cytiva

Heparin **Sepharose** 6 Fast Flow

Affinity chromatography

Instructions for Use

Heparin is a naturally occurring glycosaminoglycan which serves as an effective affinity binding and ion exchange ligand for a wide range of biomolecules, including coagulation factors and other plasma proteins, lipoproteins, protein synthesis factors, enzymes that act on nucleic acids and steroid receptors.

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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 Resin characteristics

Heparin Sepharose™ 6 Fast Flow belongs to the BioProcess™ range of 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.

Heparin is a glycosaminoglycan consisting of alternating hexuronic (D-glucuronic or L-iduronic) and D-glucosamine residues. The polymer is heavily sulphated, carrying sulphamino (N-sulphate) groups at C-2 of the glucosamine units as well as ester sulphate (O-sulphate) groups in various positions (see *Figure 1, on page 4*). The heparin ligand used in Heparin Sepharose 6 Fast Flow is isolated from porcine intestinal mucosa, and has a molecular weight distribution over the range 5 000 to 30 000.

The base matrix, Sepharose 6 Fast Flow, consists of cross-linked 6% agarose particles. It provides the resin with high physical stability and excellent flow characteristics. Heparin is linked to the Sepharose matrix by reductive amination which is a very stable binding, even in alkaline conditions. The stability of this resin is limited only by the heparin itself. The essential characteristics of Heparin Sepharose 6 Fast Flow are summarized in *Table 1, on page 5*.

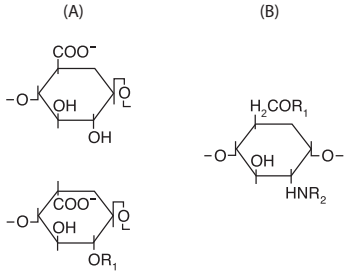


Fig 1. Heparin consists of alternating hexuronic acid (A) and D-glucosamine residues (B). The hexuronic acid can be either D-glucuronic acid (top) or its C-5 epimer, L-iduronic acid. R_1 = –H or $–SO_3^-$ or $–COCH_3$.

Table 1. Characteristics of Heparin Sepharose 6 Fast Flow

Matrix	Cross-linked agarose, 6%, spherical
Particle size, d_{50V} [1]	~ 90 µm
Ligand	Porcine heparin
Ligand concentration	~ 2 mg heparin/mL resin
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]	4 to 12
pH stability, CIP[5]	4 to 13
Chemical stability	Stable to commonly used aqueous buffers, 0.05 sodium acetate pH 4.0, 20% ethanol, 4 M NaCl, 8 M urea, 6 M guanidine hydrochloride, 0.1 M NaOH
Autoclavability	30 min at 121°C in in 0.02 M NaH_2PO_4 pH 7, 5 cycles
Delivery conditions	20% ethanol containing 0.05 M sodium acetate
Storage	20% ethanol containing 0.05 M sodium acetate, 4°C to 30°C

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.

2 Packing columns

Heparin Sepharose 6 Fast Flow is supplied preswollen. Decant the 20% ethanol solution and replace it with binding buffer before use.

Recommended lab-scale columns

Column	Bed volume (mL)	Bed height (cm)
Tricorn™ 5/20 (5 mm i.d.)	0.5	2.6
Tricorn 5/50 (5 mm i.d.)	0.2 to 1.1	0.8 to 5.6
Tricorn 10/20 (10 mm i.d.)	2.1	2.6
Tricorn 10/50 (10 mm i.d.)	4.4	5.6
Tricorn 10/100 (10 mm i.d.)	3.6 to 8.4	4.6 to 10.6
Tricorn 10/300 (10 mm i.d.)	19.4 to 24.1	24.6 to 30.6
XK 16/20 (16 mm i.d.)	30	15.5
XK 16/40 (16 mm i.d.)	16 to 70	8 to 35
XK 26/20 (26 mm i.d.)	66	12.5
XK 26/40 (26 mm i.d.)	45 to 186	8.5 to 35
XK 50/20 (50 mm i.d.)	274	14
XK 50/30 (50 mm i.d.)	0 to 559	0 to 28
HiScale™ 16/20	0 to 40	20
HiScale 16/40	16 to 80	8 to 40
HiScale 26/20	0 to 106	20
HiScale 26/40	69 to 212	13 to 40
HiScale 50/20	0 to 393	20
HiScale 50/40	274 to 785	14 to 40

Recommended large-scale columns

Column	Bed volume (L)	Bed height (cm)
AxiChrom™ (i.d. 50 to 200 mm)	Up to 16.7	Up to 50
AxiChrom (i.d. 300 to 1600 mm)	Up to 1005	Up to 50
BPG (i.d. 100 mm to 450 mm)	2.4 to 131	Up to 83
INdEX (i.d. 70–200 mm)	Up to 24.8	Up to 79
CHROMAFLOW™ variable bed columns. Inner diameters from 280–2000 mm.		

Packing lab-scale columns

Step	Action
1	Assemble the column (and packing reservoir if necessary).
2	Remove air from the column dead spaces by flushing the end-piece and adapter with packing buffer. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with packing buffer.
3	Resuspend the resin by shaking the container (avoid stirring the sedimented resin). Mix the packing buffer with the resin to form 50% to 70% slurry (sedimented bed volume/slurry volume = 0.5 to 0.7).
4	Pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
5	If using a packing reservoir, immediately fill the remainder of the column and reservoir with packing buffer. Attach the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.

Step	Action
6	Open the bottom outlet of the column and set the pump to run at the desired flow rate. Ideally, Sepharose 6 Fast Flow based resins are packed at a constant pressure of approximately 1.5 bar (0.15 MPa). If the packing equipment does not include a pressure gauge, use a packing flow velocity of approximately 500 cm/h (10 cm bed height, 25°C, low viscosity buffer). If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate the pump can deliver. This should also give a reasonably well-packed bed. ***Note:*** *Do not exceed 75% of the packing flow velocity in subsequent chromatographic procedures using the same pump.*
7	When the bed has stabilized, close the bottom outlet and stop the pump.
8	If using a packing reservoir, disconnect the reservoir and fit the adapter to the column.
9	With the adapter inlet disconnected, push down the adapter approximately 2 mm into the bed, allowing the packing solution to flush the adapter inlet.
10	Connect the pump, open the bottom outlet and continue packing. The bed will be further compressed at this point and a space will be formed between the bed surface and the adapter.

Step	Action
11	Close the bottom outlet. Disconnect the column inlet and lower the adapter approximately 2 mm into the bed. Connect the pump. The column is now ready to use.

Packing large-scale columns

General packing recommendations

Columns can be packed in different ways depending on the type of column and equipment used. Always read and follow the relevant column instruction manual carefully.

Sepharose 6 Fast Flow based resins are easy to pack since their rigidity allows the use of high flow rates (see *Figure 2, on page 10*). Three suitable types of packing method are given in this instruction:

- CHROMAFLOW packing.
- Pressure packing (for columns with adapters).
- Hydraulic pressure packing.

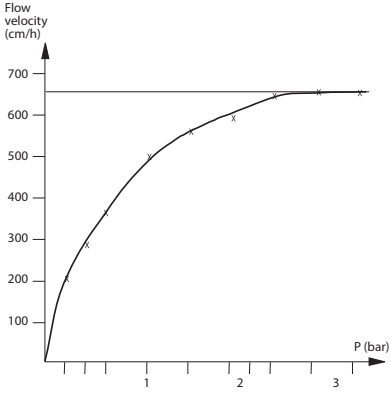


Fig 2. Pressure/flow curve for Sepharose 6 Fast Flow, bed height 10 cm.

Column packing has a major effect on the quality of separation. Therefore, it is important to pack and test the column according to the following recommendations.

Begin the packing procedure by determining the optimal packing flow velocity. Guidelines are given below for determining the optimal packing velocities rates for columns with adapters and fixed bed heights.

Determining optimal packing flow velocity

The optimal packing flow velocity is dependent on column size and type, bed height, packing solution and temperature. The optimal packing flow velocity must be determined empirically for each individual system.

Use the following procedure to determine the optimal packing flow velocity:

Step	Action
1	Calculate the exact amount of resin needed for the slurry (this is especially important for columns with fixed bed heights). The quantity of resin required is approximately 1.15 L sedimented resin per liter packed bed.
2	Prepare the column exactly as for column packing.
3	Run the column at a low flow rate (e.g., 30% of the expected maximum flow velocity) and record the flow velocity and back pressure when the bed is packed and the pressure has stabilized.
4	Increase the flow velocity in small steps and record the flow velocity and pressure at each step after the pressure has stabilized.
5	Continue recording flow and pressure until the maximum flow velocity has been reached, i.e., when the flow rate levels off to a plateau indicating bed compression or when the pressure reaches the pressure specification of the column used.
6	Plot pressure against flow as indicated in *Figure 2, on page 10*. The optimal packing flow velocity/pressure is 70% to 100% of the maximum flow velocity/ pressure.

The operational flow rate/pressure must be < 70% of the packing flow velocity/pressure.

Packing CHROMAFLOW columns

Procedure

Prepare the column for packing as described in the User Manual.

Packing from the top

Step	Action
1	Set the top nozzle to the pack position (mid-position).
2	Fully retract the bottom nozzle (run position).
3	Make sure that the top mobile phase is closed.
4	Open the bottom mobile phase.
5	Open Inlet C and start the packing pump. Adjust the flow to achieve the required packing conditions for the selected resin. Monitor column pressure and the outlet flow velocity in order to record column packing parameters. (Remember to stir the resin slurry during packing to prevent it from settling.)
6	Continue pumping until the column is fully packed and the pump stalls due to build-up of resin in the pipelines. Turn off the packing pump.
7	Fully retract the top nozzle to its run position. Close Outlet (C). Open Inlet (B) from the water/buffer tank and open Outlet (D). The pump must now be restarted to rinse the top slurry lines. (If the nozzle is full of liquid when in the packing position, make sure that the waste slurry outlet is open before retracting the nozzle.)

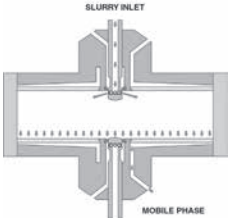
Step	Action
8	To clean-in-place, exchange the buffer tank for wash/ buffer tank containing cleaning solution.

Packing from below

To pack from the bottom, carry out the same procedure for the connections and flow path via the bottom nozzle. The column is now ready to equilibrate and test.

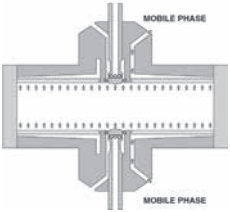
Note: *It is possible to use a different packing method with a predetermined amount of resin. In this case the complete amount of resin is packed into the column causing compression of the bed. When all resin has entered the column the pump is stopped, the top nozzle is retracted, the bottom mobile phase valve closed and the resin is allowed to decompress within the column.*

Table 2. Principle of operation – Chromaflow columns



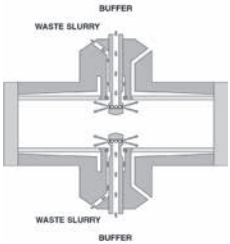
Packing position

The top nozzle is extended part of the way (mid position) into the column. The bottom nozzle is fully retracted. Slurry enters the column via the top nozzle and excess liquid exits via the bottom mobile phase outlet. After packing, the slurry lines are isolated from the mobile phase and can be cleaned independently from the rest of the column.



Running position

The bottom and top nozzles are retracted. Mobile phase enters the column directly into an annulus, immediately behind the bed support. The annulus is cut through at an angle to ensure that linear flow rate is kept constant during distribution of the mobile phase across the bed.



Unpacking position

In this position, both bottom and top nozzles are fully extended into the column, thereby exposing a third passage through which medium leaves the column.

Cleaning solution can be pumped through the nozzles and sprayed into the column. In this way the column is easily and effectively cleaned without exposing the interior or the resin to the environment, or without dismantling the column.

Pressure packing (BPG Columns)

BPG Columns are supplied with a movable adapter. They are packed by conventional pressure packing by pumping the packing solution through the bed at a constant flow rate (or back pressure).

Step	Action
1	Pour some water (or packing buffer) into the column. Make sure that there is no air trapped under the bottom bed support. Leave about 2 cm of liquid in the column.
2	Mix the packing buffer with the resin to form a 50% to 70% slurry (sedimented bed volume/slurry volume = 0.5 to 0.7). Pour the slurry into the column. Insert the adapter and lower it to the surface of the slurry, making sure no air is trapped under the adapter. Secure the adapter in place.
3	Seal the adapter O-ring and lower the adapter a little into the slurry, enough to fill the adapter inlet with packing solution.
4	Connect a pump and a pressure meter and start packing at the predetermined packing flow velocity (or pressure). Keep the flow velocity (or pressure) constant during packing and check the pressure at the column inlet. Never exceed the pressure limit for the column or the resin.
5	When the bed has stabilized, close the bottom valve and stop the pump. The bed starts rising in the column. Loosen the O-ring and lower the adapter to 0.5 to 1.0 cm above the bed surface.
6	Seal the O-ring, start the pump and continue packing. Repeat steps 5 and 6 until there is a maximum of 1 cm between bed surface and adapter when the bed has stabilized. Mark the bed height on the column tube.

Step	Action
7	Close the bottom valve, stop the pump, disconnect the column inlet and, without loosening the adapter O-ring, push the adapter down to approximately 3 mm below the mark on the column tube. The packing solution will flush the adapter inlet. Remove any trapped air by pumping liquid from the bottom (after the inlet tubing and the bottom valve have been properly filled).

Hydraulic packing (INdEX Columns)

INdEX Columns are supplied with a hydraulic function which allows an extremely simple, rapid and reproducible packing procedure. The resin is packed at the same time as the adapter is lowered into position at the correct pressure.

The adapter is pushed down by a constant hydraulic pressure, forcing water or packing buffer through the slurry and compressing it so that a packed bed is gradually built up. The hydraulic pressure can be generated using a pump and a pressure relief valve.

When the adapter reaches the surface of the settled resin, it continues downwards under hydraulic pressure compressing the resin. The extent to which the resin is compressed depends upon the pressure from the adapter and the elasticity of the resin. The quantity of resin required when packing Sepharose 6 Fast Flow based resins by hydraulic pressure is approximately 1.2 liters sedimented resin per liter packed bed.

Step	Action
1	Pour some water (or packing solution) into the column. Make sure that there is no air trapped under the bottom bed support. Leave about 2 cm of liquid in the column.
2	Pour the 75% slurry into the column. Fill the column with packing solution up to the top of the glass tube and mix the slurry. Allow the resin to sediment to just below the bevel of the glass tube (G), see *Figure 3, on page 19*.
3	Put the adapter in a resting position against the bevel of the glass tube. Avoid trapping air bubbles under the adapter by slightly tilting the adapter while mounting.
4	Lower the lid and secure it in place.
5	Connect a pump to the inlet of the hydraulic chamber (A), with a manometer and a pressure relief valve in-line between the pump and the hydraulic chamber. The manometer must be placed after the valve in the direction of the flow.
6	Open the hydraulic inlet (A), and the hydraulic outlet (C). Start the pump and flush the hydraulic chamber (E) free of air and any residual resin.
7	Close (C) and open the elution inlet/outlet (B) to allow trapped air in the adapter bed support to escape.

Step	Action
8	Close (B) and open the elution inlet/outlet (D) to start the packing, applying a predefined constant hydraulic packing pressure. When packing Sepharose 6 Fast Flow based resin in an INdEX column to a bed height of 15 cm, the recommended hydraulic packing pressure is 1.5 bar for INdEX 100 and 0.8 bar for INdEX 200.
9	When the adapter has reached the surface of the settled bed, continue to run the pump until the adapter has been lowered 5 mm into the packed bed.
10	Close (A) and (D) and stop the pump.
11	Run the column with upward flow for a few minutes to remove residual air trapped in the adapter. The column is now ready for use.
12	To unpack the column, connect the outlet from the pump to (B) and open (C) while keeping (D) closed. This will cause the adapter to rise from the bed surface.

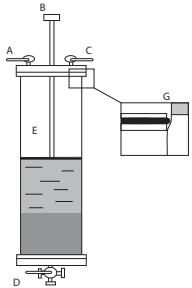


Fig 3. Schematic representation of INdEX column with a 4-port (2-way) valve mounted at the bottom outlet.

3 Evaluation of packing

To check the quality of the packing and to monitor this during the working life of the column, column efficiency should be tested directly after packing, prior to re-use, and when separation performance is seen to deteriorate.

The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate, HETP, and the asymmetry factor, As. These values are easily determined by applying a sample such as 1% (v/v) acetone solution to the column. Sodium chloride can also be used as a test substance. Use a concentration of 2.0 M NaCl in water with 0.5 M NaCl in water as eluent.

The calculated plate number will vary depending on the test conditions and it is therefore to be used as a reference value only. It is also important that conditions and equipment are kept constant so that results are comparable. Changes in solute, solvent, eluent, sample volume, flow rate, liquid pathway, temperature, etc., will influence the results.

For optimal results, the sample volume must be at max. 2.5% of the column volume and the flow velocity between 15 and 30 cm/h.

If an acceptance limit is defined in relation to column performance, the column plate number can be used as part of the acceptance criteria for column use.

Method for measuring HETP and A_s

To avoid dilution of the sample, apply it as close to the column inlet as possible.

Conditions	
Sample volume:	2.5%
Sample concentration:	1.0%
Flow velocity	15 cm/h
UV:	280 nm, 1 cm, 0.1 AU

Calculate HETP and A_s from the UV curve (or conductivity curve if NaCl is used as sample) as follows:

$$HETP = L/N$$

$$N = 5.54(V_e/W_h)2$$

where

L = Bed height (cm)

N = number of theoretical plates

V_e = Peak elution distance

W_h = Peak width at half peak height

V_e and W_h are in the same units.

To facilitate comparison of column performance the concept of reduced plate height is often used.

The reduced plate height is calculated by: HETP/d

where d is the diameter of the particle. As a guideline, a value of < 3 is normally acceptable.

The peak should be symmetrical, and the asymmetry factor as close as possible to 1 (values between 0.8–1.5 are usually acceptable). A change in the shape of the peak is usually the first indication of bed deterioration due to use.

Peak asymmetry factor calculation: $A_S = b/a$

where

a = 1st half peak width at 10% of peak height.

b = 2nd half peak width at 10% of peak height.

The figure below shows a UV trace for acetone in a typical test chromatogram in which the HETP and A_S values are calculated.

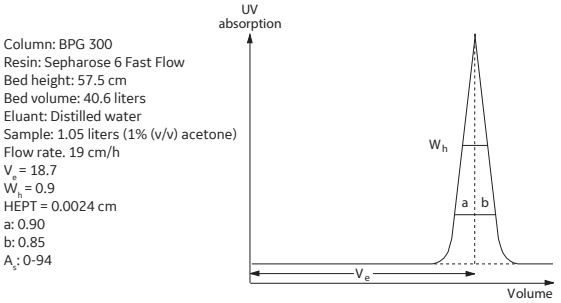


Fig 4. UV trace for acetone in a typical test chromatogram showing the HETP and A_S value calculations

4 Operation

Binding

Immobilized heparin has two main modes of interaction with proteins. Heparin Sepharose 6 Fast Flow can be used as an affinity chromatography resin; e.g., for purification of coagulation factors or nucleic binding proteins. Different substances can differ in their affinity for Heparin Sepharose 6 Fast Flow. The binding capacity of a particular protein will depend upon parameters such as buffer composition, pH, flow rate and temperature. Heparin Sepharose 6 Fast Flow might also function as a cation exchanger due to the negatively charged sulphate and carboxylate groups on the immobilized heparin ligand.

A commonly used binding buffer for the purification of plasma proteins is 10 to 20 mM sodium citrate buffer, pH 7.4. Since the heparin ligand acts as an affinity ligand in these cases, it might be advisable to include low concentration of NaCl in order to eliminate unspecific ionic interactions.

In other applications 10 mM sodium phospate, pH 7.0 or 20 mM Tris-HCl, pH 8.0 are often recommended as binding buffers.

Elution

Elution is commonly performed by increasing the ionic strength of the buffer. Elution using a continuous linear gradient or step gradient with NaCl, KCl or $(NH_4)SO_4$ up to 1.5 to 2 M is most frequently used.

5 Maintenance

For best performance from Heparin Sepharose 6 Fast Flow over a long working life, follow the procedures described below.

Cleaning-In-Place

Cleaning-in-place (CIP) is a cleaning procedure that removes contaminants that might remain in the packed column after regeneration. Regular CIP also prevents the build-up of these contaminants in the resin bed and helps to maintain the capacity, flow properties and general performance of Heparin Sepharose 6 Fast Flow.

Heparin Sepharose 6 Fast Flow withstands exposure to 0.1 M NaOH for long periods with no significant loss of binding capacity for antithrombin III. When contamination is severe, 0.5 M NaOH can be used, however, a decrease in functionality will be seen over time (*Figure 5, on page 24*). Other reagents in which the resin is stable include 8 M urea and 6 M guanidine hydrochloride.

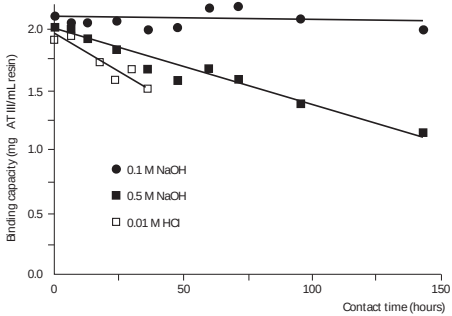


Fig 5. Functional stability of Heparin Sepharose Fast Flow was tested in three different CIP studies. CIP with 0.5 M NaOH can be used effectively over shorter periods.

Recommended cleaning-in-place procedures are summarized in the table below. Always wash the column thoroughly with equilibration buffer after cleaning-in-place.

For removal of	Wash with	Column volumes	Contact time
Ionically bound proteins	2 M NaCl	0.5	10-15 min
Precipitated or denatured proteins	0.1 M NaOH or 6 M guanidine-HCl or 8 M urea	4 approx. 2 approx. 2	1-2 h 30 min-1 h 30 min-1 h
Hydrophobically bound proteins	nonionic detergent	4	1-2 h

Sanitization

For inactivation of microbial contaminants, equilibrate the column with buffer consisting of 0.1 M NaOH and 20% ethanol and allow to stand for 1 h.

Wash the column thoroughly with running buffer after sanitization.

Sterilization

Autoclaving is the only recommended sterilization treatment. Equilibrate the resin with 0.02 M NaH_2PO_4. Dismantle the column and autoclave the resin for 30 min at 121°C. Sterilize the column parts according to the instructions in the column manual. Re-assemble the column, then pack and test it as recommended.

Storage

Store Heparin Sepharose 6 Fast Flow at 4°C to 30°C in 0.05 M sodium acetate containing 20% ethanol as preservative.

6 Ordering information

Product	Pack size	Product code
Heparin Sepharose 6 Fast Flow	50 mL	17099801
Heparin Sepharose 6 Fast Flow	250 mL	17099825
Heparin Sepharose 6 Fast Flow	1 L	17099803
Heparin Sepharose 6 Fast Flow	5 L	17099804

Related products	Pack size	Product code
HiScale 16/20	1	28964441
HiScale 16/40	1	28964424
HiScale 26/20	1	28964514
HiScale 26/40	1	28964513
HiScale 50/20	1	28964445
HiScale 50/40	1	28964444
Tricorn 5/20 column (5 mm i.d.)	1	28406408
Tricorn 5/50 column (5 mm i.d.)	1	28406409
Tricorn 10/20 column (10 mm i.d.)	1	28406413
Tricorn 10/50 column (10 mm i.d.)	1	28406414
Tricorn 10/100 column (10 mm i.d.)	1	28406415
Tricorn 10/150	1	28406416
Tricorn 10/200	1	28406417
Tricorn 10/300	1	28406418
XK 16/20 (16 mm i.d.)	1	28988937
XK 26/20 (26 mm i.d.)	1	28988948
XK 50/20 (50 mm i.d.)	1	28988952
XK 60/40	1	28988938
XK 26/40	1	28988949
XK 50/30	1	28988953
HiTrap™ Heparin HP, 1 mL	5x1 mL	17040601
HiTrap Heparin, 5mL	1x5 mL	17040701
HiPrep™ 16/10 Heparin FF	1 (20 mL)	17158901

Related literature	Code no.
Data File CHROMAFLOW columns	18113892
Data File BPG Columns 100, 140, 200, 300 and 450 series	18111523
Data File INdEX	18111561
Data File AxiChrom	28929041

Reference literature

For general advice on lab-scale use, refer to the *Handbook* 18102229.

For general advice on optimization, scaling up and other aspects relating to process chromatography we recommend:

Handbook of Process Chromatography: A Guide to optimization, scale-up and validation. Academic Press, pp 188-214 (1997). Sofer G. and Hagel, L.

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