cytiva

HiTrap™ Phenyl FF (high sub)
HiTrap™ Phenyl FF (low sub)
HiTrap™ Phenyl HP
HiTrap™ Butyl FF
HiTrap™ Butyl S-FF
HiTrap™ Butyl HP
HiTrap™ Octyl FF
HiTrap™ HIC Selection Kit

Prepacked columns

Instructions for Use

cytiva.com

11003452 AK

Table of Contents

Important

Read these instructions carefully before using the HiTrap™ columns.

Safety

For use and handling of the product in a safe way, refer to the Safety Data Sheet.

Intended use

HiTrap columns are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

1 Abstract

The products are ready-to-use 1 or 5 mL columns prepacked with hydrophobic interaction chromatography (HIC) resins. The columns provide fast, reproducible and convenient separation using a syringe, a pump, or a chromatography system like ÄKTA™.

The HiTrap HIC Selection Kit includes seven 1 mL HiTrap columns containing different HIC resins. The kit provides the possibility to screen for the most appropriate HIC resin for specific applications. The screening procedures recommended help optimize salt concentration, sample loading, resolution, and other chromatographic parameters.

2 Product description

HiTrap column characteristics

The columns are made of biocompatible polypropylene that does not interact with biomolecules.

The columns are delivered with a stopper at the inlet and a snap-off end at the outlet. *Table 1, on page 4* lists the characteristics of HiTrap columns.

Fig 1. HiTrap, 1 mL column.

Fig 2. HiTrap, 5 mL column.

Note: *HiTrap columns cannot be opened or refilled.*

Note: *Make sure that the connector is tight to prevent leakage.*

Table 1. Characteristics of HiTrap columns

Column volume (CV)	1 mL	5 mL
Column dimensions	0.7 × 2.5 cm	1.6 × 2.5 cm
Column hardware pressure limit	5 bar (0.5 MPa)	5 bar (0.5 MPa)
Recommended flow rate	1 mL/min	5 mL/min
Maximum flow rate[1]	4 mL/min	20 mL/min

[1] Room temperature, aqueous buffers.

Note: *The pressure over the packed bed varies depending on a range of parameters such as the characteristics of the chromatography medium, the sample/liquid viscosity and the column tubing used.*

Connector kit supplied with HiTrap column

Connectors	Use	Quantity
Union 1/16" male/Luer female	For connection of syringe to HiTrap column	1 or 2
Stop plug female, 1/16"	For sealing bottom of HiTrap column	2, 5, or 7

Resin properties

The HIC resins are based on the crosslinked beaded agarose matrices, Sepharose™ Fast Flow and Sepharose High Performance. The resins have excellent flow properties with high physical and chemical stabilities. All Sepharose matrices show virtually no nonspecific 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 HiTrapHiTrap HIC resins are listed in *Table 2, on page 7* and *Table 3, on page 8*, and their chemical stability is described in *Chemical stability, on page 7*.

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

Butyl Sepharose High Performance is based on a ~ 34 µm matrix. The small beads with high rigidity give high resolution at a high flow rate, and make the product excellent for polishing steps. Even though the ligand concentration is higher than for the other Butyl resins, it shows a similar selectivity as for those products for the test proteins used in the functional test.

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 medium to high hydrophobicity. The availability of two ligand concentration 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.

Butyl-S Sepharose 6 Fast Flow is based on a ~ 90 µm matrix. The main differences between Butyl-S Sepharose 6 Fast Flow and Butyl Sepharose 4 Fast Flow lie in the length of their spacer arms, the concentration of the immobilized ligands, and the type of connector atom (O-ether or S-ether) linking each ligand to the Sepharose base matrix. Butyl-S Sepharose 6 Fast Flow contains a sulfur atom as a linker between the spacer arm and the butyl ligand. It is the least hydrophobic resin in the Cytiva portfolio and is intended for purification or removal of strongly hydrophobic biomolecules at low salt concentrations, with high recovery and low risk of denaturation.

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.

Chemical stability

Thanks to good chemical stability the prepacked columns can be used with common buffers and organic solvents, like 70% ethanol, 30% isopropanol, 6 M GuHCl, and 1.0 M NaOH. Only use sodium hydroxide for cleaning purposes.

Note: *Consult local safety regulations to verify if safety precautions are required for the use of 70% ethanol.*

For some of the resins, the compatibility with other solvents is verified through a stability test with for example, 3 M $(NH_4)SO_4$. Due to instability, ammonium sulfate is unsuitable for use at pH values above 8.0.

Resin characteristics

Table 2. HiTrap HIC resin characteristics

	Phenyl Sepharose High Performance	Phenyl Sepharose 6 Fast Flow (low sub)	Phenyl Sepharose 6 Fast Flow (high sub)	Butyl Sepharose High Performance
Matrix	6% cross-linked agarose, spherical			
Ligand	Phenyl	Phenyl	Phenyl	Butyl
Ligand concentration	~ 25 µmol/ mL resin	~ 25 µmol/ mL resin	~ 45 µmol/ mL resin	~ 50 µmol/ mL resin
Particle size, d_{50v}[1]	~ 34 µm	~ 90 µm	~ 90 µm	~ 34 µm
pH stability				
Operational[2]	3 to 13			
CIP[3]	2 to 14			
Storage	20% ethanol, 4°C to 30°C			

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

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

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

Table 3. HiTrap HIC resin characteristics, cont'd

	Butyl Sepharose 4 Fast Flow	Butyl-S Sepharose 6 Fast Flow	Octyl Sepharose 4 Fast Flow
Matrix	4% cross-linked agarose, spherical	6% cross-linked agarose, spherical	4% cross-linked agarose, spherical
Ligand	Butyl	Butyl-S	Octyl
Ligand concentration	~ 40 µmol/ mL resin	~ 10 µmol/ mL resin	~ 5 µmol/ mL resin
Particle size, d_{50v}[1]		~ 90 µm	
pH stability			
Operational[2]		3 to 13	
CIP[3]		2 to 14	
Storage		20% ethanol, 4°C to 30°C	

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

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

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

3 Method optimization

Overview

This chapter gives a general description of how to use the mechanisms of HIC separation to optimize the method. Since method optimization is preferably performed with small column volumes in order to save sample and buffer, certain parameters such as the dynamic binding capacity can be optimized using shorter bed heights than those for the final scale.

Working principle of separation in HIC

Hydrophobic interaction chromatography separates proteins according to the differences in their surface hydrophobicity. The reversible interaction between the proteins and the hydrophobic ligand is affected by:

- hydrophobicity of the resin
- properties and composition of the sample
- presence and distribution of surface-exposed hydrophobic amino acid residues
- salt type and salt concentration used in the binding buffer

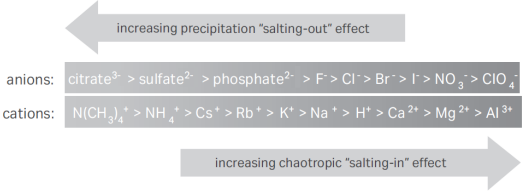
Equilibration

Add salt to the mobile phase to equilibrate the stationary phase to the desired start conditions.

Sample application and wash

Bind the target molecules to the HIC resin and wash out all unbound materials.

Binding is promoted by moderately high concentrations of anti-chaotropic salts such as ammonium sulfate and sodium sulfate. The Hofmeister series illustrated below arranges common anions and cations in order of their effects on the solubility of protein in aqueous solutions. Increasing the salting-out effect promotes hydrophobic interactions and increases the binding capacity of HIC resins for proteins. The opposite dominates when the chaotropic effect of the salts is increased.



In general, the binding process is more selective than the elution process. It is therefore important to optimize the binding buffer with respect to pH, solvent type, salt type, and salt concentration.

Elution

Decrease the salt concentration of the buffer to elute the bound solutes. Use stepwise or gradient elution to separate the target biomolecules.

Regeneration

Remove the remaining bound biomolecules to prepare the stationary phase for the next run.

Factors affecting HIC

Important factors to consider when selecting a HIC resin and optimizing its chromatographic performance are:

- type of base matrix (for example, agarose, organic copolymers)
- ligand structure
- ligand concentration
- characteristics of target protein and other sample components
- salt type
- salt concentration
- temperature
- pH
- additives

The ligand structure and ligand concentration as well as the salt type and salt concentration for the binding step determine the outcome. In general, the type of ligand determines the binding selectivity while its concentration determines the binding capacity.

Ligand choice must be determined empirically through screening experiments for each individual separation task.

For more detailed information, refer to the handbook *Hydrophobic Interaction and Reversed Phase Chromatography, Principles and Methods* (cytiva.com/handbooks).

Screening experiments

A moderate to high salt concentration promotes protein binding to HIC adsorbents and stabilizes protein structure. Elution is achieved by a linear or stepwise decrease in salt concentration.

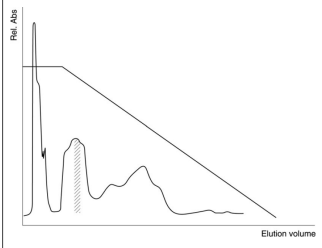
HIC resins bind the protein of interest at a high salt concentration. Binding conditions are dependent on the salt chosen. Salt concentration must be below the protein precipitation threshold.

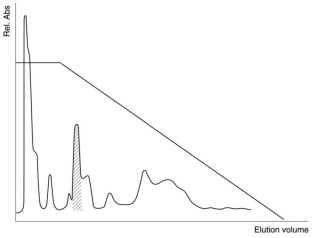
- If the protein does not bind, choose a more hydrophobic resin.
- If the protein binds so strongly that elution requires non-polar additives, choose a less hydrophobic resin.

Screening suggestions

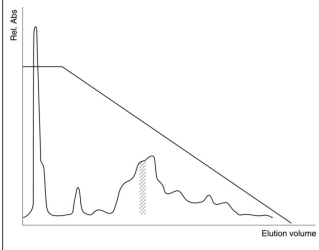
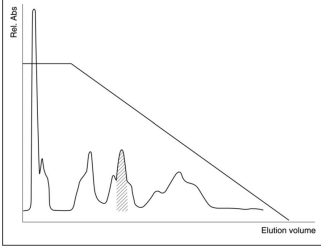
The following pages list typical elution profiles, results and suggestions for further screening. The striped regions in the chromatograms equal the elution position of the protein of interest.

Elution peak near the start of the gradient

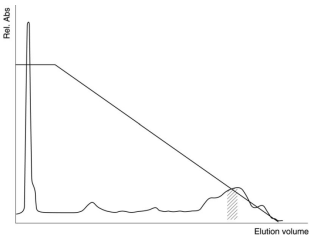
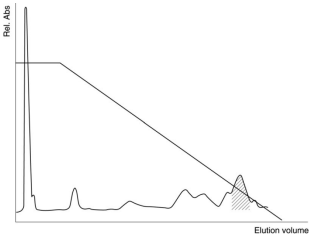
Resolution	Results and suggestions
Unsatisfactory  	Not much can be gained by changing the salt concentration of the binding buffer. Decreasing the salt concentration weakens the binding capacity and might lead to co-elution with the unbound fraction. Increasing the salt concentration, might result in co-adsorption of unwanted impurities leading to decreased selectivity. Changing the pH of the binding buffer might result in stronger binding and higher selectivity. Also, testing another salt might improve performance. • Change the pH, or choose a less chaotropic salt. If no improvement in selectivity is seen: • Try a resin with a different ligand, or a higher degree of ligand substitution.

Resolution	Results and suggestions
Satisfactory  	This can be a good choice of resin. Binding capacity might be low because the protein is eluted early in the gradient. A compensatory increase in salt concentration might lead to decreased selectivity because some unbound proteins might be adsorbed together with the protein of interest. Not much can be gained by changing the pH of the binding buffer because the resolution is satisfactory. In case of resolution loss when compensating for low binding capacity by a moderate increase in salt concentration: • Try a resin with a different ligand, or a higher degree of ligand substitution.

Elution peak near the middle of the gradient

Resolution	Results and suggestions
Unsatisfactory  	The effects of changing the salt concentration of the binding buffer are limited because the contaminants are eluted immediately before and after the protein of interest. Changing the pH of the binding buffer or testing a different salt might have a positive effect on resolution. Also, testing a different gradient slope might be an effective way to increase resolution. • Change the pH, choose a less chaotropic salt, or test a different gradient slope. If no improvement in selectivity is seen: • Try a resin with a different ligand, or a higher degree of ligand substitution.
Satisfactory  	This is a very good ligand choice with a low risk of strong binding of the most hydrophobic contaminants.

Elution peak near the end of the gradient

Resolution	Results and suggestions
Unsatisfactory  	A decrease in initial salt concentration weakens binding leading to earlier protein elution. It might not have a positive effect on selectivity, because contaminants are eluted immediately before and after the protein of interest. Changing the pH of the binding buffer or testing another salt might improve resolution. • Change the pH, or choose a more chaotropic salt. If no improvement is seen: • Try a resin with a different ligand, or a lower degree of ligand substitution.
Satisfactory  	This can be a good choice of resin. However, some of the most hydrophobic substances might bind so strongly that they are difficult to remove from the resin. Decreasing the salt concentration of the binding buffer results in earlier protein elution, a lower risk of elution difficulties with strongly bound proteins, and a reduction in cycle time. If strong binding of hydrophobic contaminants is seen: • Try a resin with a different ligand, or a lower degree of ligand substitution.

4 Operation

Buffer preparation

Water and chemicals must be of high purity. For buffers with a high salt concentration like ammonium sulfate, use a high quality salt to prevent baseline drift.

Recommended buffers

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

Elution buffer: 50 mM sodium phosphate, pH 7.0

Sample preparation

Since adsorption is carried out at high salt concentration, the composition of the sample needs to be adjusted to the pH and ionic strength of the binding buffer (high salt buffer) for consistent and reproducible results. When possible, dissolve the sample in binding buffer. Buffer exchange can be carried out using HiPrep™ 26/10 Desalting, HiTrap Desalting, or PD-10 columns for ionic strengths up to ~ 1.5 M (at higher ionic strengths the resin in the desalting column can shrink).

Another way to increase the ionic strength is by:

- addition of a solid salt

  Note: *Protein precipitation might occur due to high local salt concentrations.*

- addition of the salt as a high concentration stock solution
- dilution of the sample with binding buffer followed by pH adjustment

The sample must 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 might be due to incorrect ionic strength.

High sample viscosity causes high back pressure, instability of the sample zone and gives an irregular flow pattern with decreased resolution. High back pressure can also damage the column packing. The recommended maximum sample viscosity corresponds to a protein concentration of ~ 50 mg/mL in an aqueous solution. If lipids or other very hydrophobic substances are present in the sample, they might 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

Step	Action
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 Luer connector) or to the pump tubing.
2	Remove the snap-off end at the column outlet.
3	Wash the column with 5 column volumes (CV) of elution buffer at 1 mL/min.
4	Wash with 5 to 10 CV binding 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 to 3 mL/min can be run on a HiTrap 1 mL column packed with Sepharose High Performance resin.*

Elution with linear descending gradients

A linear decrease of the salt concentration is the most frequently used type of elution in hydrophobic interaction chromatography. Continuous gradients can be prepared in different ways depending on available equipment:

- a peristaltic pump and a gradient mixer
- a one-pump or a two-pump system

Step	Action
1	Equilibrate the column (see *Column equilibration, on page 19*).
2	Adjust the sample to the chosen starting pH and ionic strength (see *Sample preparation, on page 17*).
3	Apply the sample.
4	Wash with 5 to 10 CV binding buffer until the UV trace of the effluent returns to near baseline.
5	Start the gradient elution. A gradient volume of 10 to 20 CV is usually enough.
6	Regenerate the column by washing with 5 CV distilled water followed by 5 CV binding 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 peak 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 characterize the sample and its chromatographic behavior.

5 Determination of binding capacity

The amount of sample that 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, the starting conditions selected such as the 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:

Step	Action
1	Equilibrate the column, see *Column equilibration, on page 19*.
2	Adjust the sample to the chosen starting pH and ionic strength, see *Sample preparation, on page 17*.
3	Determine the concentration of the actual protein in the sample by UV, PAGE, ELISA, or other appropriate techniques.
4	Apply the sample 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.

Step	Action
5	Wash the column with 5 to 10 CV binding buffer until the baseline is stable.
6	Elute bound proteins with 2 to 5 CV elution buffer (low salt buffer) and collect the eluate.
7	Analyze 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 analyzing eluate from step 6.

6 Cleaning and 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 CV 0.5 to 1.0 M NaOH followed by 5 to 10 CV water at a flow rate of 1 mL/min. Strongly bound substances can be removed by washing with 5 to 10 CV of up to 70% ethanol or 30% isopropanol.

7 Scaling up

Columns and resins 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 (back pressure will increase). All HiTrap HIC columns included in HiTrap HIC Selection Kit are available as individual packages of 5 × 1 mL and 5 × 5 mL. Further scale up can be achieved using the prepacked columns HiPrep Phenyl FF (high sub) 16/10, HiPrep Phenyl FF (low sub) 16/10, HiPrep Butyl FF 16/10, HiPrep Octyl FF 16/10, or HiLoad™ Phenyl Sepharose High Performance or bulk resin packs, see *Chapter 10 Ordering information, on page 27*.

BioProcess™ chromatography resins are developed and supported for production-scale chromatography. BioProcess resins are produced with validated methods and are tested to meet manufacturing requirements. Secure ordering and delivery routines give a reliable supply of resins for production scale. Regulatory Support Files (RSF) are available to assist process validation and submissions to regulatory authorities. BioProcess resins cover all purification steps from capture to polishing.

8 Adjusting pressure limits

Overview

The pressure resulting from a flow through a column affects the packed bed and the column hardware, as shown in the image below. Pressure increases in connection with:

- high flow rate
- high-viscosity buffer or sample

- low temperature
- flow restrictor
- long and narrow tubing

Note: *Exceeding the flow limit can damage the column, see Table 1, on page 4.*

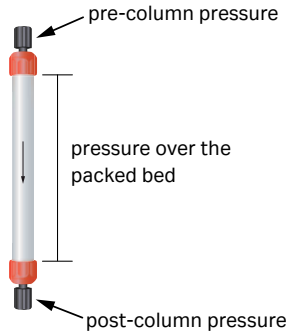


Fig 3. Pre-column and post-column measurements.

ÄKTA avant and ÄKTA pure

The system automatically monitors the pressures (pre-column pressure and pressure over the packed bed, Δp). The pre-column pressure limit is equal to the column hardware pressure limit, see *Table 1, on page 4*.

The maximum pressure for the packed bed depends on the resin characteristics and the sample/liquid viscosity. The pressure value also depends on the tubing that is used between the column and the instrument.

ÄKTA systems with a pressure sensor in the pump

Follow the steps below to adjust the pressure limit in the software for systems with a pressure sensor in the pump.

Step	Action
1	Replace the column with a piece of tubing.
2	Run the pump at the maximum intended flow rate.
3	Record the pressure as total system pressure, **P1**.
4	Disconnect the tubing and run the pump at the same flow rate used in *step 2* .
5	Note that there will be a drip from the column valve.
6	Record the pressure as **P2**.
7	Calculate the new pressure limit as a sum of **P2** and the column hardware pressure limit, see *HiTrap column characteristics, on page 3*.
8	Replace the pressure limit in the software with the calculated value. *Result:* The actual pressure over the packed bed (Δp) during the run is equal to the actual measured pressure minus the total system pressure (**P1**).

Note: *Repeat the procedure each time parameters change.*

9 Storage

Store the HiTrap HIC columns equilibrated with 5 to 10 CV 20% ethanol. The recommended storage temperature is 4°C to 30°C.

10 Ordering information

Products	Quantity	Product code
HiTrap HIC Selection Kit, 7 different HIC resins	7 × 1 mL	28411007
HiTrap Phenyl FF (high sub)	5 × 1 mL	17135501
	5 × 5 mL	17519301
HiTrap Phenyl FF (low sub)	5 × 1 mL	17135301
	5 × 5 mL	17519401
HiTrap Phenyl HP	5 × 1 mL	17135101
	5 × 5 mL	17519501
HiTrap Butyl FF	5 × 1 mL	17135701
	5 × 5 mL	17519701
HiTrap Butyl-S FF	5 × 1 mL	17097813
HiTrap Butyl HP	5 × 1 mL	28411001
	5 × 5 mL	28411005
HiTrap Octyl FF	5 × 1 mL	17135901
	5 × 5 mL	17519601

Related products

Related products	Quantity	Product code
Prepacked columns		
HiPrep Phenyl FF (high sub) 16/10	1 × 20 mL	28936545
HiPrep Butyl FF 16/10	1 × 20 mL	28936547
HiPrep Octyl FF 16/10	1 × 20 mL	28936548
Bulk resins		
Phenyl Sepharose High Performance	75 mL	17108201
	1 L[1]	17108203
Phenyl Sepharose 6 Fast Flow (low sub)	25 mL	17096510

Related products	Quantity	Product code
	200 mL	17096505
	1 L[1]	17096503
Phenyl Sepharose 6 Fast Flow (high sub)	25 mL	17097310
	200 mL	17097305
	1 L[1]	17097303
Butyl Sepharose High Performance	200 mL	17543202
	1 L[1]	17543203
Butyl Sepharose 4 Fast Flow	25 mL	17098010
	200 mL	17098001
	500 mL[1]	17098002
Butyl-S Sepharose 6 Fast Flow	200 mL	17097802
	1 L[1]	17097803
Octyl Sepharose 4 Fast Flow	200 mL	17094602
	1 L[1]	17094603

[1] Larger quantities available. Contact your local representative for further information.

Accessories

Product	Quantity	Product code
Luer female - 1/16" male	2	18111251
(For connection of syringe to top of HiTrap column)		
Tubing connector flangeless/M6 female	2	18100368
(For connection of tubing to bottom of HiTrap column)		
Tubing connector flangeless/M6 male	2	18101798
(For connection of tubing to top of HiTrap column)		

Product	Quantity	Product code
Union 1/16" female/M6 male	6	18111257
(For connection of original FPLC System through bottom of HiTrap column)		
Union M6 female - 1/16" male	5	18385801
(For connection of original FPLC System through top of HiTrap column)		
Union Luerlock female/M6 female	2	18102712
(For connection of syringe to top of HiTrap columns)		
Fingertight connector 1/16" male, narrow	8	28401081
(For connecting HiTrap and HiPrep columns to ÄKTA systems)		
Stop plug female, 1/16"	5	11000464
(For sealing bottom of HiTrap column)		
Fingertight stop plug, 1/16"	5	11000355
(For sealing a HiTrap Butyl-S FF column)		

Related literature

Handbook	Reference
Hydrophobic Interaction and Reversed Phase Chromatography, Principles and Methods	CY11248

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11003452 AK V:1 06/2023