HiPrep Phenyl FF (high sub) 16/10
HiPrep Phenyl FF (low sub) 16/10
HiPrep Phenyl HP 16/10
HiPrep Butyl FF 16/10
HiPrep Octyl FF 16/10

Prepacked columns

Instructions for Use

HiPrep™ Phenyl FF (high sub) 16/10, HiPrep Phenyl FF (low sub) 16/10, HiPrep Phenyl HP 16/10, HiPrep Butyl FF 16/10, and HiPrep Octyl FF 16/10 are prepacked, ready to use columns for hydrophobic interaction chromatography (HIC). The columns provide fast, preparative separations of proteins and other biomolecules based on their hydrophobic interaction with hydrophobic groups attached to the uncharged chromatography resin. The columns are used in an optimal way with liquid chromatography systems such as ÄKTA™.

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Read these instructions carefully before using the products.

Intended use

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

Safety

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

1 Product description

HiPrep column characteristics

HiPrep columns are made of biocompatible polypropylene that does not interact with biomolecules. The columns are delivered with stoppers at the inlet and outlet. The arrow on the column label indicates the column orientation and the recommended flow direction, see Figure 1.

Fig 1. HiPrep 16/10 column

Note: *HiPrep columns cannot be opened or refilled*

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

Table 1. Characteristics of HiPrep 16/10 column

Column volume (CV)	20 mL
Column dimensions	16 × 100 mm
Column hardware pressure limit[1]	0.5 MPa (5 bar, 72.5 psi)

[1] The pressure over the packed bed varies depending on a range of parameters such as the characteristics of the chromatography resin and the column tubing used.

Properties of HIC resins

The HIC resins are based on the cross-linked 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 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 HIC resins are listed in *Table 2, on page 6* and *Table 3, on page 7*.

Phenyl Sepharose 6 Fast Flow

Phenyl Sepharose 6 Fast Flow, with a particle size of ~ 90 µm, is ideal for initial and intermediate step purifications requiring a matrix with medium to high hydrophobicity. Two ligand concentration grades are available (high sub and low sub), which increases the possibility of finding the best selectivity and capacity for a given application.

Phenyl Sepharose High Performance

Phenyl Sepharose High Performance is based on a ~ 34 µm particle size and is ideal 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 4 Fast Flow

Butyl Sepharose 4 Fast Flow 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.

Octyl Sepharose 4 Fast Flow

Octyl Sepharose 4 Fast Flow is optimized for the separation of larger proteins in capture and intermediate purification steps. With its different selectivity it is an important complement to the other hydrophobic matrices.

Table 2. Characteristics of Phenyl Sepharose 6 FF (high and low sub) and Phenyl Sepharose HP

	Phenyl Sepharose 6 FF(high and low sub)	**Phenyl Sepharose HP**
Matrix	Cross-linked agarose, 6%, spherical	Cross-linked agarose, spherical
Ligand	Phenyl	Phenyl
Particle size (d_{50v}) [1]	~ 90 µm	~ 34 µm
Ligand concentration	~ 45 µmol/mL resin (high sub) ~ 25 µmol/mL resin (low sub)	~ 25 µmol/mL resin
Recommended operating flow rate [2]	5 mL/min (150 cm/h)	3 mL/min (90 cm/h)
Maximum operating flow rate [2]	10 mL/min (300 cm/h)	5 mL/min (150 cm/h)
pH stability, operational [3]	3 to 13	3 to 12
pH stability, CIP [4]	2 to 14	3 to 12
Chemical stability	Stable to commonly used aqueous buffers, 1.0 M NaOH[5] 8 M urea, 6 M guanidine hydrochloride, 30% isopropanol, and 70% ethanol	Stable to commonly used aqueous buffers, 0.01 M NaOH, 8 M urea, 6 M guanidine hydrochloride, 30% isopropanol, and 70% ethanol
Avoid	Solutions with pH <2, and phenol	
Storage	20% ethanol (or 0.01 M NaOH[6]), 4°C to 30°C	20% ethanol, 4°C to 30°C

1 Median particle size of the cumulative volume distribution.

2 At room temperature using buffers with the same viscosity as water. See also *Table 4, on page 15*.

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

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

5 1.0 M NaOH should only be used for cleaning purposes.

6 In most cases, no long-term stability data has been generated by Cytiva in 0.01 M NaOH. In some cases, accelerated studies at elevated temperature indicate that storage in 0.01 M NaOH can be a viable option but no guarantees can be made regarding retained function of the product.

Table 3. Characteristics of Butyl Sepharose FF and Octyl Sepharose 4 FF

	Butyl Sepharose 4 FF	Octyl Sepharose 4 FF
Matrix	Cross-linked agarose, 4%, spherical	Cross-linked agarose, 4%, spherical
Ligand	Butyl	Octyl
Particle size (d_{50v}) [1]	~ 90 µm	~ 90 µm
Ligand concentration	~ 40 µmol/mL resin	~ 5 µmol/mL resin
Recommended operating flow rate [2]	5 mL/min (150 cm/h)	5 mL/min (150 cm/h)
Maximum operating flow rate [2]	10 mL/min (300 cm/h)	10 mL/min (300 cm/h)
pH stability, operational [3]	3 to 13	3 to 13
pH stability, CIP [4]	2 to 14	2 to 14
Chemical stability	Stable to commonly used aqueous buffers, 1.0 M NaOH[5], 6 M guanidine hydrochloride, 30% isopropanol, and 70% ethanol	
Avoid	Solutions with pH <2, and phenol	
Storage	20% ethanol (or 0.01 M NaOH[6]), 4°C to 30°C	

1 Median particle size of the cumulative volume distribution.

2 At room temperature using buffers with the same viscosity as water. See also *Table 4, on page 15*.

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

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

5 1.0 M NaOH should only be used for cleaning purposes.

6 In most cases, no long term stability data has been generated by Cytiva in 0.01 M NaOH. In some cases, accelerated studies at elevated temperature indicate that storage in 0.01 M NaOH can be a viable option but no guarantees can be made regarding retained function of the product.

2 Optimization

General

Separation of biomolecules on HIC resins depends on the hydrophobicity of the resin, the nature and composition of the sample, the prevalence and distribution of surface-exposed hydrophobic amino acid residues, and the type and concentration of salt in the binding buffer. Unlike reversed phase chromatography (RPC), which is a separation method closely related to HIC, the binding of biological proteins to HIC resins is promoted, or otherwise modulated, by the presence of relatively high concentrations of anti-chaotropic salts such as ammonium sulfate and sodium sulfate (Fig 2). Elution of bound proteins is achieved simply by stepwise or gradient elution with buffers of low salt content.

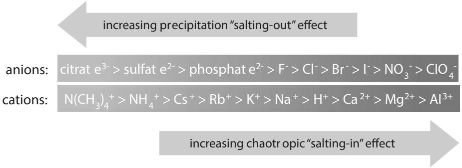

Fig 2. The Hofmeister series of some anions and cations arranged according to their effects on the solubility of protein in aqueous solutions. Increasing the salting-out effect promotes hydrophobic interactions and increases the binding capacity of the HIC resin for proteins. The opposite situation dominates when the chaotropic effect of the salts is increased.

HIC resins available from Cytiva are produced as a graded series of hydrophobic resins based on alkyl or aryl ligands attached to a hydrophilic base matrix, for example Capto™ and Sepharose. In each instance, the type and concentration of ligand has been optimized to cover the range of hydrophobicities of the proteins in a biological extract, varying from weak to moderate to strong hydrophobic proteins. This strategy results in HIC resins for “all occasions” where the emphasis is on high recovery, purity, and reduced risk for denaturation of the target proteins in a biological extract.

Factors affecting HIC

The main parameters to consider when selecting a HIC resin and optimizing its chromatographic performance is:

- The nature of the base matrix

  (e.g., agarose, organic co-polymers, etc.)
- Structure of the ligand
- Concentration of the ligand
- Characteristics of the target protein and other sample components
- Type of salt
- Concentration of salt
- Temperature
- pH

Of these parameters, the structure and concentration of ligand as well as the type and concentration of salt added during the binding step are of highest importance in determining the outcome of a HIC purification. In general, the type of immobilized ligand determines its binding selectivity toward the proteins in a sample while its concentration determines its binding capacity.

HIC resins fall into two groups, depending on their interactions with sample components:

- Straight alkyl chains (butyl, octyl) show a “pure” hydrophobic character.
- Aryl ligands (phenyl) show a mixed mode behavior, 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.

Target protein

The target protein characteristics (in a HIC context) are usually not known since minimal data are available in this respect. There are some published data regarding the hydrophobicity indices for a number of purified proteins based on amino acid composition, the number and distribution of surface-exposed hydrophobic amino acids, and the order of their elution from RPC columns but few, if any, have proved to be useful when purifying a protein in a real biological sample. For this and other reasons, the binding behavior of a protein exposed to a HIC resin has to be determined on a case-by-case basis.

Solvent

The solvent is one of the most important parameters, which influence the capacity and selectivity in HIC. In general, the binding process is more selective than the elution process. It is therefore important to optimize the start buffer with respect to pH, type of solvent, type of salt and concentration of salt.

Salts

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 might be worthwhile to test 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 sulfate often gives the best resolution of a mixture of standard proteins compared to other salts. Due to instability, ammonium sulphate is not suitable when working at pH values above 8.0. If sodium chloride is used, a concentration of up to 3 to 4 M is usually needed. Due to instability, ammonium sulfate 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 might exclude its use at high concentrations.

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 resin should bind the protein of interest at a reasonably low concentration of salt. Binding conditions are dependent on the salt chosen. The salt concentration must be below that which causes precipitation of proteins in the sample.

- If the substance does not bind, a more hydrophobic resin should be chosen.

- If the substance binds so strongly that nonpolar additives are required for elution, a column with a less hydrophobic resin should be tried.

The bound protein should be eluted from the column with high recovery.

pH

The effect of pH is not well established. In general, an increase in pH above 8.5 weakens hydrophobic interactions whereas a decrease in pH below 5.0 results in an apparent increase in the retention of proteins on HIC resins. In the range of pH 5.0 to 8.5, the effect seems to be minimal or insignificant.

Temperature

It is generally accepted that the binding of proteins to HIC resins is entropy driven, which implies that the solute-resin interaction increases with increased temperature. In some instances, the reverse effect has been observed. In practical work, you need to be aware that a purification process developed at room temperature might not be reproduced in the cold room, or vice versa. In other instances, temperature control is mandatory in order to obtain reproducible results from run to run.

Additives

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).

Automated buffer preparation

Users of ÄKTA chromatography systems with BufferPrep or BufferPro functionality can select from a range of buffer recipes to conveniently screen resins over a range of pH values and elution conditions

3 Operation

Prepare buffers

When using high salt concentration buffers, especially ammonium sulfate, use a salt of high quality to prevent baseline drift. Commonly used salts are ammonium sulfate, sodium chloride and sodium sulfate.

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

The following buffers can be used as starting points for buffer optimization:

Start buffer

1.5 M ammonium sulfate, 50 mM sodium phosphate, pH 7.0

Elution buffer

50 mM sodium phosphate, pH 7.0

Note: *Water and chemicals used for buffers must be of high purity. It is highly recommended to filter buffers through a 0.22 µm or a 0.45 µm filter before use.*

Prepare the sample

Step	Action
1	Adjust the sample to the composition of the start buffer, using one of these methods: • Dilute the sample with start buffer. ***Note:*** *The sample should be fully solubilized. If the sample starts to precipitate, reduce the ionic strength of the start buffer, or change to a different salt.* • Exchange buffer using a HiPrep 26/10 Desalting, HiTrap™ Desalting or PD-10 Desalting column ***Note:*** *Use buffer exchange if chaotropic agents, such as guanidine hydrochloride or urea have been used for initial solubilization as they will inhibit hydrophobic interaction.*
2	Filter the sample through a 0.45 µm filter or centrifuge at 10 000 × g for 10 min immediately before loading it to the column. This prevents clogging and increases the life time of the column when loading large sample volumes.

Recommended flow rates

The table below outlines recommended flow rates for the different resin types under different conditions. For viscous buffers and samples the flow rate must be optimized. Starting with a low flow rate is recommended.

Table 4. Recommended flow rates for HiPrep HIC columns.

Resin type	First time use or after long time storage in 20% EtOH	Experimental condition	Cleaning-in-place (CIP)
High performance	0.8 mL/min	3 mL/min	3 mL/min
Fast flow	2.0 mL/min	5 mL/min	5 mL/min

Purification

Flow rate: See *Table 4, on page 15*

Column tubing: Choose the optimal tubing kit for the column and the application you intend to run. (i.d.: 0.25, 0.50 or 0.75 [mm]). A tubing with wider inner diameter gives broader peaks whereas a tubing with a smaller inner diameter gives higher back pressure.

Step	Action
1	Remove the stoppers and connect the column to the system. Avoid introducing air into the column. ***Note:*** *To prevent leakage, make sure that the connectors are tight. Use fingertight 1/16" connector (28401081).*
2	Wash out the ethanol with at least 5 column volumes (CV) distilled water or elution buffer.
3	Equilibrate the column with 10 CV start buffer at recommended flow rate (see *Table 4, on page 15*).

Step	Action
4	Adjust the sample to the chosen starting pH and conductivity and load on the column. If the sample has a high viscosity, use a lower flow rate during sample loading.
5	Wash with 5 to 10 CV start buffer or until the UV signal returns to near baseline.
6	Elute, either by linear gradient elution or a step elution, see below. If required, the collected eluted fractions can be buffer exchanged or desalted. • *Linear gradient elution* Elute with 0% to 100% elution buffer in 10 to 20 CV. • *Step elution* Elute with 2 to 5 CV elution buffer at a salt concentration lower than in the start buffer. Repeat, lowering the salt content at each step until the target protein has been eluted.
7	Wash with 5 CV salt-free elution buffer to elute any remaining bound material.
8	If required, perform a CIP to clean the column.
9	Re-equilibrate with 5 to 10 CV start buffer or until the UV baseline, eluent pH, and conductivity reach the required values.

Note: *Do not exceed the maximum recommended flow and back pressure for the column.*

4 Cleaning-in-place (CIP)

Regular cleaning

Regenerate the column after each run by rinsing it with 100 mL distilled water at room temperature to elute material still bound to the column. See *Table 4, on page 15* for recommended flow rates.

Re-equilibrate the column with at least 100 mL start buffer at room temperature until the UV baseline and pH/conductivity values are stable.

Rigorous cleaning

Reverse the flow direction and run the following sequence of solutions at room temperature:

- 80 mL of a 1.0 M NaOH (0.01 M NaOH for Phenyl Sepharose HP) solution (removes precipitated proteins, hydrophobically bound proteins, and lipoproteins from the column) followed by 80 mL distilled water.
- 80 mL of 70% ethanol or 30% isopropanol (removes proteins, lipoproteins, and lipids that are strongly hydrophobically bound to the column) followed by 60 mL distilled water.

After cleaning, equilibrate the column with approximately 100 mL start buffer at room temperature before use.

5 Adjusting pressure limits

The pressure in chromatography system software is generated by the flow through a column. The pressure affects the packed bed and the column hardware, see the figure below. The pressure is increased during running/using one or a combination of the following conditions:

- High flow rates
- Buffers or sample with high viscosity
- Low temperature
- A flow restrictor

Note: *Exceeding the flow limit (see recommended flow rates in Table 2, on page 6 and Table 3, on page 7) can damage the column.*

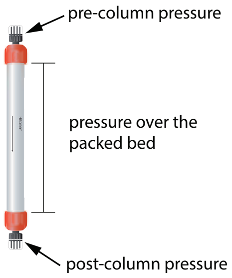

Fig 3. Precolumn and post-column measurements.

ÄKTA avant and ÄKTA pure

The system will automatically monitor the pressures (precolumn pressure and pressure over the packed bed, Δp). The precolumn pressure limit is the column hardware pressure limit (see *Table 1, on page 3* and *Table 2, on page 6*).

The maximum pressure the packed bed can withstand depends on resin characteristics and sample/liquid viscosity. The measured value also depends on the tubing used to connect the column to the instrument.

ÄKTAexplorer, ÄKTApurifier, ÄKTAFPLC, and other systems with pressure sensor in the pump

To obtain the optimal functionality in ÄKTAexplorer, ÄKTApurifier, ÄKTAFPLC, and other systems with pressure sensor in the pump, the pressure limit in the software can be adjusted as follows:

Step	Action
1	• Replace the column with a piece of tubing. • Run the pump at the maximum intended flow rate. • Record the pressure as total system pressure, P1.
2	• Disconnect the tubing and run the pump at the same flow rate used in step 1. • Note that there will be a drip from the column valve. • Record the pressure as P2.

Step	Action
3	• Calculate the new pressure limit as a sum of P2 and the column hardware pressure limit (see *Table 1, on page 3*). • 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 which is the total system pressure (P1).

Note: *Repeat the procedure each time the parameters are changed.*

6 Storage

The column is supplied in 20% ethanol. If the column is to be stored for more than two days after use, clean the column according to the procedure described in section *Cleaning-in-Place (CIP)*. Then equilibrate with at least 100 mL of 20% ethanol. Do not freeze.

Make sure that the column is tightly sealed to avoid drying out.

Note: *Never store the HiPrep HIC columns in a high salt concentration solution.*

7 Troubleshooting

Problem	Possible cause/*corrective action*
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. *Clean the column and exchange or clean the filter.* *Check pH and salt stability of sample.* Sample has changed during storage. *Prepare fresh samples.* Protein might be unstable or inactive in the elution buffer. *Determine the stability of the protein.* Column equilibration incomplete. *Repeat or prolong the equilibration step until conductivity and pH are constant.* Proteins are forming aggregates that bind strongly to the resin. *Use lower salt concentrations.*
Protein elutes later than expected or not at all.	Salt concentration too high. *Decrease salt concentration in elution buffer.* Hydrophobic interactions too strong. *Use resin with lower hydrophobicity or lower ligand density.*
Protein elutes earlier than expected (during the wash phase).	Salt concentration of sample and buffer is too low. *Increase salt in sample and buffer.* Column equilibration incomplete. *Repeat or prolong the equilibration step until conductivity is constant.*

Problem	Possible cause/*corrective action*
High back pressure during the run	The column is clogged. *Reverse the flow direction and try to pump 100 mL elution buffer through the column. Return to normal flow direction and run 100 mL buffer through the column at low flow rate. If back pressure is not decreased, reverse the flow direction again and follow the rigorous cleaning protocol in Section Cleaning-in-place (CIP).* High viscosity of solutions. *Use lower flow rate.*
Loss of resolution and/or decreased sample recovery	Insufficient elution and CIP. *Follow the rigorous cleaning protocol in Section Cleaning-in-place (CIP). Optimize the elution conditions, the CIP protocol and/or perform CIP more frequently.*
Unstable pressure curve	Air in the column. *Reverse the flow direction and pump 100 mL of well degassed start buffer through the column at room temperature.*

Note: *See Table 4, on page 15 for recommended flow rates.*

8 Ordering information

Product	Quantity	Product code
HiPrep Phenyl FF (high sub) 16/10	1 × 20 mL	28936545
HiPrep Phenyl FF (low sub) 16/10	1 × 20 mL	28936546
HiPrep Phenyl HP 16/10	1 × 20 mL	29018184
HiPrep Butyl FF 16/10	1 × 20 mL	28936547
HiPrep Octyl FF 16/10	1 × 20 mL	28936548

Related products	Quantity	Product code
HiTrap HIC Selection Kit,	7 × 1 mL	28411007
7 different HIC resins		
HiTrap Phenyl FF (high sub)	5 x 1 mL	17135501
	5 x 5 mL	17519301
HiTrap Phenyl FF (low sub)	5 x 1 mL	17135301
	5 x 5 mL	17519401
HiTrap Phenyl HP	5 x 1 mL	17135101
	5 x 5 mL	17519501
HiTrap Octyl FF	5 x 1 mL	17135901
	5 x 5 mL	17519601
HiTrap Butyl FF	5 x 1 mL	17135701
	5 x 5 mL	17519701
HiTrap Butyl-S FF	5 x 1 mL	17097813
	5 x 5 mL	17097814
HiTrap Butyl HP	5 x 1 mL	28411001
	5 x 5 mL	28411005
HiPrep 26/10 Desalting	1 x 53 mL	17508701
	4 x 53 mL	17508702

Accessories	Quantity	Product code
HiTrap/HiPrep, 1/16" male connector for ÄKTA *(For connection of columns with 1/16" fittings to ÄKTA)*	8	28401081

Related literature	Product code
Hydrophobic Interaction Chromatography and Reversed Phase Chromatography, Principles and Methods	11001269

Related literature	Product code
Prepacked chromatography columns for ÄKTA systems, Selection Guide	28931778

cytiva.com/protein-purification

For local office contact information, visit cytiva.com/contact

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