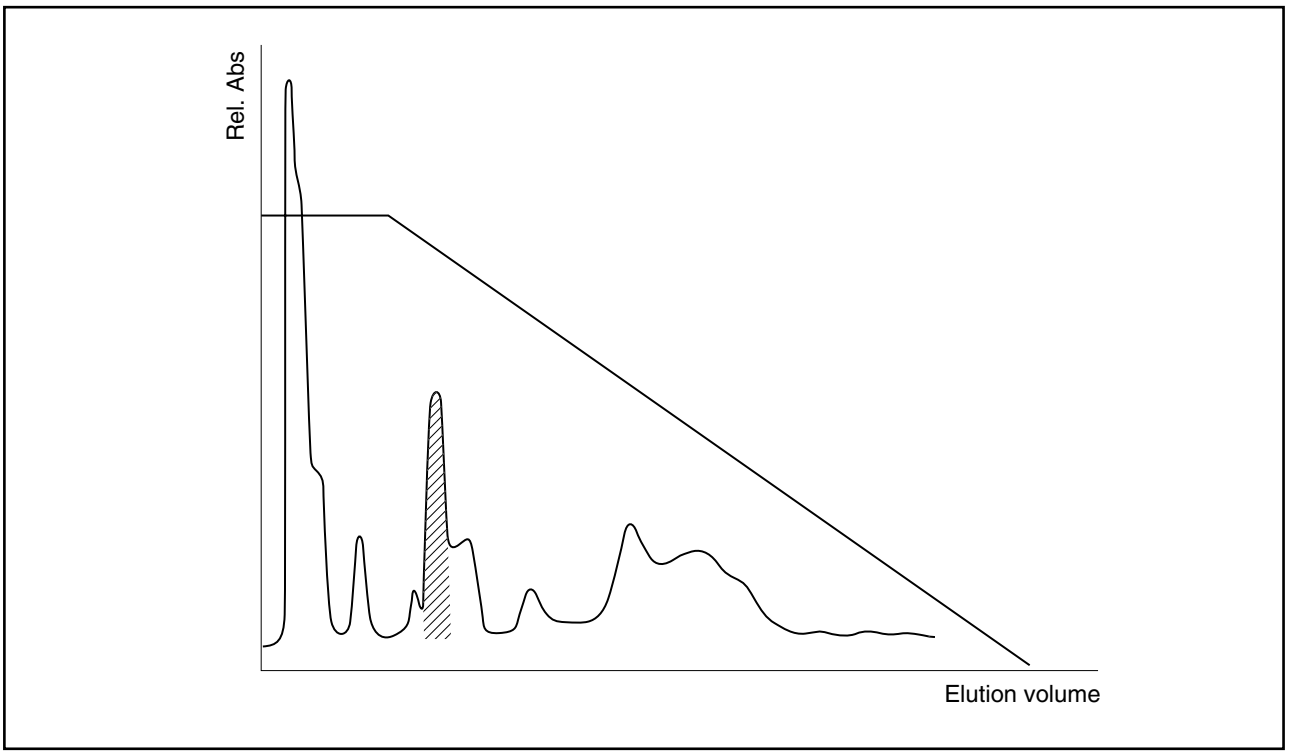


Result: The protein is eluted in the middle of the gradient. Resolution is not satisfactory.

Discussion: Changing the concentration of salt in the start buffer will have a limited effect since the contaminants are eluted very close to, both before and after, the protein of interest. Changing the pH of the start buffer or changing to a different salt may have impact on resolution and should be tried. Trying a different gradient slope may also be an effective way to increase the resolution.

Next step: Repeat the experiment at a lower or higher pH or with a salt with higher salting-out effect. If no improvement in selectivity is obtained, try a medium with a different ligand or with a higher degree of ligand substitution.

Fig. 4.

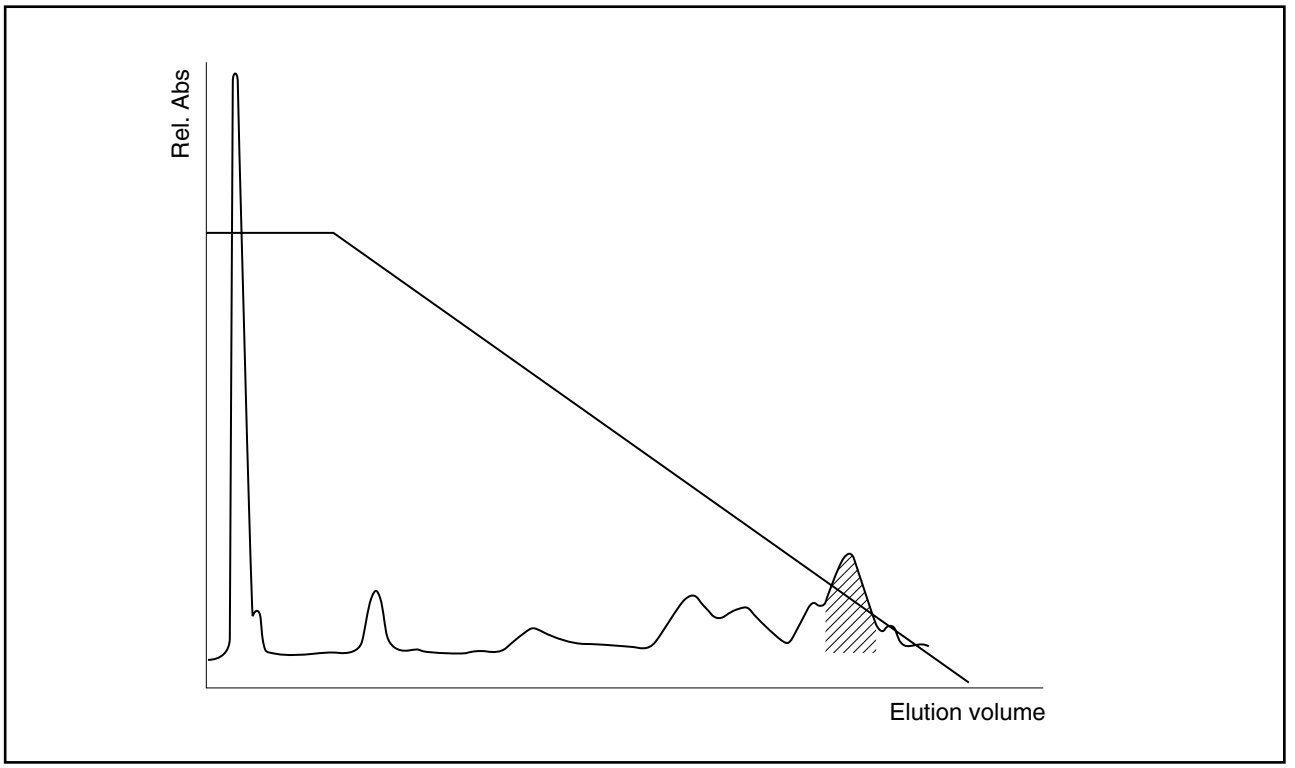


Result: The protein is eluted early in the gradient. Resolution is satisfactory.

Discussion: This can be a good choice of medium. The fact that the protein is eluted early in the gradient indicates that the binding capacity might be low. An increase in salt concentration to compensate for this may lead to a decrease in selectivity since some of the unbound proteins might be adsorbed together with the protein of interest. Not much can be gained by changing the pH of the start buffer since the resolution was considered satisfactory.

Next step: If low capacity is a problem and a moderate increase in salt concentration leads to loss in resolution, try a medium with a different ligand or a medium with a higher degree of ligand substitution.

Fig. 5.

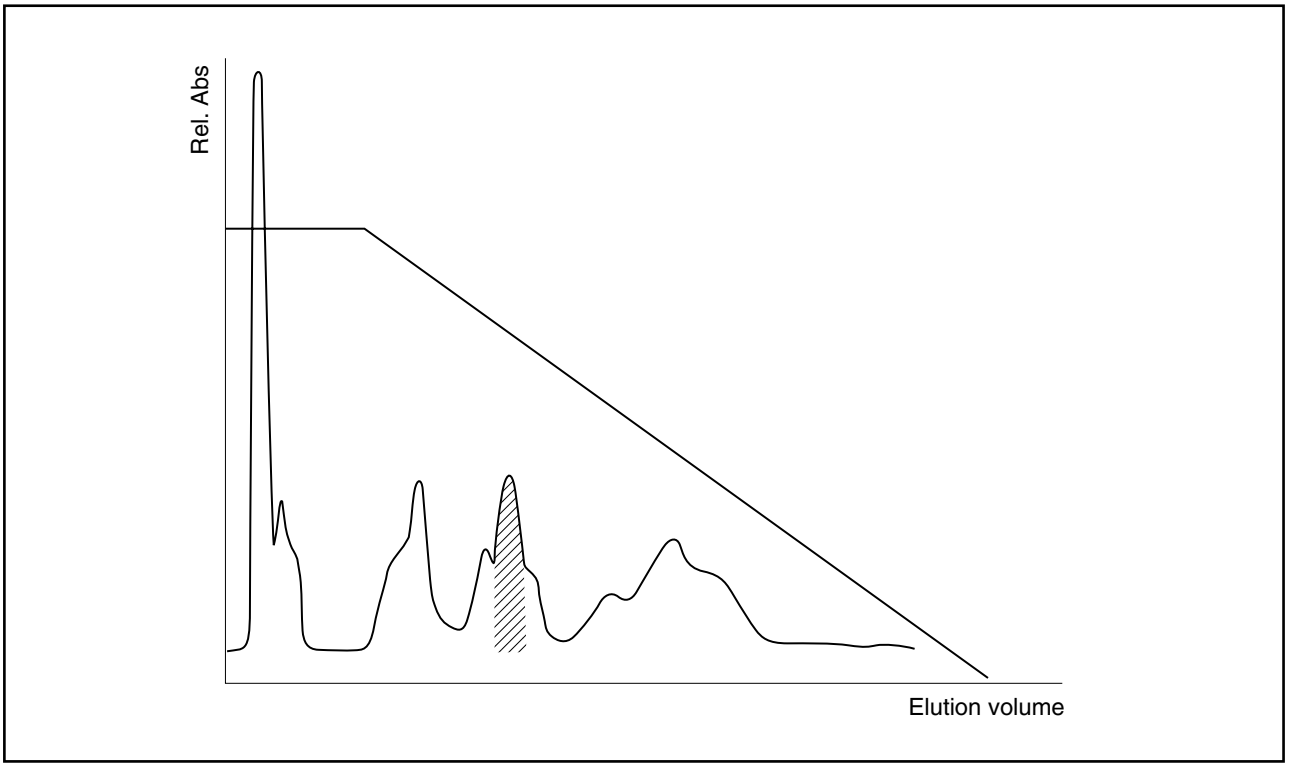


Result: The product is eluted near the end of the gradient. Resolution is satisfactory.

Discussion: This can be a good choice of medium. A disadvantage might be that some of the most hydrophobic substances bind so strongly that they are difficult to remove from the media. Decreasing the concentration of salt in the start buffer will give earlier elution of the protein and reduce the risk that strongly bound proteins are difficult to elute. It also leads to a reduction in cycle time.

Next step: If problems with strong binding of contaminants are discovered, try a medium with a different ligand or with a lower degree of ligand substitution.

Fig. 6.



Result: The protein is eluted in the middle of the gradient. Resolution is satisfactory.

Discussion: The choice of ligand is very good and there is little risk for strong binding of the most hydrophobic contaminants.

Operation

Buffer preparation

Water and chemicals should be of high purity. When using high salt concentration buffers, especially ammonium sulphate, it is possible to treat the buffer with activated charcoal for approx. 2 hours and filter through 0.45 µm filter to avoid UV baseline drift.

Sample preparation

Since adsorption is carried out at high salt concentration, the composition of the sample should be adjusted to the pH and ionic strength of the start buffer (high salt buffer) for consistent and reproducible results. When possible, dissolve the sample in start buffer. Buffer exchange can be carried out using HiTrap Desalting or PD-10 columns for ionic strengths up to ~1.5 M, (at higher ionic strengths there is a risk that the gel will shrink). Another way to increase the ionic strength is by addition of solid salt (Note: precipitation may occur due to high local salt concentrations), by addition of the salt as a high concentration stock solution or by dilution of the sample with start buffer followed by pH adjustment.
The sample should be fully solubilized. We recommend centrifugation or filtration immediately before loading on the column to remove particulate material (0.45 µm filter). Never apply turbid solutions to the column. Turbidity indicates sample insolubility which may be due to incorrect ionic strength.
High sample viscosity causes high back pressure, instability of the sample zone and gives irregular flow pattern with decreased resolution. High back pressure can also damage the column

packing. Recommended maximum sample viscosity corresponds to a protein concentration of ~50 mg/ml in aqueous solution. If lipids or other very hydrophobic substances are present in the sample, they may interact very strongly with the HIC column, diminishing capacity and being very difficult to remove. In such cases, using a slightly less hydrophobic column as a pre-column can be very efficient. The pre-column should be chosen to bind the most hydrophobic material and allow the substance of interest to pass through under starting conditions.

Column equilibration

1. Fill the syringe or pump tubing with elution buffer (low salt buffer). Remove the stopper. To avoid introducing air into the column, connect the column ”drop to drop” to either the syringe (via the adaptor) or to the pump tubing.
2. Remove the snap-off end at the column outlet.
3. Wash the column with 5 column volumes elution buffer at 1 ml/min (HiTrap 1 ml column) or 5 ml/min (HiTrap 5 ml column).
4. Wash with 5-10 column volumes start buffer (high salt buffer).

If air is trapped in the column, wash with buffer until the air disappears.

Note: If a P1-pump is used a max flow rate of 1-3 ml/min can be run on a HiTrap 1 ml column packed with Sepharose High Performance media.

Elution with linear descending gradients

A linear decrease of the salt concentration is the most frequently used type of elution in hydrophobic interaction chromatography. Recommended buffers are 50 mM sodium phosphate, 1.0 M ammonium sulphate, pH 7.0 as start buffer and 50 mM sodium phosphate, pH 7.0 as elution buffer. Continuous gradients can be prepared in different ways depending on available equipment:

– A peristaltic pump and a gradient mixer e.g. pump P-1, gradient mixer GM-1
– A one pump system, e.g. ÄKTAprime™
– A two pump system, e.g. ÄKTAdesign or FPLC

1. Equilibrate the column (see column equilibration).
2. Adjust the sample to the chosen starting pH and ionic strength (see sample preparation).
3. Apply the sample.
4. Wash with 5-10 column volumes of the start buffer until the UV trace of the effluent returns to near baseline.
5. Start the gradient elution. A gradient volume of 10-20 column volumes is usually enough.
6. Regenerate the column by washing with 5 column volumes of distilled water followed by 5 column volumes of start buffer. The column is now ready for a new sample. Avoid storage of the column or the chromatography system in high salt buffer to prevent crystal build-up in the equipment.

Elution with stepwise descending gradients

Stepwise elution is the sequential use of the same buffer at different ionic strengths. It is technically simple and fast and suitable for syringe operation. It is often used for sample concentration and sample clean up. Stepwise elution gives small peak volumes and the resolution depends on the difference in elution power between each step. When stepwise elution is applied, one has to keep in mind the danger of artifactual peaks when a subsequent step is executed too early after a tailing peak. For this reason it is recommended to start with a continuous gradient to characterise the sample and its chromatographic behaviour.

Determination of binding capacity

The amount of sample which can be applied to a column depends on the capacity of the column and the degree of resolution required. The capacity is dependent on the sample composition, chosen starting conditions such as pH, ionic strength, buffer salts and the flow rate at which the adsorption is done. The dynamic capacity can be determined by frontal analysis using real sample:

1. Equilibrate the column (see column equilibration).
2. Adjust the sample to the chosen starting pH and ionic strength (see sample preparation).
3. Determine the concentration of the actual protein in the sample by UV, PAGE, ELISA or other appropriate techniques.

4. Apply the sample solution to the column with a pump or a syringe at the flow rate to be used in the purification method. Collect fractions and continue to apply sample until the column is saturated.
5. Wash the column with 5-10 column volumes start buffer until the baseline is stable.
6. Elute bound proteins with 2-5 column volumes of elution buffer (low salt buffer) and collect the eluate.
7. Analyse fractions and eluates from steps 4 and 6 for the protein in question and determine the breakthrough profile. The practical capacity is the amount that can be applied without any breakthrough and the total capacity available is determined by analysing eluate from step 6.

Scaling up

Columns and media for scale-up are available. For quick scale-up of purifications, two or three HiTrap HIC columns of the same type can be connected in series (backpressure will be higher). Further scale up can be achieved using the prepacked columns HiPrep™ 16/10 Phenyl FF (high sub), HiPrep 16/10 Phenyl FF (low sub), HiPrep 16/10 Butyl FF, HiPrep 16/10 Octyl FF or HiLoad™ Phenyl Sepharose High Performance or bulk media packs. See Ordering Information.

Regeneration

HIC adsorbents can normally be regenerated by washing with distilled water. To prevent gradual build-up of contaminants

on the column, regular cleaning is recommended. Precipitated proteins can be removed by washing with 5 column volumes of 0.5-1.0 M NaOH followed by 5 column volumes of water at a flow rate of 1 ml/min (HiTrap 1 ml column) or 5 ml/min (HiTrap 5 ml column). Strongly bound substances can be removed by washing with 5-10 ml of up to 70% ethanol or 30% isopropanol.

Storage

Store the HiTrap HIC columns equilibrated with 5-10 column volumes 20% ethanol or 0.01 M NaOH. The recommended storage temperature is +4 to +30 °C.

Further information

Visit *www.hitrap.com* and *www.chromatography.amershambiosciences.com* for more information. Useful handbooks are also available, see ordering information.

Ordering Information

Designation	No. Supplied	Code No.
Pre-packed columns		
HiTrap HIC Selection Kit, 5 different HIC media	5 x 1 ml	17-1349-01
HiTrap Phenyl FF (high sub)	5 x 1 ml	17-1355-01
HiTrap Phenyl FF (high sub)	5 x 5 ml	17-5193-01
HiTrap Phenyl FF (low sub)	5 x 1 ml	17-1353-01
HiTrap Phenyl FF (low sub)	5 x 5 ml	17-5194-01
HiTrap Phenyl HP	5 x 1 ml	17-1351-01
HiTrap Phenyl HP	5 x 5 ml	17-5195-01
HiTrap Butyl FF	5 x 1 ml	17-1357-01
HiTrap Butyl FF	5 x 5 ml	17-5197-01
HiTrap Octyl FF	5 x 1 ml	17-1359-01
HiTrap Octyl FF	5 x 5 ml	17-5196-01
HiPrep 16/10 Phenyl FF (high sub)	1 x 20 ml	17-5095-01
HiPrep 16/10 Phenyl FF (low sub)	1 x 20 ml	17-5094-01
HiPrep 16/10 Butyl FF	1 x 20 ml	17-5096-01
HiPrep 16/10 Octyl FF	1 x 20 ml	17-5097-01
HiLoad 16/10 Phenyl Sepharose High Performance	1 x 20 ml	17-1085-01
HiLoad 26/10 Phenyl Sepharose High Performance	1 x 53 ml	17-1086-01

Bulk media	Quantity	Code No.
Phenyl Sepharose High Performance	75 ml[1]	17-1082-01
Phenyl Sepharose 6 Fast Flow (low sub)	25 ml	17-0965-10
	200 ml[1]	17-0965-05
Phenyl Sepharose 6 Fast Flow (high sub)	25 ml	17-0973-10
	200 ml[1]	17-0973-05
Butyl Sepharose 4 Fast Flow	25 ml	17-0980-10
	200 ml[1]	17-0980-01
Octyl Sepharose 4 Fast Flow	25 ml	17-0946-10
	200 ml	17-0946-02

[1] Larger quantities are available. Please contact your local representative for further information.

Accessories

Designation	No. Supplied	Code No.
1/16" male/luer female	2	18-1112-51
Union luerlock female/M6 female	2	18-1027-12
Union 1/16" female/M6 male	6	18-1112-57
Tubing connector flangeless/M6 male	2	18-1017-98
Tubing connector flangeless/M6 female	2	18-1003-68
Union M6 female /1/16" male	5	18-3858-01
Stop plug female, 1/16"	5	11-0004-64
Fingertight stop plug, 1/16"	5	11-0003-55

Literature

Hydrophobic Interaction Chromatography & Reversed Phased Chromatography, Principles and Methods, Handbook	1	11-0012-69
Convenient Protein Purification, HiTrap Column Guide	1	18-1129-81

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