GE Healthcare

Instructions 56-1191-01 AD **Hydrophobic interaction**

Phenyl Sepharose 6 Fast Flow (low sub)
Phenyl Sepharose 6 Fast Flow (high sub)
Butyl-S Sepharose 6 Fast Flow
Butyl Sepharose 4 Fast Flow
Octyl Sepharose 4 Fast Flow

Sepharose™

Contents

1. Introduction

Phenyl Sepharose™ 6 Fast Flow (low sub), Phenyl Sepharose 6 Fast Flow (high sub), Butyl Sepharose 4 Fast Flow and Octyl Sepharose 4 Fast Flow are media for hydrophobic interaction chromatography (HIC). Substances are separated on the basis of their varying strength of their hydrophobic interaction with hydrophobic groups attached to the uncharged matrix.

Sepharose Fast Flow HIC media belong to the BioProcess™ media family. With their high quality and high batch-to-batch reproducibility they are ideal for all stages of an operation – from process development through scale-up and into production. Characteristics of the different media can be found in Appendix B, Table 1.

The instructions that follow are based upon packing Sepharose Fast Flow HIC media in the recommended XK 16/20 Column. To modify these instructions for columns of different dimensions, refer to Appendix A. For pratical movie instruction in good packing techniques, Column Packing Packing The Movie is recommended

Detailed information on the technique of HIC can be found in the handbook ”Hydrophobic Interaction & Reversed Phase Chromatography: Principles and Methods”, from GE Healthcare.

2. Material needed

Sepharose Fast Flow HIC media
XK 16/20 column
HiLoad Pump P-50
Gradient maker
Injection valve (LV-3 or LV-4)
Graduated cylinder or beaker
Vacuum flask and pump
5 ml syringe
Glass rod
Packing buffer.

Note: The packing buffer should be the same as the binding buffer. See the section on ”Binding” for buffer recommendations. High viscosity buffers should not be used during packing. If such buffers are required for the separation, equilibrate the column in the high viscosity buffer at a reduced flow rate when packing is completed.

3. Preparing the medium

1. Equilibrate all material to room temperature.
2. Sepharose Fast Flow HIC media are supplied pre-swollen in 20% ethanol. Decant the ethanol solution and replace it with packing buffer to a total volume of 32.5 ml (75% settled medium: 25% buffer).
3. Degas the slurry under vacuum.

4. Assembling the column

Details of the column parts can be found in the instructions supplied with the column. Before packing ensure that all parts, particularly the nets, net fasteners and glass tube, are clean and intact.

1. Connect the column bottom end piece to a pump or syringe. Submerge the end piece in buffer and fill it using the pump or syringe. Ensure that there are no air bubbles trapped under the net. Close the tubing with a stopper and mount the end piece on the column.
2. Flush the column with buffer, leaving a few ml at the bottom. Mount the column vertically on a laboratory stand.

5. Packing the column

These instructions are based for packing Sepharose Fast Flow HIC media in the recommended XK 16/20 Column. To modify these instructions for columns of different dimensions, refer to Appendix A.

1. Pour the medium slurry into the column in one continuous motion. Pouring down a glass rod held against the wall of the column helps prevent the introduction of air bubbles. Fill the remainder of the column with buffer.

2. Wet the column adaptor by submerging the plunger end in buffer, and drawing buffer through with a syringe or pump. Ensure that all bubbles have been removed. Disconnect the pump or syringe. Insert the adaptor into the top of the column at an angle, taking care not to trap air under the net. Tighten the adaptor O-ring to give a sliding seal on the column wall.
3. Fit a syringe barrel to the sample application valve and connect the valve between the adaptor and the pump. With the valve in the sample application position, slide the adaptor down into the column. This will displace all air in the tubing as far as the sample application valve. Switch the valve a few times to remove any trapped bubbles. Continue inserting the adaptor until it reaches the medium slurry. Tighten the O-ring and lock the adaptor in position.
4. Open the bottom outlet of the column and start the pump.
   Sepharose 6 Fast Flow media – Pack the medium at a flow rate of 17 ml/min until the bed height is constant (normally 4 to 5 minutes).
   Sepharose 4 Fast Flow media – Pack the medium at a flow rate of 12–14 ml/min until the bed height is constant (normally 4 to 5 minutes).
5. Stop the pump, close the column outlet, loosen the adaptor O-ring to give a sliding seal and re-position the adaptor on the surface of the medium bed. Press the adaptor into the surface of the medium an additional 1–2 mm. Lock the adaptor in position, open the column outlet and start the pump at the column packing flow rate.

If the bed continues to pack, repeat step 5. When the medium bed is stable, the column is packed equilibrated and ready for use.

6. Equilibration

To equilibrate, pump approximately 100 ml of start buffer through the column at a flow rate of 3.5 ml/min for Sepharose 6 Fast Flow and 2.5 ml/min for Sepharose 4 Fast Flow. The column is fully equilibrated when the pH and/or conductivity of the effluent is the same as the start buffer.

7. Sample Preparation

The amount of sample that can be applied to the column differs considerably, depending on the degree of substitution of the medium, the nature of the sample and on start buffer conditions. See Table 1 in Appendix B for some capacity guidelines.

High ligand density does not necessarily correspond to high capacity for adsorption of protein, but a high ligand density can encourage multi-point attachment of proteins which otherwise might have difficulty adsorbing to lower ligand densities. A moderate ligand density allows selective binding of the protein of interest by adjustment of the binding buffer concentration.

The sample should be dissolved in start buffer. Alternatively the sample may be transferred to start buffer by dialysis or by buffer exchange using a HiTrap Desalting or a PD-10 Desalting columns. The viscosity of the sample should not exceed that of the buffer. For normal aqueous buffer systems, this corresponds to a protein concentration of approximately 50 mg/ml.

Before application the sample should be centrifuged or filtered through a 0.45 µm filter to remove any particulate matter.

8. Operating Flow rates

The flow rate used for sample binding and subsequent elution will depend on the degree of resolution required, but is normally within the range 2.5–5 ml/min for Sepharose 4 Fast Flow media and 5–10 ml/min for Sepharose 6 Fast Flow media. The lower the flow rate, the better the resolution.

9. Binding

The binding of proteins to hydrophobic media is influenced by:

- the structure of the ligand (e.g., carbon chain or an aromatic ligand)
- the ligand density
- the ionic strength of the buffer
- the salting-out effect (see The Hofmeister series below)
- the temperature

Those salts which cause salting-out (e.g., ammonium sulphate) also promote binding to hydrophobic ligands. The column is equilibrated and the sample is applied in a solution of high ionic strength. A typical starting buffer is 1.7 M (NH4)2SO4, which is just below the concentration employed for salting out proteins.

Hydrophobic interactions are weaker at lower temperatures. This must be taken into account if chromatography is done in a cold room.

Elution

Bound proteins are eluted by reducing the strength of 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)
- increasing the concentration of chaotropic ions in the buffer with an increasing gradient (linear or step)
- eluting with a polarity-reducing organic solvent (e.g., ethylene glycol) added to the buffer
- eluting with detergent added to the buffer

The Hofmeister series

← Increasing salting-out effect

Anions: PO_4^{3-} SO_4^{2-} CH_3COO^- Cl^- Br^- NO_3^- ClO_4^- I^- SCN^-

Cations: NH_4^+ Rb^+ K^+ Na^+ Cs^+ Li^+ Mg^{2+} Ba^{2+}

Increasing chaotropic effect →

Increasing the salting-out effect strengthens hydrophobic interactions; increasing the chaotropic effect weakens hydrophobic interactions.

A suggested starting gradient is a linear gradient from 0 to 100% B with:

Buffer A: 50 mM phosphate buffer, pH 7.0 + 1.7 M $(NH_4)_2SO_4$*

Buffer B: 50 mM phosphate buffer, pH 7.0

* When working with proteins which have a tendency to aggregate, start with a lower $(NH_4)_2SO_4$ concentration to avoid protein precipitation.

10. Regeneration

After every run, elute reversibly bound material with low ionic strength buffer at a flow rate of 3.5 ml/min for Sepharose 6 Fast Flow and 2.5 ml/min for Sepharose 4 Fast Flow. Monitor the UV absorbance during regeneration to determine when bound substances have been completely washed out of the column. Wash the column with 40–60 ml of distilled H_2O and re-equilibrate with 100 ml of starting buffer.

In some applications, substances such as denatured proteins or lipids do not elute in the regeneration procedure. These can be removed by cleaning-in-place procedures.

11. Cleaning-in-place (CIP)

Remove precipitated proteins by washing the column with 80 ml 1 M NaOH solution at a flow rate of 1.2–1.4 ml/min, followed immediately with 40–60 ml of distilled H_2O. and re-equilibrate with 100 ml of starting buffer.

Remove strongly hydrophobically bound proteins, lipoproteins and lipids by washing the column with 80 ml of 70% ethanol or 30% isopropanol. Apply increasing concentration gradients to avoid air bubble formation, when using high concentrations of organic solvents. Wash the column with distilled water and re-equilibrate.

Alternatively, wash the column with two 40 ml of 0.1–0.5% detergent in a basic or acidic solution. For example, wash with 0.1–0.5% non-ionic detergent in 0.1 M acetic acid at a flow rate of 1.2–1.4 ml/min in the reversed flow direction, for a total contact time of 1–2 hours. After treatment with detergent always remove residual detergent by washing with 100 ml of 70% ethanol. Wash the column with distilled water and re-equilibrate.

12. Sanitization

Sanitization reduces microbial contamination of the medium bed to a minimum.

Wash the column in the reversed flow direction for 30–60 minutes with 0.5–1 M NaOH, at a flow rate of 1.2–1.4 ml/min. Re-equilibrate the column with approximately 100 ml sterile start buffer.

13. Storage

For column storage, wash with 5 column volumes of distilled water followed by 5 column volumes of 20% ethanol. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid overpressuring the column. Ensure that the column is sealed well to avoid drying out. Whenever possible use a storage and shipping device, if supplied by the manufacturer. Store columns and unused media at +4 °C to +30 °C in 20% ethanol. Do not freeze.

Appendix A

Converting to columns of different dimensions

Flow rates

Flow rates quoted in this instruction are for an XK 16/20 column. To convert flow rates for columns of different dimensions:

1. Divide the volumetric flow rates (ml/min) quoted by a factor of 2 (the cross-sectional area in cm^2 of the XK 16/20) to give the linear flow rate in cm/min.
2. Maintain the same linear flow rate and calculate the new volumetric flow rate according to the cross-sectional area of the specific column to be used

$$\text{Linear flow rate} = \frac{\text{Volumetric flow rate}}{\text{Column cross-sectional area}}$$

Volumes

Volumes (buffers, gradients, etc.) quoted in this instruction are for an XK 16/20 column that has a bed volume of 20 ml (bed height × cross-sectional area). To convert volumes for columns of different dimensions, increase or decrease in proportion to the new column bed volume.

$$\text{New volume} = \text{Old volume} \times \frac{\text{New bed volume}}{\text{Old bed volume}}$$

Appendix B

Table 1. Media characteristics

Degree of substitution	Ligand density per ml medium
Phenyl Sepharose 6 Fast Flow low sub	25 µmol
Phenyl Sepharose 6 Fast Flow high sub	40 µmol
Butyl-S Sepharose 6 Fast Flow	10 µmol
Butyl Sepharose 4 Fast Flow	40 µmol
Octyl Sepharose 4 Fast Flow	5 µmol
Mean particle size	90 µm
Bead size range	45–165 µm
Bead structure	Spherical highly cross-linked agarose, 6 or 4%
Max volumetric flow rate (XK 16/20)	
Sepharose 6 Fast Flow	15 ml/min
Sepharose 4 Fast Flow	8 ml/min
Max linear flow rate	
Sepharose 6 Fast Flow	450 cm/hour
Sepharose 4 Fast Flow	240 cm/hour

Max operating pressure	
Sepharose 6 Fast Flow	0.15 MPa (1.5 bar)
Sepharose 4 Fast Flow	0.10 MPa (1.0 bar)
pH stability*	
Long term	3–13
Short term	2–14
Chemical stability, 40 °C for 7 days in:	1 M NaOH, 70% ethanol, 30% iso-propanol, 6 M guanidine hydrochloride, 8 M urea
Autoclavable	121 °C for 20 min in H_2O

* pH stability, long term refers to the pH interval where the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance.

pH stability, short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures. All ranges given are estimates based on our knowledge and experience.

Ordering Information

Description	Pack size	Code No.
Phenyl Sepharose 6 Fast Flow low sub	25 ml	17-0965-10
Phenyl Sepharose 6 Fast Flow high sub	25 ml	17-0973-10
Butyl-S Sepharose 6 Fast Flow	25 ml	17-0978-10
Butyl Sepharose 4 Fast Flow	25 ml	17-0980-10
Octyl Sepharose 4 Fast Flow	25 ml	17-0946-10

Related Products		
XK 16/20 column		18-8773-01
Valve LV-3		19-0016-01
Valve LV-4		19-0017-01
HiLoad Pump P-50		19-1992-01
HiTrap Desalting column	5 × 5 ml	17-1408-01
PD-10 Desalting columns	30	17-0851-01
Hydrophobic Interaction & Reversed Phase Chromatography:		
Principles and Methods		11-0012-69
Column packing – The Movie		18-1165-33

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