Sephacryl™ High Resolution

Sephacryl S-100 High Resolution
Sephacryl S-200 High Resolution
Sephacryl S-300 High Resolution
Sephacryl S-400 High Resolution
Sephacryl S-500 High Resolution

Table of Contents

Please read these instructions carefully before using the products.

Safety

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

1 Introduction

Sephacryl High Resolution (HR) is one of the BioProcess™ range of size exclusion chromatography resins. These resins meet the industrial bioprocessing demands for reproducibility, scalability, chemical and physical stability, security of supply, and prompt delivery.

These instructions contain information about the characteristics of the resins, process operation (including packing), process optimization, maintenance, equipment and troubleshooting.

2 Characteristics

Structure and selectivities

Sephacryl HR is a cross-linked copolymer of allyldextran and N,N'-methylene bisacrylamide.

Sephacryl HR is a hydrophilic resin for minimized non-specific adsorption. It gives high recoveries and performs well at laboratory and industrial scales. The particle size, d_{50V} [1] ~50 µm, together with the rigidity of the matrix, ensures fast flow characteristics and high resolution.

Sephacryl HR is available in five different selectivities, see Table 1, covering a wide molecular weight range from peptides to very large biomolecules.

[1] Median particle size of the cumulative volume distribution.

Table 1. Characteristics of Sephacryl HR chromatography resins

Sephacryl	S-100 HR	S-200 HR	S-300 HR	S-400 HR	S-500 HR
Fractionation range (M_r)					
Globular proteins	~1×10^3– ~1×10^5	~5×10^3– ~2.5×10^5	~1×10^4– ~1.5×10^6	~2×10^4– ~8×10^6	–
Dextrans	–	~1×10^3– ~8×10^4	~2×10^3– ~4×10^5	~1×10^4– ~2×10^6	~4×10^4– ~2×10^7
Exclusion limit DNA (base pairs)	–	~30	~118	~271	~1078
Particle size, d_{50v}[1]	~ 50 µm	~ 50 µm	~ 50 µm	~ 50 µm	~ 50 µm
Matrix	Cross-linked copolymer of allyl dextran and N,N'-methylene bisacrylamide				
Chemical stability	Stable in commonly used buffers: 0.2 M NaOH, 0.1 M HCl, 1 M acetic acid, 8 M urea, 6 M guanidine hydrochloride, 1% SDS, 2 M NaCl, 20% ethanol, 30% propanol, 30% acetonitrile, 0.5 M NaOH (only for cleaning-in-place)				
pH stability					
operational[2]	3–11	3–11	3–11	3–11	3–11
CIP[3]	2–13	2–13	2–13	2–13	2–13
Physical stability	Negligible volume variation due to changes in pH or ionic strength				
Autoclavability	At 121°C, pH 7 for 5 × 20 min (resins only)				
Storage	20% ethanol	20% ethanol	20% ethanol	20% ethanol	20% ethanol

1 *Median particle size of the cumulative volume distribution.*
2 *pH range where resin can be operated without significant change in function.*
3 *pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.*

Stability

Cross-linking of the copolymer matrix gives Sephacryl HR resins high chemical and physical stabilities, see Table 1 and Figure 2. The resins are unaffected by the solutions commonly used in process chromatography and cleaning, see Table 1.

Note: *Chromatographic resins should never be exposed to chemical or physical extremes for longer time than necessary.*

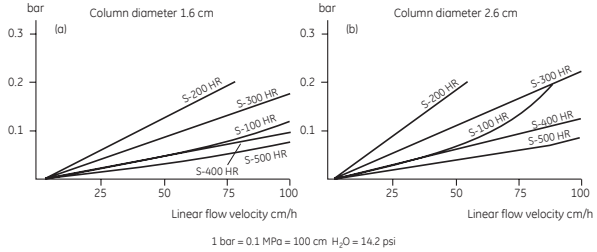

Fig 1. Pressure drop as a function of linear flow velocity for Sephacryl HR. Bed height: approximately 60 cm, eluent: distilled water, temperature: 25°. To calculate the volumetric flow, multiply the linear flow velocity by the cross-sectional area of the column (2 cm^2 for XK 16 or 5.3 cm^2 for XK 26).

3 Column packing guidelines

Sephacryl HR is supplied in 20% ethanol.

Recommended columns

Table 2. Recommended columns for Sephacryl HR.

Column	Inner diameter (mm)	Bed volume [1]	Bed height (cm)
Lab scale			
XK 16/100	16	20–191 mL	max 95
XK 26/100	26	53–504 mL	max 95
XK 50/100	50	196–1864 mL	max 95
Production scale			
AxiChrom™ Pilot	50–200	0.2–16 L	max 50
AxiChrom Process	300–1600	7–1005 L	max 50
BPG	100–450	1–112 L	max 72

[1] Bed volume range calculated from 10 cm bed height to maximum bed height.

All large-scale columns can be supplied as variable bed height columns. Do not choose large diameter columns if the bed height is low.

For information on packing of process scale columns, please contact your local GE representative.

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.

- Pressure/flow packing (for columns with adaptors).
- Suction packing (for large columns with fixed bed heights).

How well the column is packed will have a major effect on the result of the separation. It is therefore very important to pack and test the column according to recommendations.

Slurry preparation

Packing solution can be either water or running buffer. Packing in running buffer saves later equilibration time. If running buffer is used, it is recommended to include 0.15 M NaCl to prevent any non-specific interactions during run.

Follow the instructions below how to prepare a slurry:

Step	Action
1	Determine the desired packed bed volume by multiplying the cross-sectional area of the column by the desired bed height.
2	Gently shake the bottle of Sephacryl HR to make an even slurry.
3	Measure out the required volume of resin slurry, 1.2 × the desired packed resin volume, using a measuring cylinder and pour it into a beaker. Leave to settle and decant the 20% ethanol storage solution.
4	Dilute the resin suspension with packing solution to form a 50% to 70% slurry (sedimented bed volume/slurry volume= 0.5 to 0.7).
5	Stir gently with a glass rod to make a homogeneous suspension free from aggregates. Never use a magnetic stirrer.

Pressure/flow packing of XK columns

XK columns are supplied with a movable adaptor. They are packed by conventional pressure packing by pumping the packing solution through the chromatographic bed at constant flow velocity (or back pressure).

The recommended flow rates for packing XK columns with aqueous buffers at room temperature are shown in Table 2.

Table 3. Recommended flow rates during column packing

Column	Bed height (cm)	Step 1 (mL/min)	Step 2 (mL/min)
XK 16/40	35	1 to 2	2 to 4
XK 16/70	65	1 to 2	2 to 4
XK16/100	95	1 to 2	2 to 4
XK 26/40	35	2 to 4	4 to 8
XK 26/70	65	2 to 4	4 to 8
XK 26/100	95	2 to 4	4 to 8
XK 50/60	55	8 to 10	4 to 8
XK 50/100	95	8 to 10	4 to 8

Step	Action
1	Pour some water (or buffer used for packing) into the column. Make sure that there is no air trapped under the bottom net. Leave about 2 cm of liquid in the column
2	Prepare a 50% to 70% resin slurry according to Sub section *Slurry preparation, on page 7*. When slurry volume is greater than the total volume of the column, connect a second glass column to act as a reservoir (see *Section 9 Ordering information, on page 21* for details). Pour the slurry into the column. Insert the adaptor and lower it to the surface of the slurry, making sure no air is trapped below the adaptor. Secure the adaptor in place.
3	Seal the adaptor O-ring and lower the adaptor a little into the slurry, enough to fill the adaptor inlet with packing solution.

Step	Action
4	Connect a pump and a pressure meter and start packing at the recommended flow velocity for XK-columns (Step 1, Table 2). Keep the flow velocity constant during packing and check the pressure at the column inlet. Never exceed the pressure limit for column or resin.
5	When the resin has settled, mark the bed height on the column tube, close the bottom valve and stop the pump. The bed starts rising in the column. Loosen the O-ring and lower the adaptor to about 0.5 to 1.0 cm from the resin surface.
6	Seal the O-ring, start the pump and continue packing. at the recommended packing flow rate for XK columns (Step 2, Table 2). Repeat steps 5 and 6 until there is a maximum of 1 cm between resin surface and adaptor when the resin has stabilized.
7	Close the bottom valve, stop the pump, disconnect the column inlet and push the adaptor down to approximately 3 mm below the mark on the column tube, without loosening the adaptor O-ring. The packing solution will flush the adaptor inlet. Remove any trapped air by pumping liquid from the bottom (after the inlet tubing and the bottom valve have been properly filled).

4 Evaluation of column packing

Intervals

Test the column efficiency to check the quality of packing. Testing should be done after packing and at regular intervals during the working life of the column and also when separation performance is seen to deteriorate.

Column efficiency testing

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 (A_s). These values are easily determined by applying a sample such as 1% acetone solution to the column. Sodium chloride can also be used as a test substance. Use a concentration of 0.8 M NaCl in water as sample and 0.4 M NaCl in water as eluent.

For more information about column efficiency testing, consult the application note *Column efficiency testing* (28-9372-07).

Note: *The calculated plate number will vary according to the test conditions and it should only be used as a reference value. It is important that test conditions and equipment are kept constant so that results are comparable. Changes of solute, solvent, eluent, sample volume, flow velocity, liquid pathway, temperature, chromatography system, etc. will influence the results.*

Sample volume and flow velocity

For optimal column efficiency results, the sample volume should be approximately 1% of the column volume and the flow velocity 30 cm/h. If an acceptance limit is defined in relation to column performance, the column plate number can be used as one of the acceptance criteria for column use.

Method for measuring HETP and A_S

Calculate HETP and A_S from the UV curve (or conductivity curve) as follows:

$$HETP = \frac{L}{N}$$

L = bed height (cm)
N = number of theoretical plates

$$N = 5.54 \times \left(\frac{V_R}{W_h}\right)^2$$

V_R = volume eluted from the start of sample application to the peak maximum
W_h = peak width measured as the width of the recorded peak at half of the peak height
V_R and W_h are in the same units

The concept of reduced plate height is often used for comparing column performance.

The reduced plate height, h, is calculated as follows:

$$h = \frac{HETP}{d_{50v}}$$

d_{50v} = mean diameter of the beads (cm)

As a guideline, a value of < 3 is very good.

The peak should be symmetrical, and the asymmetry factor as close to 1 as possible (a typical acceptable range could be $0.8 < A_S < 1.8$).

A change in the shape of the peak is usually the first indication of bed deterioration due to excessive use.

Peak asymmetry factor calculation:

$$A_s = \frac{b}{a}$$

a = ascending part of the peak width at 10% of peak height
b = descending part of the peak width at 10% of peak height

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

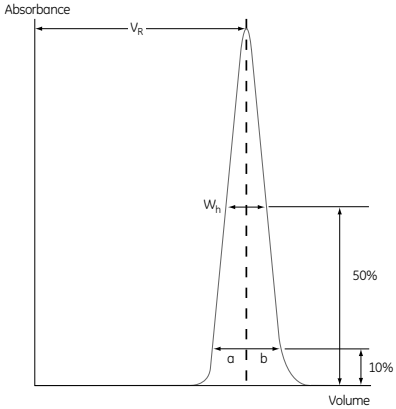

Fig 2. A typical test chromatogram showing the parameters used for HETP and A_s calculations.

5 Performing a separation run

The method below is recommended as a start. If further optimization is needed, see *Section 7 Method design and optimization, on page 15*.

Step	Action
1	Use a linear flow rate of 15 cm/h.
2	Equilibrate the column with 2 column volumes of running buffer (0.05 M sodium phosphate, 0.15 M NaCl, pH 7.4).
3	Apply the sample (sample volume: 1% of the column volume).
4	Perform the elution using running buffer until the separation is complete.

6 Maintenance

For best performance of Sephacryl HR column over a long time, follow the procedures described below:

Equilibration

After packing, and before a chromatographic run, equilibrate with at least 2 column volumes of running buffer.

Regeneration

After each separation, elute any reversibly bound material either with a high ionic strength solution (e.g. 1 M NaCl in buffer) or by increasing pH. Regenerate the resin by washing with at least 2 column volumes of buffer, or until the column effluent shows stable conductivity and pH values.

Cleaning-in-place (CIP)

CIP is a procedure that removes contaminants such as lipids, precipitates, or denatured proteins that may remain in the packed column after regeneration. Such contamination is especially likely when working with crude materials. 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 the resin.

A specific CIP protocol should be designed for each process according to the type of contaminants present. Wash the column with 0.2 to 0.5 M NaOH or a solution of a non-ionic detergent at a flow rate of 15 to 20 cm/h. The total contact time with the cleaning solution should be 1 to 2 h. After washing always reequilibrate the column with 2 column volumes of buffer. The frequency of CIP depends of the nature and the condition of the starting material, but one CIP cycle is generally recommended every 5 separation cycles.

Sanitization

Sanitization is the use of chemical agents to inactivate microbial contaminants in the form of vegetative cells. Sanitization also helps maintain a high level of both process hygiene and process economy. An example of effective sanitization is given below.

Recommended CIP and sanitization protocol

Purpose	Procedure
Removal of contaminants	Wash the resin in the column with 0.1 M NaOH at 10 cm/h with reversed flow direction. Contact time 1 h.

Sterilization

Autoclaving is the only recommended sterilization treatment. Equilibrate the resin with 0.5 M NaCl, pH 7. Dismantle the column and autoclave the resin at 121°C for 5 × 20 minutes.

Sterilize the column parts according to the instructions in the column instructions. Re-assemble the column, then pack and test it as recommended.

Storage

Sephacryl HR: 20% ethanol.

Storage temperature: 4°C to 30°C.

Store unused resin in the container at a temperature of 4°C to 30°C.

Packed columns should be equilibrated in 20% ethanol before storage.

Note: *Use a well de-gassed water/ethanol mixture.*

7 Method design and optimization

Introduction

Size exclusion chromatography (SEC) is widely used in process chromatography, particularly for polishing of the final product, i.e. removal of product aggregates, transfer of product to formulation buffer or desalting. Since molecules are separated according to differences in their size, resins for a SEC step is selected on the basis of its selectivity for the molecular weight of the molecule of interest. See Table 1.

To achieve maximum productivity and maximum purity in a large scale SEC process there are three steps to complete:

- Optimization of the method to ensure best resolution
- Optimization of the process for highest productivity
- Scale-up

Optimization for best resolution

For best resolution the molecule of interest should have an elution volume which corresponds to a K_{av} between 0.1 and 0.6. The resolution (R_s) should be about 1.25. See Figure 4.

Resolution is affected by flow velocity, column efficiency and bed height. Too high flow velocity will decrease resolution. The flow velocity at which optimal efficiency is obtained is dependent on the molecular weight of the molecule of interest. As a rule-of-thumb, larger molecules normally require lower flow velocity.

Column efficiency is dependent upon how well the column is packed. This can be measured by determining column efficiency, see *Section 4 Evaluation of column packing, on page 10*. The number of theoretical plates obtained (N) should be as high as possible. A typical value for Sephacryl HR is >5000/m.

A poorly packed column will give rise to uneven flow, zone broadening and loss of resolution.

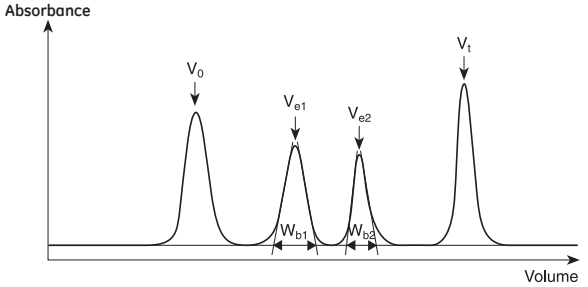

Fig 3. SEC chromatogram showing substances eluting at different elution volumes

$K_{av} = (V_e - V_0/(V_t - V_0)$

$R_s = 2(V_{e2} - V_{e1})/(W_{b1} + W_{b2})$

where:

V_e = elution volume, V_t = total liquid volume
V_t = total column volume, W_b = peak width at base
V_0 = void volume

Bed height also affects the resolution, the higher the bed height the better the resolution. A typical bed height for Sephacryl HR is 60 to 90 cm.

Column size and sample volume are interdependent. Recommended sample volumes for Sephacryl HR resins are between 0.5% and 4% of the total column volume. The lower sample volume, the higher resolution.

As with all SEC resins, some pH-dependent interactions can occur with both acidic and basic proteins at very low salt concentrations. These, however, can be completely avoided by using buffers with a salt concentration of at least 0.15 M.

Process optimization

It is advisable to optimize the product at laboratory scale; this will save both time and material. GE offers a range of columns suitable for method development or small scale production such as XK columns or BPG columns. All have compatible bed heights and are suitable for scale up to process scale.

A convenient alternative for method development is to use the range of prepacked HiPrep™ columns, see *Section 9 Ordering information, on page 21*.

When optimizing a SEC step for maximum productivity, the following parameters need careful consideration:

- Sample volume
- Flow velocity
- Sample concentration

Conditions which lead to maximum resolution are often in conflict with other experimental objectives. The parameters that are optimized for maximum productivity also influence resolution. Therefore, in any SEC step, there is usually a compromise between resolution and productivity.

Sample volume greatly influences resolution in SEC techniques and is thus usually limited to maximum 4% of the total column volume.

Flow velocity influences resolution. Flow velocity that are too high decrease resolution. For each different SEC resin and sample there is an optimal flow velocity range. As a rule-of-thumb, smaller molecules can be separated at higher flow velocity.

The optimal sample concentration varies with the applications at hand. High sample concentrations can be used but note that too high viscosity decreases the resolution.

It is often suitable to use SEC directly after an absorption technique that gives a highly concentrated feed (for example affinity chromatography).

For a test run, the following conditions are appropriate:

Linear flow velocity:	15 cm/h
Sample volume:	1% of the column volume

Scaling up

After the SEC step has been optimized at laboratory scale, the process can be scaled up, usually in the order of 100-fold. Scale up is carried out by increasing the diameter of the column. When scaling up, some parameters remain constant while others are increased.

Maintain:

- Bed height
- Linear flow velocity (cm/h)
- Sample concentration and volume (in relation to column volume)
- Efficiency in terms of N

Increase:

- Volumetric flow (mL/min)
- Column diameter

The larger equipment needed when scaling up can also cause some deviations from the results at small scale. Check the buffer delivery system and monitoring system for time delays or volume changes. Different lengths and diameters of outlet tubing can cause zone spreading on larger systems.

8 Troubleshooting guide

High back pressure

Note: *Use a lower flow rate to avoid high back pressure when working at low temperatures, like in a cold room, or when the column is used with 20% ethanol or other viscous solutions.*

Step	Action
1	Check if equipment up to and after the column is generating any back pressure. (For example valves and tubing of incorrect dimensions.)
2	Perform CIP to remove tightly bound material from the resin.
3	Check column parts such as filters, nets etc., according to the column instruction.

Unexpected chromatography results

Step	Action
1	Check the UV detector cell.
2	Check the flow velocity.
3	Check the buffers.
4	Check that there are no gaps between the adaptor and the resin bed, or back mixing of the sample before application.
5	Check the efficiency of the column packing, see *Section 4 Evaluation of column packing, on page 10*.
6	Check if there have been any changes in the pretreatment of the sample.

Trapped air

Step	Action
1	Check that the buffers are equilibrated to the same temperature as the packed column.
2	Check that there are no loose connections or leaking valves.

If air has entered the column, the column should be repacked. However, if only a small amount of air has been trapped on top of the bed, or between the adaptor net and head, it can be removed by pumping eluent in the opposite direction. After this, check the efficiency of the packed bed (see *Section 4 Evaluation of column packing, on page 10*) and compare the result with the original efficiency values.

9 Ordering information

Product	Quantity	Product Code
Sephacryl S-100 HR	150 mL	17061210
	750 mL	17061201
	10 L	17061205
	60 L	17061260
Sephacryl S-200 HR	150 mL	17058410
	750 mL	17058401
	10 L	17058405
	60 L	17058460
Sephacryl S-300 HR	150 mL	17059910
	750 mL	17059901
	10 L	17059905
Sephacryl S-400 HR	150 mL	17060910
	750 mL	17060901
	10 L	17060905
Sephacryl S-500 HR	150 mL	17061310
	750 mL	17061301
	10 L	17061305

Related products

Product	Quantity	Product Code
HiPrep 16/60 Sephacryl S-100 HR	1 × 120 mL	17116501
HiPrep 26/60 Sephacryl S-100 HR	1 × 320 mL	17119401
HiPrep 16/60 Sephacryl S-200 HR	1 × 120 mL	17116601
HiPrep 26/60 Sephacryl S-200 HR	1 × 320 mL	17119501
HiPrep 16/60 Sephacryl S-300 HR	1 × 120 mL	17116701
HiPrep 26/60 Sephacryl S-300 HR	1 × 320 mL	17119601
HiPrep 16/60 Sephacryl S-400 HR	1 × 120 mL	28935604
HiPrep 26/60 Sephacryl S-400 HR	1 × 320 mL	28935605
HiPrep 16/60 Sephacryl S-500 HR	1 × 120 mL	28935606
HiPrep 26/60 Sephacryl S-500 HR	1 × 320 mL	28935607

Accessories

Column	Quantity	Product Code
XK 16/40	1	28988938
XK 16/70	1	28988946
XK 16/100	1	28988947
XK 26/40	1	28988949
XK 26/70	1	28988950
XK 26/100	1	28988951
XK 50/60	1	28988964
XK 50/100	1	28988965

Adapter [1]	Quantity	Product Code
XK 16 (for all XK columns with diameter 16 mm)	1	28989876
XK 26 (for all XK columns with diameter 26 mm)	1	28989877
XK 50 (for all XK columns with diameter 50 mm)	1	28989880

[1] Each XK column is delivered with one XK adapter and one bottom piece.

Packing reservoir	Quantity	Product Code
RK 16/26 (for all XK columns with diameter 16 mm and 26 mm)	1	28989858
RK 50 (for all XK columns with diameter 50 mm)	1	28989861

Literature

Product	Product Code
Handbook: Size exclusion chromatography, Principles & Methods	18102218
Data file: Sephacryl High Resolution media, HiPrep Sephacryl HR columns	18106088

10 Further information

For further information visit www.gelifesciences.com or contact your local GE representative.

For local office contact information, visit
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