Phenyl **Sepharose** 6 Fast Flow (low sub)
Phenyl **Sepharose** 6 Fast Flow (high sub)

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

Phenyl Sepharose™ 6 Fast Flow (low substitution) and Phenyl Sepharose 6 Fast Flow (high substitution) are part of the Cytiva range of resins for hydrophobic interaction chromatography (HIC) (Fig 1). Both resins satisfy process chromatography requirements in terms of performance, stability, scaleability, and bulk availability. As part of the BioProcess™ range of resins, Phenyl Sepharose 6 Fast Flow (low sub) and Phenyl Sepharose 6 Fast Flow (high sub) carry comprehensive technical and regulatory support for production scale applications. The resins have long track records of use in downstream processing of biopharmaceuticals.

- High dynamic binding capacity and stability
- Fast Flow matrix gives high flow rates
- Highly hydrophilic base matrix making true hydrophobic interaction chromatography possible without interfering secondary interactions influencing protein conformation or binding
- Suitable for a wide range of applications from research to production scale

HIC technology

HIC is widely used for purification of macromolecules (proteins and peptides). Substances are separated on the basis of their varying strength of hydrophobic interaction with hydrophobic groups attached to an uncharged resin matrix. This technique is usually performed in the presence of moderately high concentrations of anti-chaotropic salts (salt-promoted adsorption chromatography).

Several factors influence the chromatographic behavior of proteins and peptides on hydrophobic resins. Some of these factors are crucial for developing an optimized purification procedure. Purification protocols for analytical or preparative separations in small scale, with emphasis on resolution, are different from those in a manufacturing process where the emphasis is on the highest possible productivity.

Fig 1. Phenyl Sepharose 6 Fast Flow (low sub) and Phenyl Sepharose 6 Fast Flow (high sub) for rapid purification.

Parameters that influence binding, resolution, selectivity, and recovery include:

- Ligand structure (aliphatic or aromatic)
- Ligand concentration
- Sample characteristics
- Flow rate
- Salting-out effect
- Ionic strength
- Temperature
- pH

The dynamic binding capacity of HIC resins, which is affected by the type of buffer ion used, decreases with increasing flow rate.

Resolution is also affected by the substitution level. For adsorption of proteins, high ligand concentration does not necessarily correspond to high capacity, but can encourage multipoint attachment of proteins that otherwise might not adsorb to a resin with a lower ligand concentration.

A moderate ligand concentration can enable the user to adjust the binding buffer concentration and bind the protein of interest selectively. During desorption, parameters such as buffer composition, gradient volume, flow rate, and gradient shape (linear or step-wise) play an important role.

HIC is compatible with other chromatographic techniques commonly used in purification schemes. For example, after ammonium sulphate precipitation, ion exchange, and affinity chromatography, the sample is often left in a high salt concentration. With HIC as the next step, it is usually possible to transfer the sample directly, eliminating the need for prior dialysis or size exclusion chromatography. As elution of the protein of interest from a HIC column often leaves the substance at low ionic strength, HIC is a practical step before techniques such as size exclusion chromatography, ion exchange, reversed phase, and affinity chromatography.

Phenyl Sepharose 6 Fast Flow

Phenyl Sepharose 6 Fast Flow (low sub) and Phenyl Sepharose 6 Fast Flow (high sub) are based on cross-linked 6% agarose. Sepharose 6 fast Flow is a rigid matrix that enables rapid processing of large volumes. Both media are suitable for applications at all scales from laboratory to production.

At process scale, Cytiva Fast Flow HIC resins are particularly useful during the initial and intermediate stages of a separation process when high flow rates are required. At production scale, the resins meet the requirements for reliable and economic purification of biological material. Phenyl Sepharose 6 Fast Flow (high sub) and Phenyl Sepharose 6 Fast Flow (low sub) were originally developed and tested in cooperation with leading large-scale manufacturers and are used today in routine commercial pharmaceutical production. For information on additional Cytiva HIC chromatography resins suitable for large-scale manufacturing, please visit cytiva.com/capto.

Stability

Phenyl Sepharose 6 Fast Flow (low sub) and Phenyl Sepharose 6 Fast Flow (high sub) are compatible with commonly used aqueous buffers. The resins have high chemical and mechanical stability and withstand high concentrations of denaturing agents such as urea and guanidine hydrochloride.

Both resins have high thermal stability and are autoclavable at 121°C for 20 minutes. Table 1 summarizes the resin characteristics.

Table 1. Characteristics of Phenyl Sepharose 6 Fast Flow (low sub) and Phenyl Sepharose 6 Fast Flow (high sub)

Matrix	Cross-linked agarose, 6%, spherical
Type of ligand	Phenyl: R-O-CH_2-CH(OH)-CH_2-O-C_6H_5
Particle form	Rigid, spherical, macro porous
Particle size, d_{50V}[1]	~ 90 µm
Ligand concentration	~ 25 µmol phenyl/mL resin (low sub) ~ 45 µmol phenyl/mL resin (high sub)
Pressure/flow characteristics	250-400 cm/h at < 0.1 MPa in a XK 50/60 column with 5 cm diameter and 25 cm bed height (at 20°C using buffers with the same viscosity as water)[2,3]
pH stability, operational[4]	3 to 13
pH stability, CIP[5]	2 to 14
Operating temperature	4°C to 30°C
Chemical stability	Stable to commonly used aqueous buffers, 1.0 M NaOH[6], 3 M ammonium sulphate[7], 70% ethanol, 30% isopropanol, 0.5% SDS, 6 M guanidine hydrochloride, 8 M urea, 10% ethylene glycol
Autoclavability	20 min at 121°C in distilled water pH 7, 5 cycles
Delivery conditions	20% ethanol
Storage	20% ethanol, 4°C to 30°C Do not freeze

[1] Median particle size of the cumulative volume distribution.
[2] The pressure/flow characteristics describes the relationship between pressure and flow under the set circumstances. The pressure given shall not be taken as the maximum pressure of the resin.
[3] Pressure/flow test performed on the base matrix.
[4] pH range where resin can be operated without significant change in function.
[5] pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.
[6] 1.0 M NaOH should only be used for cleaning purposes.
[7] Due to instability, ammonium sulphate is not suitable when working at pH values above 8.0.

Production-scale use

Figure 2 shows pressure/flow curves for the resin packed in a BPG 300 Column.

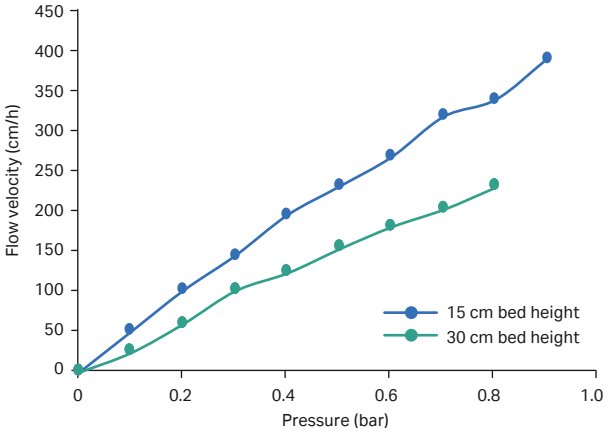


Fig 2. Typical pressure/flow curves for Phenyl Sepharose 6 Fast Flow High sub/Low sub, packed bed in a BPG 300 column with bed heights 15 cm and 30 cm.

Application

Human alpha-fetoprotein (αFP) plays an important role in fetal development and in cancer. The chromatograms in Figure 3 show a successful two-step purification procedure for a monoclonal antibody (IgG_1) raised against αFP. The hybridoma was cultured in a hollow fibre reactor and the monoclonal antibody isolated, in high yield, on Phenyl Sepharose 6 Fast Flow (high sub). In the second step, contaminating albumin was removed by size exclusion chromatography using Superdex™ 200 prep grade resin.

Process hygiene

Good process hygiene enables safety and integrity of the final product by removing or controlling any unwanted substances that might be present or generated in the raw material, or derived from the purification system itself. Good process hygiene also has a positive effect on process economy by preventing successive build-up of contaminating material on the separation resin, thus prolonging the life of the packed column.

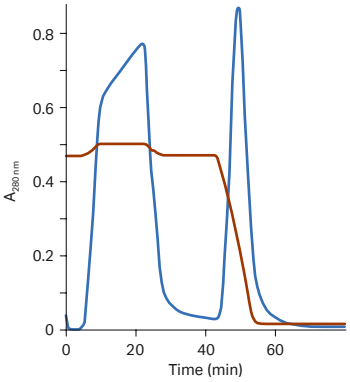
Regeneration

Wash with two bed volumes of water, followed by two to three bed volumes of starting buffer. A complete cleaning-in-place (CIP) procedure is recommended after approximately five runs, depending on the starting material.

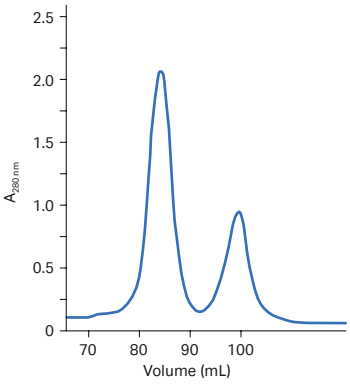
CIP

A CIP procedure is the removal from the purification system of very tightly bound precipitated or denatured substances generated in previous production runs. In some applications, substances such as lipids or denatured proteins may remain in the column bed, and not be eluted by the regeneration procedure. A specific CIP protocol has to be designed according to the type of contaminants known to be present in the feed stream. Recommended procedures for the removal of these contaminants without dismantling the column are described below. Column performance is not significantly changed by CIP procedures for at least 100 CIP cycles.

1. **Protocol to remove precipitated proteins:**
   - Wash the column with 4 bed volumes of 0.5 to 1.0 M NaOH solution at a flow velocity of 40 cm/h, followed by 2 to 3 bed volumes of water
2. **Protocol to remove strongly bound hydrophobic proteins, lipoproteins and lipids:**
   - Wash the column with 4 to 10 bed volumes of up to 70% ethanol or 30% isopropanol. (Apply gradients to avoid air bubble formation when using high concentration of organic solvents.)
   - Alternatively, wash the column with detergent in a basic or acidic solution. Wash at a flow velocity of 40 cm/h. Residual detergent can be removed by washing with 5 bed volumes of 70% ethanol

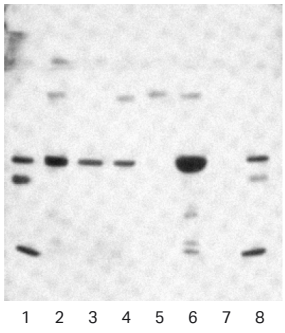


Resin: Phenyl Sepharose 6 Fast Flow (high sub)
Sample: Harvest from hollow fiber containing 4% fetal calf serum
Column: XK 16/30, 20 mL resin volume
Loading: 4 mg mAb/mL resin
Starting buffer: 0.6 M $(NH_4)_2SO_4$ + 20 mM K_2HPO_4, pH 7.0
Elution buffer: 20 mM K_2HPO_4, pH 7.0
Flow rate: 10 mL/min (300 cm/h)
Gradient volume: 10 column volumes



Resin: Superdex 200 prep grade
Sample: Gradient eluted material from Phenyl Sepharose 6 Fast Flow (high sub)
Column: XK 50/100, 1700 mL resin volume
Loading: 7 mL (11 mg protein/mL)
Buffer: 0.9% NaCl
Flow rate: 10 mL/min (30 cm/h)

Electrophoresis: PhastGel™ 10% to 15% polyacrylamide, SDS, nonreducing conditions



Lane
1. Low molecular weight markers
2. Harvest from hollow fibre
3. Flow through material from Phenyl Sepharose 6 Fast Flow (high sub)
4. Eluted material from Phenyl Sepharose 6 Fast Flow (high sub)
5. mAb from Superdex 200 prep grade polishing
6. Peak #2 from Superdex 200 prep grade polishing
7.
8. Low molecular weight markers

Fig 3. Purification procedure for a monoclonal antibody (mAb).

Figure 4 shows the results of a clearance study, a test which determines the amount of water required to replace the storage buffer containing 20% ethanol.

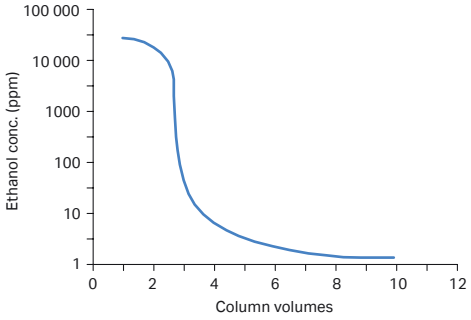


Fig 4. Removal of 20% ethanol from Phenyl Sepharose 6 Fast Flow (high sub) in an HR 10/10 Column, bed volume 8 mL; mobile phase H_2O; flow rate 1 mL/min.

Sanitization and sterilization

Sanitization using NaOH reduces microbial contamination of the resin bed to a minimum, without dismantling the column. The CIP procedures recommended above also sanitize Phenyl Sepharose Fast Flow resins effectively. A concentration of 0.5 to 1.0 M NaOH with a contact time of 30 to 60 min has proved effective for most microbial contaminations.

For sterilization of the chromatography resins, dismantle the column and autoclave the resin at 121°C for 20 min. Remember to sterilize the column parts before re-assembling and packing the column.

Operation

Phenyl Sepharose 6 Fast Flow (low sub) and
Phenyl Sepharose 6 Fast Flow (high sub) are supplied preswollen in 20% ethanol.

Equilibrate the HIC resin in three volumes of working buffer before packing.

We recommend that the sample be passed through a 0.22 to 0.45 µm filter to prolong the life of the separation resins.

Elution conditions have to be optimized for different samples to obtain maximum purity and throughput.

Storage

For longer periods of storage (e.g., weeks), the recommendation is that the unused resin shall be stored at 4°C to 30°C in 20% ethanol. Used resin can be stored at 2°C to 8°C.

References

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Ordering information

Product	Pack size	Code number
Phenyl Sepharose 6 Fast Flow (low sub)	25 mL	17096510
Phenyl Sepharose 6 Fast Flow (low sub)	200 mL	17096505
Phenyl Sepharose 6 Fast Flow (low sub)	1 L	17096503
Phenyl Sepharose 6 Fast Flow (low sub)	5 L	17096504
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 (high sub)	10 L	17097306
Phenyl Sepharose 6 Fast Flow (high sub)	1 L	17097303
Phenyl Sepharose 6 Fast Flow (high sub)	5 L	17097304
Phenyl Sepharose 6 Fast Flow (high sub)	60 L*	17097360

* Pack size available upon request

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