



Phenyl Sepharose™ CL-4B

Hydrophobic interaction chromatography resin

Instructions for Use

Phenyl Sepharose™ CL-4B is a hydrophobic interaction chromatography (HIC) resin. Substances are separated on the basis of their different hydrophobicity.

The phenyl group is covalently coupled to a cross-linked 4% agarose matrix by ether linkage, giving a hydrophobic resin with minimal leakage and no ionic properties.

Phenyl Sepharose CL-4B belongs to the Cytiva range of BioProcess™ resins. 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.

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Read these instructions carefully before using the products.

Safety

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

1 Characteristics

Table 1. Characteristics of Phenyl Sepharose CL-4B

Matrix	Cross-linked agarose, 4%, spherical
Particle size, d_{50v}¹	~ 90 μm
Ligand concentration	~ 29 $\mu\text{mol/mL}$ resin
Total binding capacity ²	3 to 5 mg β -lactoglobulin/mL resin
Maximum operating flow velocity	150 cm/h ^{3,4}
pH stability, operational ⁵	3 to 13
pH stability, CIP ⁶	3 to 14
Chemical stability	Stable to commonly used aqueous buffers
Physical stability	Negligible volume variation due to changes in pH or ionic strength
Storage	20% ethanol 4°C to 30°C

¹ Median particle size of the cumulative volume distribution.

² Protein in excess is loaded in 0.01 M phosphate buffer at pH 7 containing 4 M NaCl on an XK 16/20 column. The binding capacity is obtained by measuring the amount of bound and eluted protein in 0.01 M phosphate buffer at pH 7 containing 50% ethylene glycol.

³ Relates to a column with 5 cm diameter and 10 cm bed height. The corresponding flow rate must not exceed 75% of the packing flow rate.

⁴ Flow velocity measured on the base matrix.

⁵ pH range where resin can be operated without significant change in function.

⁶ pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.

2 Preparing the resin

Phenyl Sepharose CL-4B is supplied in 20% ethanol.

General recommendations for large-scale packing

Packing solution	10% to 20% ethanol or water
Resin slurry concentration	50%
Packing pressure	0.025 MPa (0.25 bar)
Packing flow velocity	60 to 100 cm/h

General recommendations for lab-scale packing

Wash the resin with at least 10 volumes of distilled water.
Decant the resin and prepare a slurry with binding buffer.

Packing solution	Equilibration and binding buffer, see below.
Resin slurry concentration	50% to 75%
Packing flow velocity	30 to 150 cm/h, depending on bed height and column diameter
Packing flow rate	Use a flow rate that corresponds to the maximum operating flow velocity given in Table 1 or choose a sufficient flow rate resulting in an operating flow rate that is up to 75% of the packing flow rate.

Use the following equation for conversion between linear flow rate (cm/h) and volumetric flow rate (mL/min):

$$\text{Linear flow rate} = \frac{\text{volumetric flow rate (cm}^3\text{/h)}}{\text{column cross-sectional area (cm}^2\text{)}}$$

The binding buffer must not contain agents which increase viscosity significantly. The column can be equilibrated with viscous buffers at reduced flow rates after packing.

3 Packing the resin

Step	Action
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| 1 | Equilibrate all material to the temperature at which the chromatography will be performed. |
| 2 | Degas the resin slurry. |
| 3 | Eliminate air from the column dead spaces by flushing end pieces with ethanol or water. Make sure that no air is trapped under the column net. Close the column outlet with a few centimeters of ethanol or water remaining in the column. |
| 4 | Pour the slurry into the column in one continuous motion. Pouring the slurry down a glass rod held against the wall of the column will minimize the introduction of air bubbles. |
| 5 | Immediately fill the remainder of the column with ethanol or water, assemble the column top piece onto the column and connect the column to a pump. |
| 6 | Open the bottom outlet of the column and set the run at the desired flow rate. Use the general recommendations for the packing flow velocities given in the previous chapter. Note that the packing flow velocity also depends on the column diameter and the bed height to be packed. |

Note:

If you have packed at the maximum flow velocity, do not exceed 75% of this in subsequent chromatographic procedures.

Step	Action
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| 7 | Maintain the packing flow rate for 3 column volumes after a constant bed height is reached. |
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4 Using an adapter

Step	Action
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| 1 | After the resin has been packed as described above, close the column outlet and remove the top piece from the column. Carefully fill the rest of the column with ethanol or water to form an upward meniscus at the top. |
| 2 | Insert the adapter at an angle into the column, ensuring that no air is trapped under the net. |
| 3 | Make all tubing connections at this stage. There must be a bubble-free liquid connection between the column and the pump, and column and the sample application system. |
| 4 | Slide the plunger slowly down the column so that the air above the net and in the capillary tubings is displaced by eluent. Valves on the inlet side of the column must be turned in all directions during this procedure to make sure that air is removed. |

Step	Action
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| 5 | Lock the adapter in position on the resin surface, open the column outlet and start the eluent flow. Pass eluent through the column at the packing flow rate until the resin bed is stable. Re-position the adapter on the resin surface as necessary. |
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5 Equilibration

Before applying the sample, equilibrate the column with at least 2 column volumes at chosen binding conditions until the baseline is stable.

A common binding buffer for hydrophobic interaction chromatography is 0.05 M phosphate buffer, 1.7 M ammonium sulphate, pH 7.0.

6 Binding

The binding of proteins to the hydrophobic resin is influenced by the following:

- The structure of the ligand, such as a carbon chain or an aromatic ligand. A phenyl group, for example, is less hydrophobic than an octyl group.
- The concentration and salting-out effect of the binding buffer, see the Hofmeister series in [Table 3, on page 9](#).

- Salts that cause salting-out, for example ammonium sulphate, also promote binding to hydrophobic ligands. The sample is applied in a solution with a high salt concentration. A salt concentration between 0.5 and 2.0 M ammonium sulphate is commonly used. The column must be equilibrated at the same concentration.

Note: *When working with proteins that tend to aggregate, start with a lower ammonium sulphate concentration to avoid the risk of precipitation.*

- Temperature, as hydrophobic interactions usually decrease with decreasing temperature.
- Ethanol reduces hydrophobic interaction significantly. Remove any ethanol traces by washing the resin thoroughly before applying the sample.

7 Elution

Bound proteins are eluted by reducing the hydrophobic interaction. This can be done by:

- reducing the concentration of salting-out ions in the buffer with a decreasing salt gradient (linear or step)
- eluting with a nonpolar organic solvent
- including detergent in the eluent

Table 3. The Hofmeister series. Increasing the salting-out effect strengthens the hydrophobic interactions, whereas increasing the chaotropic effect weakens them.

← Increasing salting-out effect

Anions: $\text{PO}_4^{3-} < \text{SO}_4^{2-} < \text{CH}_3\text{COO}^- < \text{Cl}^- < \text{Br}^- < \text{NO}_3^- < \text{ClO}_4^- < \text{I}^- < \text{SCN}^-$

Cations: $\text{NH}_4^+ > \text{Rb}^+ > \text{K}^+ > \text{Na}^+ > \text{Cs}^+ > \text{Li}^+ > \text{Mg}^{2+} > \text{Ba}^{2+}$

Increasing chaotropic effect →

As a start, we suggest a linear gradient from 0 to 100% elution buffer with:

- Start buffer (A): 0.05 M phosphate buffer, pH 7.0 + 1.7 M ammonium sulphate
- Elution buffer (B): 0.05 M phosphate buffer, pH 7.0

8 Maintenance

Regeneration

Depending on the nature of the sample, regeneration is normally performed by washing with 2 to 3 column volumes of an aqueous solution of 30% isopropanol and with 3 column volumes of distilled water, followed by re-equilibrating in start buffer. In some applications, substances such as denatured proteins or lipids do not elute in this regeneration procedure. These can be removed by cleaning-in-place procedures.

Cleaning-in-place (CIP)

CIP is the removal of very tightly bound, precipitated, or denatured substances from the purification system generated in previous purification cycles. If such contaminants accumulate on the column, they can affect the chromatographic properties of the column. If fouling is severe, the column can be blocked which can result in increased back pressure and reduced flow rate.

Remove precipitated proteins, tightly bound proteins, lipids, and lipoproteins by washing the column, in reversed flow direction, with 4 column volumes of 0.5 to 1.0 M NaOH at 40 cm/h.

Wash with distilled water, at least 2 to 3 column volumes, until the pH of the effluent is neutral. Re-equilibrate with at least 3 column volumes of binding buffer.

Alternatively, wash the column with 4 column volumes of up to 70% ethanol or 30% isopropanol or with 2 column volumes of detergent in a basic or acidic solution. Wash at a low flow velocity of approximately 40 cm/h, contact time 1 to 2 hours, reversed flow direction. When using high concentrations of organic solvents, apply increasing gradients to avoid air bubble formation.

Detergents are difficult to remove and recommended only if the other procedures fail to give satisfactory results. After treatment with detergent, wash with at least 5 column volumes of 70% ethanol to remove residual detergents.

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

Re-equilibrate the column with at least 3 column volumes of binding buffer.

Sanitization

For inactivation of microbial contaminants, equilibrate the column with 0.5 to 1.0 M NaOH at a flow velocity of approximately 40 cm/h, contact time 30 to 60 minutes. Wash the column thoroughly with running buffer after sanitization.

Re-equilibrate with at least 3 column volumes of binding buffer.

General considerations regarding CIP and sanitization

Use the CIP protocols above as guidelines for formulating a cleaning protocol specific for the raw material used. Normally, column performance is not affected significantly by the CIP or sanitization procedures described above.

CIP frequency depends on the raw material applied to the column. However, it is recommended to perform a CIP procedure once every 5 cycles during normal use. Depending on the nature of the contaminants, a combination of protocols might be required. If fouling is severe, further protocol optimization might be necessary. During CIP, the flow direction through the column must be reversed.

Sometimes longer contact times are required for sufficient cleaning or sanitization, for example, to remove endotoxins. Note that prolonged exposure to harsh conditions can shorten column lifetime. It is recommended to monitor column performance over time to make sure that functionality is not affected.

9 Storage

Store the resin in 20% ethanol at 4°C to 30°C.

10 Ordering information

Product	Quantity	Product code
Phenyl Sepharose CL-4B	10 L	17081005
Phenyl Sepharose CL-4B	200 mL	17081001

Related products	Quantity	Product code
Phenyl Sepharose 6 Fast Flow (high sub)	25 mL	17097310
Phenyl Sepharose 6 Fast Flow (high sub)	200 mL	17097305
Phenyl Sepharose 6 Fast Flow (low sub)	25 mL	17096510
Phenyl Sepharose 6 Fast Flow (low sub)	200 mL	17096505
Phenyl Sepharose High Performance	75 mL	17108201
HiTrap™ Phenyl FF (high sub)	5 × 5 mL	17519301
HiTrap Phenyl FF (low sub)	5 × 1 mL	17135301
HiTrap Phenyl FF (low sub)	5 × 5 mL	17519401
HiTrap Phenyl HP	5 × 1 mL	17135101
HiTrap Phenyl HP	5 × 5 mL	17519501
HiTrap HIC Selection Kit	7 × 1 mL	28411007

Related literature	Reference
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Application notes

Increasing productivity in hydrophobic interaction chromatography (HIC) using Capto resins	CY14112
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Data files

Capto Phenyl (high sub), Capto Butyl, Capto Octyl, and Capto Butyl-S	CY13568
Capto Phenyl ImpRes and Capto Butyl ImpRes	CY13700

Handbooks

Hydrophobic interaction and reversed phase chromatography, Principles and methods	CY11248
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