GE Healthcare

Instructions 71-6028-00 AH **High Performance Columns**

HiLoad 16/10 Phenyl Sepharose High Performance

HiLoad 26/10 Phenyl Sepharose High Performance

Introduction

HiLoad™ 16/10 and 26/10 Phenyl Sepharose™ High Performance are prepacked XK columns designed for preparative hydrophobic interaction chromatography. Phenyl Sepharose HP is based on a matrix of highly cross-linked agarose beads, which have been derivatized with phenyl groups. These are attached to the agarose matrix via uncharged, chemically stable ether linkages. The result is a truly hydrophobic medium with no ionic properties.

High mechanical strength of the matrix allows for very high flow rates, together with superior resolution.

Capacities vary according to the protein applied and its concentration. Loading capacity will also vary depending on the salt concentration, pH, and temperature at which the protein is applied.

Column data

Matrix	6% highly cross-linked spherical agarose
Mean particle size	34 µm (24–44 µm)
Ligand	Phenyl
Ligand concentration	25 µmol/ml medium
Binding capacity	45 mg α-chymotrypsinogen/ml medium
Column volume[1]	20–22 ml (XK 16/10) 53–58 ml (XK 26/10)
Theoretical plates	>12 000 m^{-1}
Recommended linear flow rate	Up to 150 cm/h
Maximum flow rate	150 cm/h (5 ml/min for XK 16/10, or 13 ml/min for XK 26/10)
Maximum pressure over the packed bed during opertation[2]	0.3 MPa, 3 bar, 42 psi
HiLoad column hardware pressure limit[2]	0.5 MPa, 5 bar, 73 psi
pH stability	
long term and working range	3–13
short term	2–14
Storage	20% ethanol

1 The medium surface is not directly visible at the bottom piece. Therefore, when calculating the total column volume, measure the height from the lowest part of the bottom piece to the medium/adaptor surface. For HiLoad 16/10 deduct 30 mm, and for HiLoad 26/10 deduct 36 mm.

2 In exceptional circumstances, HiLoad columns can withstand pressures of up to 0.4 MPa, 4 bar, 56 psi for short periods.

Many chromatography systems are equipped with pressure gauges to measure pressure at a particular point in the system, usually just after the pumps. The pressure measured here is the sum of pre-column pressure, pressure drop over the medium bed, and post-column pressure. This is always higher than the pressure drop over the bed alone. Keeping the pressure drop over the bed below 3 bar is recommended. Setting the upper limit of the pressure gauge to 3 bar will ensure that the pump shuts down before the medium is over-pressured. If necessary, post-column pressure of up to 2 bar can be added to the limit without exceeding the column hardware limit. To determine post-column pressure, proceed as follows:

To avoid breaking the column, post-column pressure must never exceed 2 bar.

1. Connect a piece of tubing in place of the column.
2. Run the pump at the maximum flow you intend to use for chromatography.
   Use a buffer with the same viscosity as you intend to use for chromatography.
   Note the backpressure as total pressure.
3. Disconnect the tubing and run at the same flow rate used in step 2.
   Note this backpressure as pre-column pressure.
4. Calculate post-column pressure as total pressure minus pre-column pressure.

If post-column pressure is higher than 2 bar, take steps to reduce it (shorten tubing, clear clogged tubing, or change flow restrictors), and perform steps 1–4 again until the post-column pressure is below 2 bar. Note post-column pressure when this has reached a satisfactory level, add 3 bar to this value, and set this as the upper pressure limit on the chromatography system.

First-time use

Connecting the column

1. Before connecting the column to a chromatography system, start the pump to remove all air from the system, particularly in tubing and valves.
2. Stop the pump.
3. Mount the column vertically, remove the domed nut, and connect the inlet tubing to the system "drop-to-drop".
4. Remove the transport syringe and connect the column outlet tubing to, for example, a monitor cell. Save the transport syringe for use when storing the column. The column is now ready for use.

Equilibration of the column

Ensure an appropriate pressure limit has been set.

Equilibrate the column for first-time use, or after long-term storage as follows:

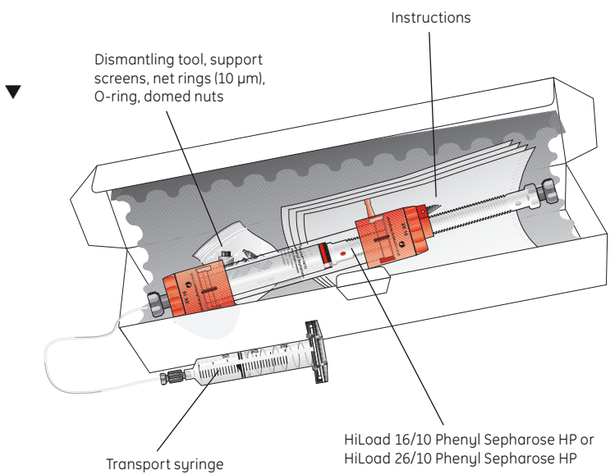
1. One column volume of low ionic strength buffer, e.g. 100 mM sodium phosphate, pH 7 at 50 cm/h, (1.6 ml/min for XK 16/10, or 4.3 ml/min for XK 26/10).
2. Two column volumes of start buffer (e.g. 1.7 M ammonium sulphate, 100 mM sodium phosphate, pH 7) at 100 cm/h, (3.2 ml/min for XK 16/10, or 8.5 ml/min for XK 26/10).

When using high salt buffers, always use highest quality of salt to avoid UV baseline drift.

Try these conditions first

Flow rate:	100 cm/h, (3.2 ml/min for XK 16/10, or 8.5 ml/min for XK 26/10)
Gradient volume:	10–20 column volumes
Sample load:	Up to 600 mg for XK 16/10 Up to 1500 mg for XK 26/10
Start buffer:	1–2 M $(NH_4)_2SO_4$, 100 mM Na_2HPO_4, pH 7.0
Elution buffer:	100 mM Na_2HPO_4, pH 7.0

Read the section "Optimization" for information on how to optimize a separation.



Equilibration before a new run

Regenerate the column after each run by rinsing with five column volumes of distilled water at a flow rate of 90 cm/h (3 ml/min for XK 16/10, or 8.5 ml/min for XK 26/10) at room temperature. Re-equilibrate the column with at least five column volumes of start buffer at a flow rate of 90 cm/h (3 ml/min for XK 16/10, or 8.5 ml/min for XK 26/10) at room temperature until the UV base-line, pH, and conductivity values have stabilized.

Buffers and solvent resistance

De-gas and filter all solutions through 0.22 µm filter to increase column lifetime. Buffers and solvents with increased viscosity will affect the backpressure and flow rate.

Daily use

All commonly used aqueous buffers, pH 3–13
Urea, up to 8 M
Acetonitrile, up to 30% in aqueous buffers

Cleaning

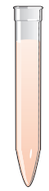
Acetonitrile, up to 30%
Sodium hydroxide, up to 1 M
Ethanol, up to 70%
Acetic acid, up to 1 M
Isopropanol, up to 30%
Guanidine hydrochloride, up to 6 M
Urea, up to 8 M

Avoid

Oxidizing agents
Unfiltered solutions

Sample recommendations

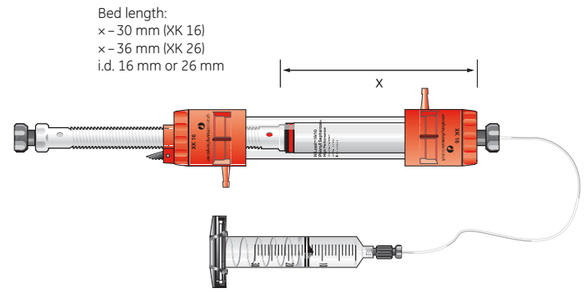
Recommended sample load	< 600 mg for XK 16/10 < 1500 mg for XK 26/10
Preparation	Dissolve the sample in start buffer, filter through 0.22 µm filter, or centrifuge at 10 000 × g for 10 min.



Delivery and storage

The column is supplied in 20% ethanol. If the column is to be stored more than two days after use, wash the column with four column volumes of distilled water, and then equilibrate with four column volumes of 20% ethanol.

To avoid air bubble formation in the column, use the transport syringe. Connect the transport syringe to the transport tubing at the column outlet. Start the pump, and fill the syringe to approximately 50% of the total syringe volume.



Choice of buffer

In hydrophobic interaction chromatography (HIC), biomolecules are separated according to their degree of surface hydrophobicity, which differs from protein to protein. This difference can therefore be utilized for separations. Use of HIC can also be advantageous following ammonium sulphate precipitation, since samples are normally applied in high salt concentration.

Elution of hydrophobic biomolecules bound to the medium requires a reduction in the degree of hydrophobic interaction. The most common method for elution is by applying a linear gradient of decreasing salt concentration. In certain separations, however, an isocratic step within the gradient may improve the separation between two sample components. Elution can also be performed by changing to a buffer containing a salt with lower salting-out capacity, see Table 1. Finally, if the proteins are not eluted with water, consider using chaotropic ions, detergents or mild organic solvents.

Table 1. Hydrophobic interaction can be increased or decreased using salts. 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+}	

Decreasing salting-out effect →

HiLoad

Optimization

Perform a first run as described in the section "Try these conditions first". If the obtained results are unsatisfactory, consider the following:

Action	Effect
Change salt concentration	Higher salt concentration increases retention times Lower salt concentration decreases retention times
Increase gradient volume	May improve resolution
Decrease flow rate	Improves resolution
Increase temperature	Increases retention times
Change pH	Changes selectivity
Increase salt concentration	May increase capacity

Cleaning-in-place (CIP)

Regular cleaning

Pass one-half to one column volume of 1 M NaOH through the column at a flow rate of 50 cm/h (1.6 ml/min for XK 16/10, or 4.3 ml/min for XK 26/10) to remove most proteins non-specifically adsorbed to the medium.

After cleaning, immediately equilibrate the column with two column volumes of water followed by two column volumes of start buffer. Further equilibration is necessary if your buffer contains detergent. Wait until the UV baseline has stabilized before applying next sample.

More rigorous cleaning

Reverse the flow rate and wash the column at a flow rate of 50 cm/h (1.6 ml/min for XK 16/10, or 4.3 ml/min for XK 26/10) at room temperature with the following solutions:

1. Two column volumes of water and four column volumes of 1 M NaOH (removal of precipitated proteins), followed by four column volumes of water.

Do not store the column in 1 M NaOH.

2. Two column volumes of water and two column volumes of 30% isopropanol or 30% acetonitrile (removal of hydrophobic proteins, lipoproteins, or lipids), followed by two column volumes of water.

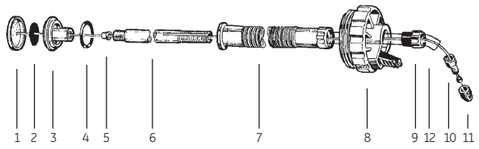
   If a new purification is to be run, equilibrate the column after cleaning with at least five column volumes of start buffer.

Changing the adaptor net ring

If, after following the cleaning procedures, backpressure remains too high, change the net ring in the column adaptor.

Follow the instructions below thoroughly since column efficiency is easily impaired if careless measures are taken. Use distilled water as eluent.

1. Close the outlet tubing of the column with a domed nut, and mark the level of the medium surface on the glass tube using a coloured pen.
2. Slacken the adaptor O-ring slightly by turning the black adjusting knob counter-clockwise. Note: It should still seal against the glass wall but allow the adaptor to slide.
   Unscrew the top piece from the column.
3. Connect the adaptor to the pump and start pumping at a flow rate of 30 cm/h (1 ml for XK 16/10, or 3 ml/min for XK 26/10). Let the flow push the adaptor upwards.
4. When the glass tube is completely full, take out the adaptor and stop the pump. The glass tube should remain completely full of liquid while changing the adaptor net ring.
5. To avoid getting air bubbles under the net, injection of 20% ethanol through the adaptor by a syringe is recommended.
6. Insert the adaptor into the column at an angle of 45 °, avoiding air bubbles. Slide the plunger 1–2 cm down and tighten the O-ring. Remove excess liquid completely before screwing the top piece onto the column end piece.
7. Remove the syringe and slide down the adaptor until it touches the medium surface. Tighten the O-ring and re-connect the inlet tubing to the system, avoiding air bubbles.
8. Remove the domed nut and start the pump. Increase the flow rate until the medium surface is approximately 3 mm above the pen mark. Stop the pump and close the outlet tubing with the domed nut again. Note: Step 8 requires a pump with high flow rate capacity up to a pressure of 0.5 MPa, 5 bar.
9. Disconnect the inlet tubing and slacken the adaptor O-ring slightly by turning the adjusting knob counter-clockwise. Press the adaptor downward to the pen mark. Tighten the O-ring. Note: Do not to loosen the O-ring too much as this will result in medium passing the O-ring.
10. Re-connect the inlet tubing, and avoid introducing air into the system.



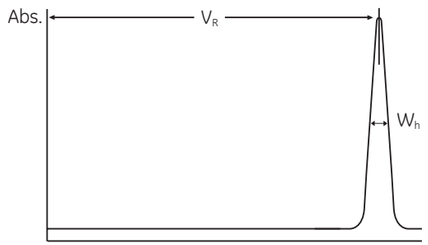
1. Net ring
2. Support screen
3. Plunger
4. O-ring
5. Sealing plug
6. Inner adjusting
7. Outer adjusting tube
8. Top piece
9. Adjusting knob
10. Capillary tubing
11. Domed nut
12. Protection tubing

Troubleshooting

Symptom	Remedy
Increased backpressure over the column	Clean the column according to the section "Cleaning-in-place (CIP)".
Loss of resolution and/or decreased sample recovery	Clean the column according to the section "Cleaning-in-place (CIP)".
Air in the column	Reverse flow direction and pump five column volumes of well de-gassed water through the column at a flow rate of 150 cm/h (5 ml/min for XK 16/10, or 13 ml/min for XK 26/10).
Space between adaptor and medium	Close the outlet tubing with the domed nut and then disconnect the inlet tubing. Slacken the O-ring slightly by turning the adjusting knob counter-clockwise and push or screw the adaptor down until it touches the and medium surface. Tighten the O-ring. To maintain an airtight system, reconnect the inlet tubing immediately.

Column efficiency test

GE Healthcare packs columns to the highest standards, and each column is thoroughly tested, with regard to the number of theoretical plates per metre (H^{-1}), see Figure 1.



Sample	Acetone 20 mg/ml
Sample volume	200 µl (XK 16/10) and 500 µl (XK 26/10)
Eluent	Distilled water
Linear flow rate	60 cm/h

Fig 1. Column efficiency test

Column efficiency is calculated using the equation:

$$H^{-1} = 5.54 (V_R/w_h)^2 \, 1000/L$$

where,

V_R = peak retention (elution) volume
w_h = peak width at half peak height
L = bed height (mm)

V_R and w_h in same units

Ordering information

Product	No. per pack	Code No.
HiLoad 16/10 Phenyl Sepharose High Performance	1 (20 ml)	17-1085-01
HiLoad 26/10 Phenyl Sepharose High Performance	1 (53 ml)	17-1086-01

Companion products

Product	No. per pack	Code No.
HiTrap™ HIC Selection Kit, 6 different media	6 × 1 ml	11-0034-53
HiTrap Phenyl HP	5 × 1 ml	17-1351-01
HiTrap Phenyl HP	5 × 5 ml	17-5195-01

Accessories

Product	No. supplied	Code No.
Dismantling tool*	1	–
Support screen XK 16*	2	19-0651-01
Support screen XK 26*	2	18-9377-01
Net ring (10 µm) XK 16*	2	18-8761-01
Net ring (10 µm) XK 26*	2	18-8760-01
Transport syringe*	1	18-1017-61
O-ring XK 16*	1	19-0163-01
O-ring XK 26*	1	19-0688-01
Domed nut*	2	18-2450-01
Union M6 female/1/16" male (for connecton to ÄKTA™ systems)	2	18-3858-01

* included in HiLoad 16/10 and/or 26/10 Phenyl Sepharose HP

Related printed literature

Product	No. per pack	Code No.
Handbook, Hydrophobic Interaction and Reversed Phase Chromatography, Principles and Methods	1	11-0012-69

Further information

For more information, please check
www.gehealthcare.com/protein-purification
or refer to the different handbooks available from GE Healthcare.

www.gehealthcare.com

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

GE Healthcare Europe GmbH
Munzinger Strasse 5, D-79111 Freiburg, Germany

GE Healthcare UK Ltd
Amersham Place, Little Chalfont, Buckinghamshire, HP7 9NA, UK

GE Healthcare Bio-Sciences Corp
800 Centennial Avenue, P.O. Box 1327, Piscataway, NJ 08855-1327, USA

GE Healthcare Bio-Sciences KK
Sanken Bldg, 3-25-1, Hyakunincho, Shinjuku-ku, Tokyo 169-0073, Japan

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