

# Phenyl Sepharose High Performance Butyl Sepharose High Performance

## HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Phenyl Sepharose™ High Performance and Butyl Sepharose High Performance are hydrophobic interaction chromatography (HIC) resins. Their performance is characterized by:

- High-resolution, high-capacity separations with high recovery
- Reliable and reproducible
- High chemical stability for effective CIP and sanitization
- Available in laboratory and BioProcess™ scale quantities
- Easy to scale up

The resins occupy a key position in the intermediate and final purification stages of a wide variety of proteins and peptides. Their availability in prepacked columns such as HiPrep™, HiScreen™, and HiTrap™ plus laboratory and BioProcess pack sizes makes purification at all scales simple and convenient (Fig 1).

## Hydrophobic interaction chromatography

Hydrophobic interaction chromatography separates and purifies biomolecules based on differences in their surface hydrophobicity. The technique is versatile and offers specific selectivity. Many proteins and peptides, as well as other hydrophobic biomolecules have sufficient numbers of exposed hydrophobic groups to allow interaction with hydrophobic ligands coupled to chromatographic matrices.


Compared with Reversed Phase Chromatography (RPC) adsorbents, HIC resins display milder elution conditions and consequently better retention of biological activity after separation. HIC is well suited for use in the middle or end of protein purification strategies that also employ other chromatographic techniques such as ion exchange and size exclusion chromatography. For example, HIC makes an ideal "next step" when purifying material that has been precipitated with ammonium sulfate or eluted in high salt concentrations during ion exchange.



**Fig 1.** Phenyl Sepharose High Performance and Butyl Sepharose High Performance, available in a variety of formats, allow high-resolution, high-capacity purification of biomolecules.

HIC is usually performed in moderate to high concentrations of salts in the start buffer, which promotes binding and helps to stabilize the protein structure. The bound molecule is eluted by decreasing the salt concentration in a linear or stepwise manner. Continuous gradient elution is most frequently used when high resolution is needed and stepwise gradient elution is recommended for sample preparation and concentration. Several factors influence the behavior of proteins and peptides on HIC resins. These include sample characteristics, flow rate, temperature, type and concentration of salt, and pH.

**Table 1.** Main characteristics of Phenyl Sepharose High Performance and Butyl Sepharose High Performance

	<b>Pheny Sepharose High Performance</b>	<b>Butyl Sepharose High Performance</b>
Functional group	Phenyl —O— 	Butyl —O—(CH <sub>2</sub> ) <sub>3</sub> —CH <sub>3</sub>
Matrix	cross-linked agarose, spherical	cross-linked agarose, spherical
Particle size, d <sub>50v</sub> <sup>1</sup>	~34 μm	~34 μm
Exclusion limit [M] Globular proteins <sup>2</sup>	~ 1 × 10 <sup>7</sup>	~ 1 × 10 <sup>7</sup>
Ligand concentration	~ 25 μmol Phenyl/mL resin	~ 50 μmol Butyl/mL resin
Total binding capacity	~ 45 mg α-chymotrypsinogen/mL resin <sup>3</sup>	~ 38 mg β-lactoglobulin/mL resin
Recommended operating flow velocity (large scale) <sup>4</sup>	≤ 100 cm/h	≤ 100 cm/h
Chemical stability	Stable to commonly used aqueous buffers, 0.01 M NaOH, 1.0 M acetic acid, 8 M urea, 6 M guanidine hydrochloride, 30% acetonitrile, 70% ethanol, 1 mM HCl, 30% isopropanol, 3 M (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> <sup>5</sup> , and 2 % SDS	
pH stability, operational <sup>6</sup>	3 to 12	3 to 12
pH stability, CIP <sup>7</sup>	3 to 13	2 to 14
Storage	20% ethanol, 4°C to 30°C	20% ethanol, 4°C to 30°C

<sup>1</sup> Median particle size of the cumulative volume distribution.

<sup>2</sup> Tested on the base matrix.

<sup>3</sup> Protein in excess is loaded in 0.1 M potassium phosphate, 2.0 M ammonium sulphate at pH 7.0 on a Tricorn 10/100 column. The binding capacity is obtained by measuring the amount of bound and eluted protein in 0.1 M potassium phosphate at pH 7.0.

<sup>4</sup> 10 cm diameter, 20 cm bed height, at room temperature using buffers with the same viscosity as water.

<sup>5</sup> Due to instability, ammonium sulphate is not suitable when working at pH values above 8.0.

<sup>6</sup> pH range where resin can be operated without significant change in function.

<sup>7</sup> pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.

## Resin characteristics

Phenyl Sepharose High Performance and Butyl Sepharose High Performance are high-resolution, high-capacity resins based on cross-linked, ~ 34 μm agarose beads modified with phenyl or butyl groups via uncharged, chemically stable ether linkages. They are truly hydrophobic resins displaying a minimum of ionic interactions. In addition, they tolerate detergents, polar organic solvents, and chaotropic agents, as well as agents for cleaning-in-place and sanitization. Table 1 lists the main characteristics of the two resins.

## Laboratory and BioProcess pack sizes

Phenyl Sepharose High Performance and Butyl Sepharose High Performance are supplied in laboratory pack sizes as well as BioProcess pack sizes for flexible scale-up and process development. The laboratory packs are for those who prefer packing columns of their choice and include straightforward instructions and tips for packing, operation, and maintenance. Empty high-resolution columns from the Tricorn™ and XK ranges are available in a variety of sizes for users who want to pack their own columns. For a complete overview of the HIC High Performance product line, refer to the Ordering information.

## Prepacked columns

By providing added speed, convenience, and reproducibility, prepacked columns extend the usefulness of Phenyl Sepharose High Performance and Butyl Sepharose High Performance. The columns can be used with simple pump configurations or a wide variety of systems. ÄKTA™ systems include preset method templates based on these prepacked columns, which further improves separation results, particularly reproducibility, and the speed at which they are achieved. Phenyl Sepharose High Performance is supplied in three types of prepacked columns, HiPrep Phenyl HP 16/10 (20 mL), HiScreen Phenyl HP (4.7 mL), and HiTrap Phenyl HP, 1 mL and 5 mL. Butyl Sepharose High Performance is supplied in prepacked HiTrap Butyl HP, 1 mL and 5 mL, and HiScreen Butyl HP (4.7 mL).

### HiPrep Phenyl HP 16/10

HiPrep columns are made of polypropylene, a material that is biocompatible with biomolecules. The columns are easily connected to a wide variety of chromatographic systems, including simple pump-based configurations and ÄKTA systems. Column characteristics are summarized in Table 2. The columns are not designed to be opened or repacked.

**Table 2.** Characteristics of prepacked HiPrep Phenyl HP 16/10 columns

Column dimensions	1.6 × 10.0 cm
Bed volume	20 mL
Recommended flow rate <sup>1</sup>	2 to 5 mL/min (60 to 150 cm/h)
Maximum flow rate <sup>1</sup>	5 mL/min (150 cm/h)
Column hardware pressure limit	5 bar (0.5 MPa, 73 psi)
Storage	20% ethanol

<sup>1</sup> Water at room temperature.

### HiScreen Phenyl HP columns

Phenyl Sepharose High Performance resin is also available prepacked in HiScreen columns. These columns are made of biocompatible polypropylene that does not interact with biomolecules. They can be run on peristaltic pumps or on chromatography systems such as ÄKTA. The columns are delivered with a stopper at the inlet and at the outlet. Table 3 lists the characteristics of HiScreen columns. Note that HiScreen columns cannot be opened or repacked.

**Table 3.** Characteristics of HiScreen columns

Column dimensions	0.77 × 10 cm
Column volume	4.7 mL
Column hardware pressure limit	8 bar (0.8 MPa, 117 psi)

<sup>1</sup> 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.

### HiTrap Phenyl HP and HiTrap Butyl HP columns

HiTrap Phenyl HP and HiTrap Butyl HP are small, prepacked columns made of biocompatible polypropylene. The columns are delivered with stoppers on the inlets and a snap-off ends on the outlets. Note that HiTrap columns cannot be opened or repacked. Two sizes are available: 1 mL and 5 mL. The 1 mL column is often used for method screening to quickly establish optimal binding and elution conditions for specific applications. Its fast and simple operation is well-suited to this role, as well as to small-scale purifications. The larger 5 mL column is an excellent choice when the purification method has been established and larger amounts of protein need to be purified. Two or three columns can be connected in series for easy scale-up. Table 4 lists key characteristics of HiTrap Phenyl HP and HiTrap Butyl HP columns.

**Table 4.** Characteristics of HiTrap Phenyl HP and HiTrap Butyl HP

Column dimensions	0.7 × 2.5 cm (1 mL), 1.6 × 2.5 cm (5 mL)
Column volumes	1 mL and 5 mL
Rec. flow rate	1.0 mL/min (1 mL), 5 mL/min (5 mL)
Max. flow rate <sup>1</sup>	4.0 mL/min (1 mL), 20 mL/min (5 mL)
Column hardware pressure limit	5 bar (0.5 MPa, 73 psi)
Storage	20% ethanol

<sup>1</sup> Room temperature, aqueous buffers.

### Operating HiTrap Phenyl HP and HiTrap Butyl HP

Using HiTrap Phenyl HP and HiTrap Butyl HP columns is easy. Complete, easy-to-follow instructions are included for fast start-up and method optimization. Whether you use a syringe and the provided Luer adapter, a peristaltic pump, or a chromatography system such as ÄKTA systems, operation is straightforward.

### HIC Selection Kit

HiTrap Phenyl HP and HiTrap Butyl HP are two components of the HiTrap HIC Selection Kit. This kit comprises seven HIC resins with different hydrophobic characteristics, each prepacked in a 1 mL HiTrap column. The remaining five resins are: 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, and Octyl Sepharose 4 Fast Flow. The kit helps users screen factors that have an influence on the behavior of proteins and peptides, and select the most appropriate HIC resin to use for a specific separation. The HiTrap HIC Selection Kit is well suited to this task (Fig 2). More information about the kit is available in Data File 18114321.

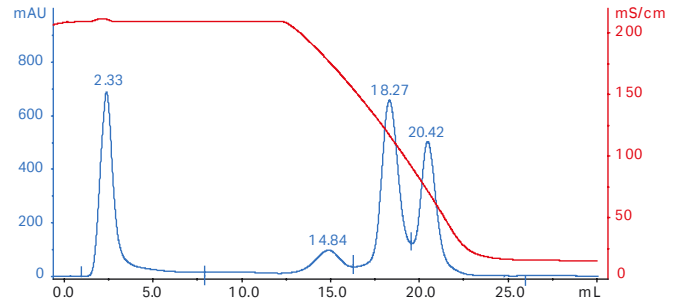
## Chemical stability

Good chemical stability (Table 1) allows the use of effective cleaning-in-place (CIP) schemes that result in high recoveries over many purification cycles. Regular CIP and sanitization hinders microbial growth and maintains a high level of hygiene to promote good economy. For CIP, regular washing with sodium hydroxide should be sufficient to remove most contaminating material, although very hydrophobic molecules may bind so

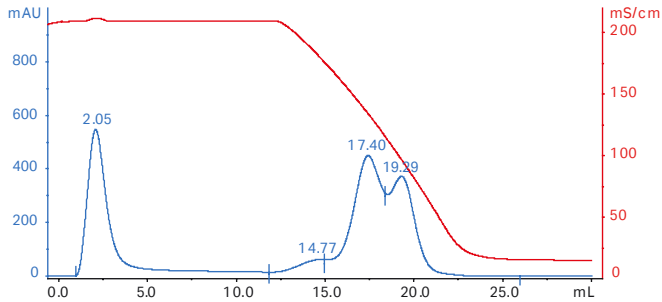
**Sample:** Cytochrome C, Ribonuclease A, Lysozyme,  $\alpha$ -chymotrypsinogen 6 mg protein/mL, (1:3:1:1) in start buffer  
**Column volume:** 1 mL, HiTrap HIC Selection Kit  
**Sample volume:** 1 mL  
**Sample load:** 6 mg protein/mL resin  
**Flow rate:** 1.0 mL/min, (150 cm/h)  
**Start buffer (A):** 0.1 M  $\text{Na}_2\text{HPO}_4$ , 1.7 M  $(\text{NH}_4)_2\text{SO}_4$ , pH 7.0  
**Elution buffer (B):** 0.1 M  $\text{Na}_2\text{HPO}_4$ , pH 7.0  
**Gradient:** 0% to 100% Elution buffer in 10 mL  
**System:** ÄKTA<sub>FPLC</sub>

tightly that they must be eluted with organic solvents agents like 70% ethanol or 30% isopropanol, or strong detergents. CIP and sanitization protocols for Phenyl Sepharose High Performance and Butyl Sepharose High Performance are included in their respective packages. Note that specific protocols should be developed according to the nature and condition of the starting material.

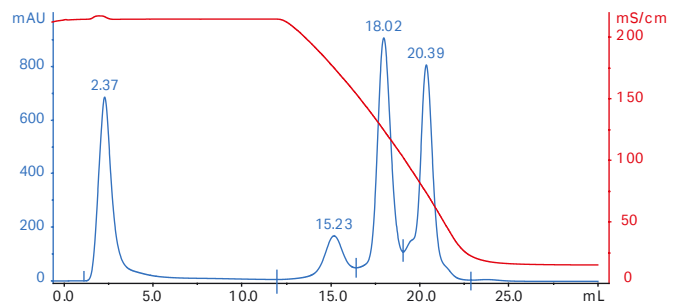
Phenyl Sepharose High Performance



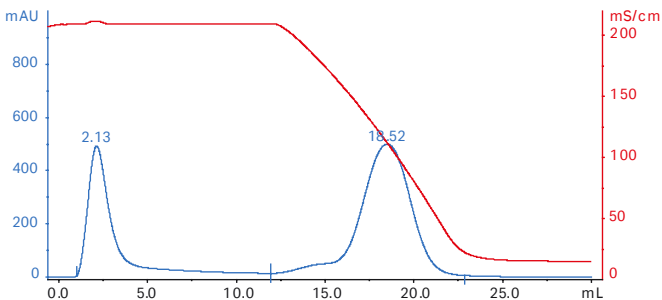
Butyl Sepharose 4 Fast Flow



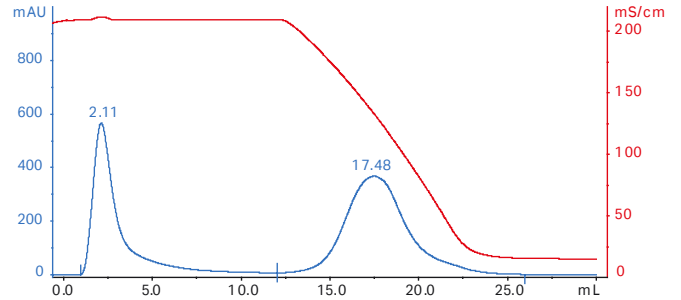
Butyl Sepharose High Performance



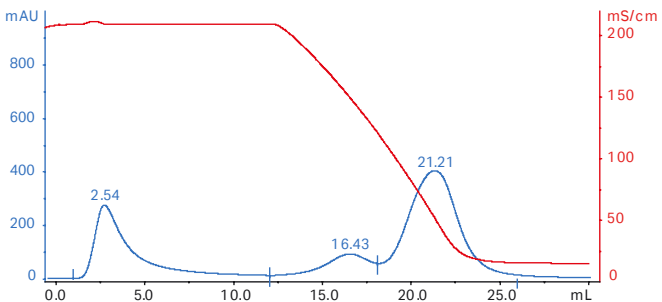
Phenyl Sepharose 6 Fast Flow (low sub)



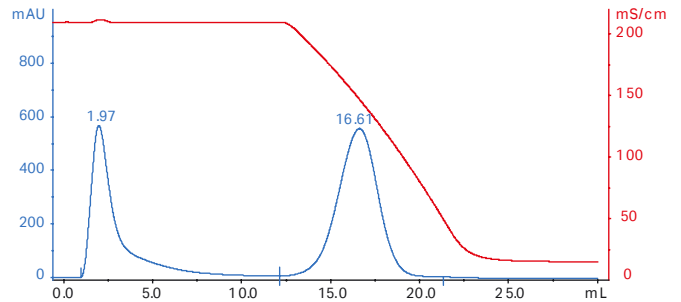
Octyl Sepharose 4 Fast Flow



Phenyl Sepharose 6 Fast Flow (high sub)



Butyl-S Sepharose 6 Fast Flow



**Fig 2.** Comparison of the selectivity characters of the different resins in HiTrap HIC Selection Kit. Elution volumes are shown at each peak.

# Applications

## Screening

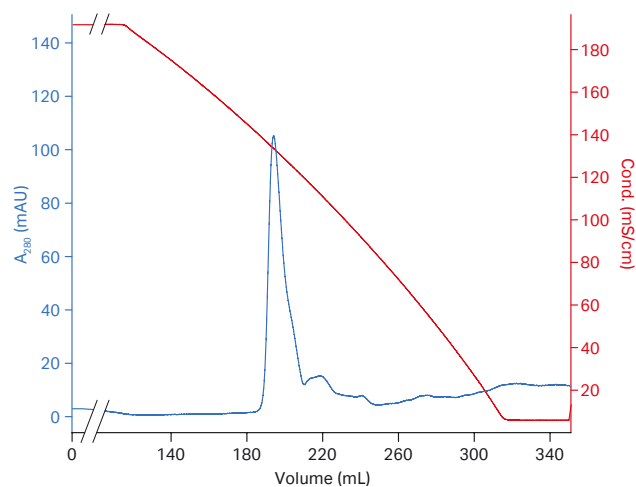
The separation result shown in Figure 2 demonstrates how important method screening is when working with HIC. Model proteins were separated using the same method and buffers. After sample injection, the bound proteins were eluted with a decreasing gradient over 10 mL.

## Purification of carbonic anhydrase

High resolution at high sample loads and flow rates, plus first class chemical and physical stability, make Phenyl Sepharose High Performance and Butyl Sepharose High Performance natural choices for preparative separations during the intermediate and final steps of a protein purification scheme. As pointed out previously, HIC is an ideal technique to further purify material that has been precipitated with ammonium sulfate or eluted in high salt concentrations during ion exchange.

Figure 3 shows a two-step approach to purification of recombinant Bovine Carbonic Anhydrase (BCA) using HiPrep 16/10 Phenyl Sepharose High Performance following an IEX purification step by HiTrap Q HP. This two-step purification approach results in a homogenous carbonic anhydrase sample, as verified by SDS-PAGE (data not shown).

**Column:** HiPrep Phenyl HP 16/10  
**Sample:** Pooled fractions from the ion exchange step. Sample was diluted 1:2 with buffer to reach an end concentration of 50 mM ammonium phosphate, 1.7 M ammonium sulphate, pH 7.0. The pool of 63 mL was then loaded on the HiPrep Phenyl HP column.  
**Start buffer:** 50 mM ammonium sulphate, 1.7 M ammonium sulphate, pH 7.0  
**Elution buffer:** 50 mM ammonium sulphate, pH 7.0. Sample was eluted by a linear gradient of 10 CV elution buffer (0% to 100%).  
**Flow rate:** 3 mL/min

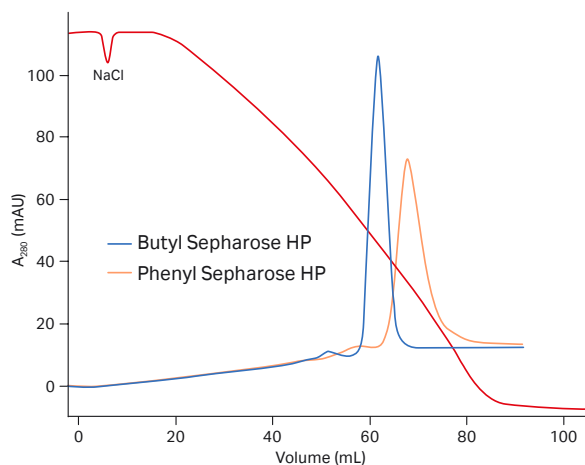


**Fig 3.** Purification of recombinant bovine carbonic anhydrase on HiPrep Phenyl HP 16/10. This two-step approach led to an almost homogenous protein (see text for details).

# mAb Purification

Butyl Sepharose High Performance is an effective second or third step in a mAb purification process due to its ability to separate monomeric mAb from aggregates and other impurities. In this case, a partly purified IgG<sub>1</sub> antibody eluted from MabSelect SuRe™ was purified on Butyl Sepharose High Performance and Phenyl Sepharose High Performance. Both resins were packed in Tricorn 5/100 and tested under similar conditions. Figure 4 shows that Butyl Sepharose High Performance and Phenyl Sepharose High Performance remove impurities equally well but with slightly different elution volumes.

**Column:** Tricorn 5/100 packed with Butyl Sepharose High Performance/ Phenyl Sepharose High Performance  
**Sample:** IgG1 purified by affinity (protein A) and anion exchange chromatography  
**Sample volume:** 1 mL  
**Sample load:** 1.37 mg/mL  
**Start buffer:** 50 mM sodium phosphate, 3 M sodium chloride, pH 6.8  
**Elution buffer:** 50 mM sodium phosphate, pH 6.8  
**Flow rate:** 0.6 mL/min (180 cm/h)  
**Gradient:** 0% to 100% elution buffer, linear gradient in 20 CV  
**System:** ÄKTExplorer 100



**Fig 4.** Separation of monomeric mAb from truncated mAb and other impurities on Butyl Sepharose High Performance and Phenyl Sepharose High Performance.

## Process development and scale-up

The excellent performance of Phenyl Sepharose High Performance and Butyl Sepharose High Performance for laboratory-scale preparative applications can be extended to process development and scale-up of HIC separations. The resins are well supported for these tasks, with special services and documentation to facilitate the development, scale-up, and routine operation of production applications. Validated manufacture, secure supply, and regulatory support comprise just part of this package. For more information, please contact your Cytiva representative.

## Ordering information

Product	Quantity	Code number
HiPrep Phenyl HP 16/10	1 (20 mL)	29018184
HiScreen Butyl HP	1 × 4.7 mL	28978242
HiScreen Phenyl HP	1 × 4.7 mL	28950516
HiTrap Butyl HP	5 × 1 mL	28411001
	5 × 5 mL	28411005
HiTrap Phenyl HP	5 × 1 mL	17135101
	5 × 5 mL	17519501

### Bulk resin

Butyl Sepharose High Performance	25 mL	17543201
	200 mL	17543202
	1 L	17543203
	5 L	17543204
Phenyl Sepharose High Performance	60 L**	17108260
	75 mL	17108201
	1 L	17108203
	5 L	17108204
	60 L**	17543260

### Related products

HiTrap HIC Selection Kit, seven different HIC resins	7 × 1 mL	28411007
HiTrap Desalting	1 × 5 mL	29048684
	5 × 5 mL	17140801
HiPrep 26/10 Desalting	1 (53 mL)	17508701
	4 (53 mL)	17508702

Related literature	Code number
Hydrophobic Interaction and Reversed Phase Chromatography Handbook: Principles & Methods	11001269
HiTrap column guide	18112981

Accessories	Quantity	
1/16" male/Luer female*	2	18111251
Tubing connector flangeless/M6 female	2	18100368
Tubing connector flangeless/M6 male	2	18101798
Union 1/16" female/M6 male	6	18111257
Union M6 female /1/16" male	5	18385801
Union Luerlock female/M6 female	2	18102712
HiTrap/HiPrep, 1/16" male connector for ÄKTA systems	8	28401081
Stop plug female, 1/16"†	5	11000464
Fingertight stop plug, 1/16"‡	5	11000355

\* One connector included in each HiTrap package.

\*\* Pack size available upon request.

† Two, five, or seven stop plugs female included in HiTrap packages depending on products.

‡ One fingertight stop plug is connected to the top of each HiTrap column at delivery.

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