GE Healthcare

Instructions 71-5002-42 AD **Gel filtration media**

Sephacryl High Resolution

Contents

1. Introduction

Sephacryl™ High Resolution is one of the GE Healthcare range of BioProcess™ Media, separation media that meet the demands of today's industrial bioprocessing for reproducibility, scalability, chemical and physical stability, security of supply, and prompt delivery.

These instructions contain information about media characteristics, process operation (including packing), process optimization, maintenance, equipment and trouble-shooting.

To ensure best performance and trouble-free operation, please read these instructions before using Sephacryl High Resolution.

2. Media characteristics

Sephacryl High Resolution is a cross-linked copolymer of allyldextran and N,N'-methylene bisacrylamide, see Figure 1.

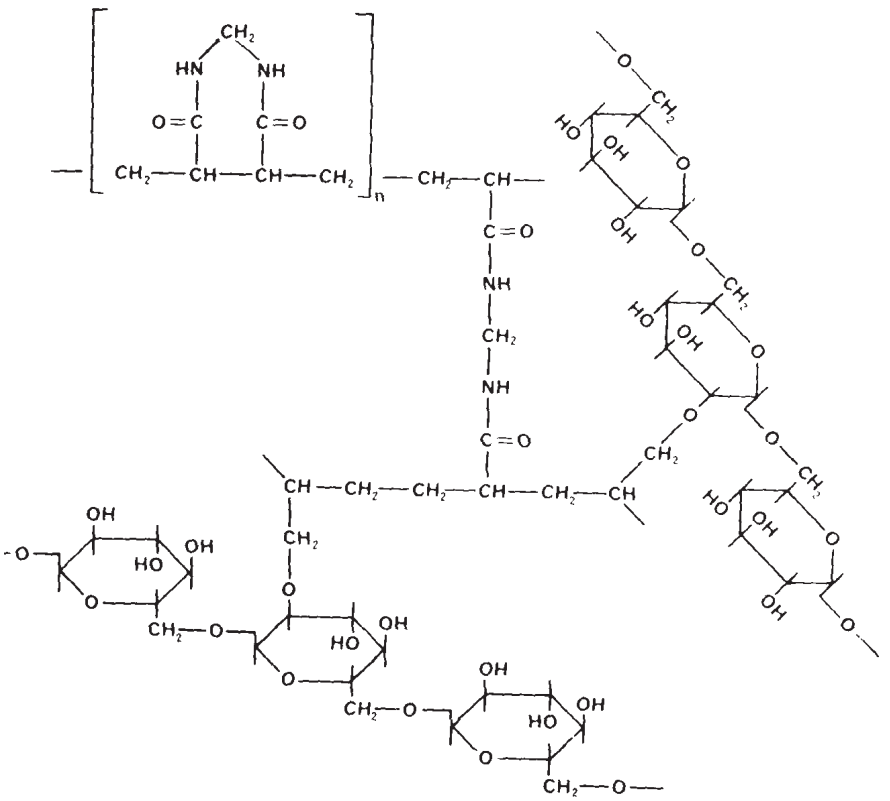


Fig 1. Partical structure of Sephacryl High Resolution media.

The mean particle size of 50 µm, together with increased rigidity of the matrix, ensures fast flow characteristics and high resolution.

Sephacryl High Resolution is a hydrophilic media that is easy to use; it gives high recoveries and performs well at all scales of operation, including commercial production.

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

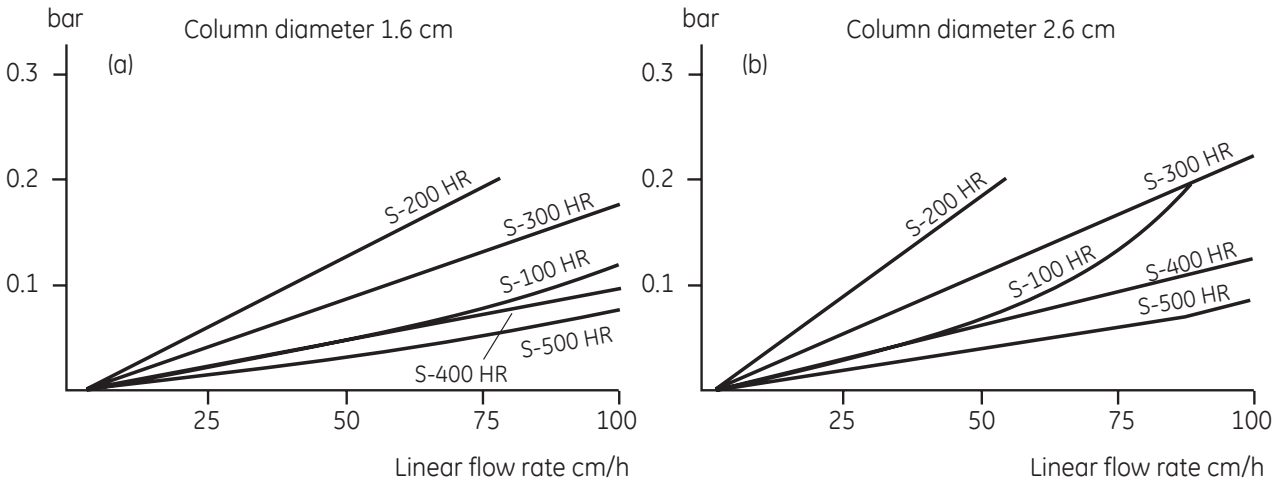
Table 1. Fractionation ranges in daltons for Sephacryl HR media.

Separation range	**(MW)**
Sephacryl S-100 High Resolution	1 000–100 000
Sephacryl S-200 High Resolution	5 000–250 000
Sephacryl S-300 High Resolution	10 000–1 500 000
Sephacryl S-400 High Resolution	20 000–8 000 000
Sephacryl S-500 High Resolution	40 000–20 000 000

Stability

Cross-linking of the copolymer matrix gives Sephacryl High Resolution media high chemical and physical stabilities, see Figure 2. They are unaffected by the solutions commonly used in process chromatography and cleaning, see Table 2.

* Chromatographic media should never be exposed to chemical or physical extremes for longer than is necessary.



1 bar =* 0.1 MPa = 100 cmH_2O = 14.2 psi

Fig 2. Pressure drop as a function of linear flow velocity for Sephacryl High Resolution. 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).

Table 2. Media characteristics.

Property	**Description**
Mean particle size	50 µm
Bead size range	25–75 µm
Bead structure	Allyl dextran and N,N'-methylene bisacrylamide
Solutions in which the media are stable	all commomly used buffers, 1 M acetic acid, 8 M urea, 6 M guanidine hydrochloride, 30% isopropanol, 30% acetonitrile, 24 % ethanol
pH stability	
Working range	3–11
Cleaning-in-place	2–13
Autoclavable	at 121 °C, 0.5 M NaCl pH 7 for 30 min

3. Column packing guidelines

Sephacryl High Resolution is supplied in suspension in 20% ethanol. Decant the 20% ethanol solution and replace with starting buffer before use.

3.1 Recommended columns

- XK™ (16/20-16/100, 26/20-26/100, 50/20-50/100) variable bed, glass columns; inner diameters from 16–50 mm, bed volumes from 2–1880 ml; bed height max 95 cm.
- BPG™, variable bed, glass columns: inner diameters from 100–450 mm, bed volumes from 2.4–43 litres; bed height max 30 cm (27 cm for BPG 450).
- BioProcess Stainless Steel fixed bed columns: inner diameters from 400–1400 mm; fixed bed volumes from 19–230 litres; fixed bed height 15 cm.

3.2 Slurry preparation

Decant the ethanol solution from the media and add enough packing solution to fill the packing equipment completely. Packing solution can be either water or starting buffer. If water is chosen, one of the following additives should be used: 0.01 M NaOH, 0.15 M NaCl, or 0.05% Tween 20. If starting buffer is used, the ionic strenght of the buffer should be adjusted to a level that corresponds to 0.15 M NaCl.

3.3 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.

- Flow velocity/pressure 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 the following recommendations.

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

3.4 Determining optimal packing flow velocity

The optimal packing flow is dependent on temperature, column size and type, media batch and volume. Consequently, the optimal packing flow must be determined empirically for each individual system.

To determine the optimal packing flow velocity, proceed as follows:

1. Calculate the exact amount of media needed for the slurry (this is especially important for columns with fixed bed heights). The quantity of media required per litre packed volume is approximately 1.15 litres sedimented media.
2. Set up the column as for packing according to the instructions in section 3.2.
3. Begin packing the media at a low flow velocity (30 cm/h).
4. Increase the pressure in increments and record the flow velocity when the pressure has stabilized. Do not exceed the maximum pressure of the column, or the maximum flow velocity for the media.
5. The maximum flow velocity is reached when the pressure/flow curve levels off or the maximum pressure of the column is reached. Stop the packing and do not exceed this flow velocity. The optimal packing flow velocity/pressure is 70–100% of the maximum flow velocity/pressure.
6. Plot the pressure/flow velocity curve as in Fig. 2 and determine the optimal packing flow velocity.

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

Note: For BPSS columns, pack the column according to instructions in section 3.6.

3.5 Flow velocity/pressure packing

XK and BPG columns

XK and BPG 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).

1. Pour some water (or packing buffer) 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. Mix the packing buffer with the medium to form a 50–70% slurry. (Sedimented bed volume/slurry volume = 0.5–0.7.)

   Pour the slurry into the column. Insert the adaptor and lower it to the surface of the slurry, making sure no air is trapped under 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.
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 column or medium.
5. When the medium 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 0.5–1.0 cm from the medium 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 medium surface and adaptor when the medium 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).

3.6 Suction packing

BioProcess Stainless Steel (BPSS) Columns

BioProcess Stainless Steel Columns are supplied with fixed end pieces. They are packed by suction, i.e. by sucking packing solution through the chromatographic bed at a constant flow velocity.

1. Fit a packing device on top of the column tube.
2. Pour some water (or packing solution) into the column. Make sure that there is no air trapped under the bottom net. Leave about 23 cm of liquid in the column.
3. Mix the packing buffer with the medium to form a 50% slurry (sedimented bed volume/slurry = 0.5). Pour the slurry into the column. Stir gently to give a homogeneous slurry.
4. Connect the column outlet valve to the suction side of a pump and start packing the bed by suction through the bed at the predetermined flow velocity. Keep the flow velocity constant during packing.
5. When the bed has stabilized, the top of the bed should be just below the junction of the column and the packing device.

If, when stabilized, the level of the bed is incorrect, add or remove slurry. Always stir the slurry thoroughly before packing.

6. Just before the last of the solution has entered the packed bed (before the surface starts to dry), close the valve at the column outlet, stop the pump, quickly remove the packing device and replace it with the lid.

This final operation should be completed as quickly as possible because the bed will expand when the flow stops.

7. Start pumping buffer with upward flow through the column to remove any air bubbles trapped under the lid.

4. 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, at regular intervals afterwards, and when separation performance is seen to deteriorate.

The best method of expressing the efficiency of a packed column is in terms of height equivalent to a theoretical plate, HETP, and the peak asymmetry factor, As. These values are easily determined by applying a sample such as 1% acetone solution to the column. (Coloured compounds and salt solutions should be avoided since they may interact with the media.)

It is of utmost importance to realize that the calculated plate number will vary depending on the test conditions and it should therefore 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 velocity, liquid pathway, temperature, etc. will influence the results.

For optimal results, the sample volume should be at maximum 2.5% of the column volume and the linear 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:	1.0% of bed volume
Sample conc.:	1.0% (v/v) acetone in water, 2.0 M NaCl or10x buffer
Eluent:	water, 0.5 M NaCl in water or dilute buffer
Flow velocity:	30 cm/h
Detection:	
Acetone:	UV 280 nm;
NaCl, buffer:	Conductivity
UV:	280 nm, 1 cm, 0.1 AU

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

$$HETP = L/N$$

and $$N = 5.54(V_e/Wh)^2$$

where V_e = Peak elution distance

W_h = Peak width at half peak height

L = Bed height (cm)

N = Number of theoretical plates

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 as

$$\frac{HETP}{d}$$

where d is the diameter of the bead. 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

Figure 3 shows a UV trace for acetone in a typical test chromatogram in which the **HETP** and A_s values are calculated.

Column: BPG 300
Media: Sepharose 6 Fast Flow
Bed height: 57.5 cm
Bed volume: 40.6 litres
Eluent: Distilled water
Sample: 1.05 litres (1% acetone)
Flow velocity: 19 cm/h
V_e =18.7
W_h =0.9
HEPT=0.024 cm
a: 0.90
b: 0.85
A_s: 0.94

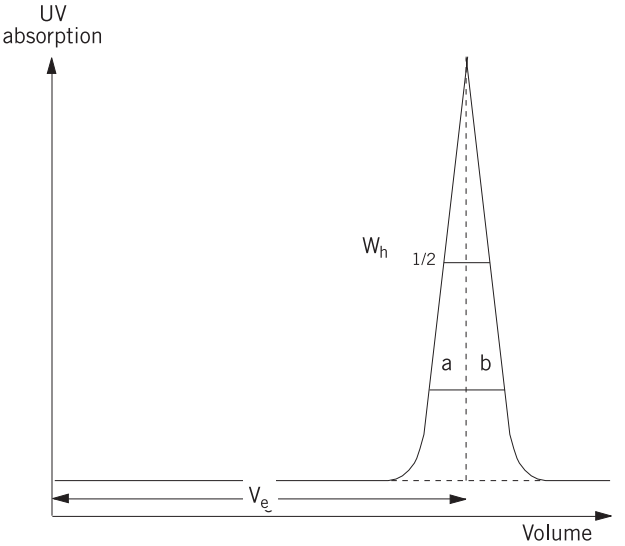


Fig 3. UV trace for acetone in a typical test chromatogram showing the HETP and A_s value calculations.

5. Maintenance

For best performance from Sephacryl High Resolution over a long working life, follow the procedures described below.

Equilibration

After packing, and before a chromatographic run, equilibrate with working buffer by washing with at least 5 bed volumes.

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 media by washing with at least 5 bed volumes of buffer, or until the column effluent shows stable conductivity and pH values.

Cleaning-In-Place

Cleaning-in-place (CIP) is a cleaning 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 media bed and helps to maintain the capacity, flow properties and general performance of the media.

A specific CIP protocol should be designed for each process according to the type of contaminants present. The frequency of CIP depends of the nature and the condition of the starting material, but one CIP cycle is generally recommended every 1–5 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 media in the column with 0.1 M NaOH at 10 cm/h, reversed flow, contact time of 1 h.

After treatment with sodium hydroxide, wash the column with 2 column volumes of buffer at 20 cm/h, normal flow direction, before applying the sample.

Sterilization

Autoclaving is the only recommended sterilization treatment. Equilibrate the media with 0.5 M NaCl, pH 7. Dismantle the column and autoclave the media at 120 °C for 30 minutes.

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

Storage

Unused media can be stored in the container at +4 °C. Ensure that the screw-top is fully tightened. Packed columns should be equilibrated in working buffer containing 20% ethanol to prevent microbial growth.

Thimerosal (e.g. Merthiolate*) should not be used.

* This trademark is owned by Eli Lilly & Co.

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

6. Method design and optimization

Gel filtration 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, media for a gel filtration 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 gel filtration process there are three steps to complete.

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

6.1 Optimization for best resolution

For best resolution the molecule of interest should have an elution volume which corresponds to a Kav between 0.1-0.6. The resolution (Rs) should be about 1.25. See Figure 4.

Resolution is affected by flow velocity, column efficiency and bed height. Flow velocity that are too high 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; higher flow rates can be used with smaller molecules with maintained resolution.

Column efficiency is dependent upon how well the column is packed. This can be measured by determining HETP, see section 4. The number of theoretical plates obtained (N) should be as high as possible. A typical value for Sephacryl High Resolution is 6700 m^{-1}.

$$N=L/HETP$$

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

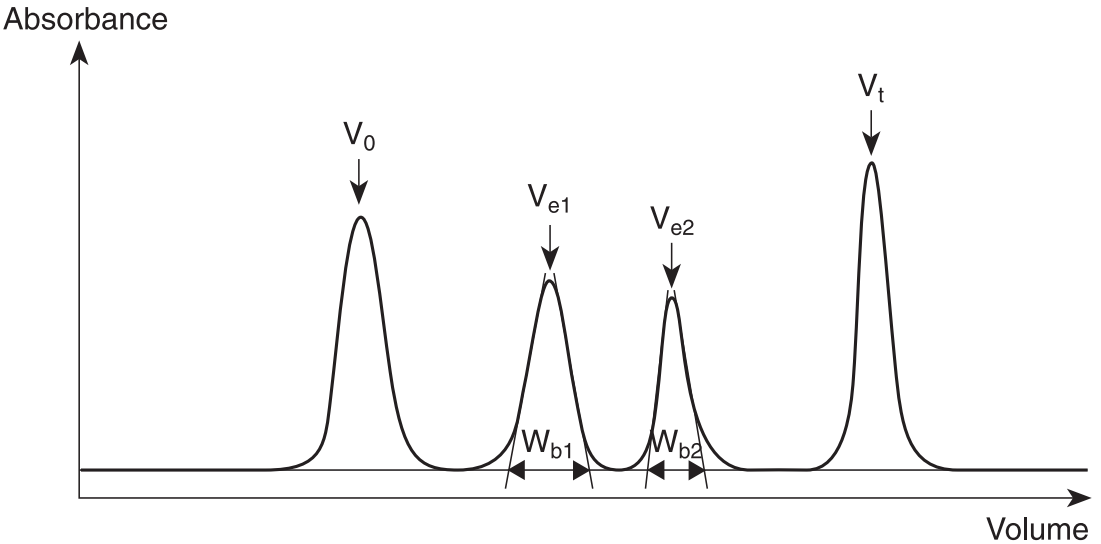


Fig 4. Gel filtration chromatogram showing substances eluting at different elution volumes.

$K_{aw} = (V_e - V_0)/(V_c - V_0)$ $R_s = 2(V_{e2} - V_{e1})/(W_{b1} + W_{b2})$

where: V_e =elution volume, V_t =total liquid volume
V_c =geometric column volume, W_b =peak width at base
V_0 =void volume.

Bed height also effects the resolution, the higher the bed height the better the resolution. A typical bed height for Sephacryl High Resolution is 60–90 cm.

Column size and sample volume are interdependent. Recommended sample volumes for Sephacryl High Resolution media lie between 0.5–4% of the total bed volume.

As with all gel filtration media, 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.

6.2 Process optimization

It is advisable to optimize the product at laboratory scale; this will save both time and material. GE Healthcare 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 our range of prepacked HiPrep™ Columns. (Ordering information can be found on the last page.)

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

a) feed concentration

b) flow velocity

c) feed volume

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 gel filtration step, there is usually a compromise between resolution and productivity.

Feed concentration should be as high as possible without increasing viscosity too much. High feed concentrations decrease the resolution, the optional concentration varies with the applications at hand.

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

Feed volume greatly influences resolution in gel filtration techniques and is thus usually limited to approximately 4% of the total column volume.

It is often suitable to use gel filtration directly after an absorption technique that gives a highly concentrated feed (for example ion exchange chromatography).

For a test run, the following are appropriate:

Linear flow velocity:	15 cm/h
Feed volume:	1% of the bed volume

To achieve the required resolution, it is advisable to use a high feed concentration, as high a flow velocity as possible, and to adjust feed volume.

6.3 Scaling up

After the gel filtration step has been optimized at laboratory scale, the process can be scaled up, usually in the order of 100-old. 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
- Sample concentration and volume (in relation to bed volume)
- Efficiency in terms of N

Increase:

- Volumetric flow velocity
- 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 pipes can cause zone spreading on larger systems.

7. Trouble-shooting guide

High back pressure

1. Check that all valves between the pump and the collection vessel are fully open.
2. Check that all valves are clean and free from blockage.
3. Check if equipment in use up to and after the column is generating any back pressure. (For example valves and flow cells of incorrect dimensions.)
4. Perform CIP to remove tightly bound material from the media.
5. Check column parts such as filters, nets etc., according to the column instruction manual.

Unexpected chromatographic results

1. Check the recorder speed/signal.
2. Check the flow velocity.
3. Check the buffers.
4. Check that there are no gaps between the adaptor and the media bed, or back mixing of the sample before application.
5. Check the efficiency of the column packing, see page 11.
6. Check if there have been any changes in the pretreatment of the sample.

Infections

1. Check the connections and prefilters.
2. Check the in-going components such as buffers, sample components, etc.
3. Check that the column has been properly sanitized.

Trapped air

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 page 11) and compare the result with the original efficiency values.

8. Ordering information

Product	Pack size	Code No.
Sephacryl S-100 HR	150 ml	17-0612-10
	750 ml	17-0612-01
	10 l	17-0612-05
	60 l	17-0612-60
Sephacryl S-200 HR	150 ml	17-0584-10
	750 ml	17-0584-01
	10 l	17-0584-05
	60 l	17-0584-60
Sephacryl S-300 HR	150 ml	17-0599-10
	750 ml	17-0599-01
	10 l	17-0599-05
Sephacryl S-400 HR	150 ml	17-0609-10
	750 ml	17-0609-01
	10 l	17-0609-05
Sephacryl S-500 HR	150 ml	17-0613-10
	750 ml	17-0613-01
	10 l	17-0613-05
Pre-packed columns:		
HiPrep 16/60 Sephacryl S-100 HR	1 x 120 ml	17-1165-01
HiPrep 26/60 Sephacryl S-100 HR	1 x 320 ml	17-1194-01
HiPrep 16/60 Sephacryl S-200 HR	1 x 120 ml	17-1166-01
HiPrep 26/60 Sephacryl S-200 HR	1 x 320 ml	17-1195-01
HiPrep 16/60 Sephacryl S-300 HR	1 x 120 ml	17-1167-01
HiPrep 26/60 Sephacryl S-300 HR	1 x 320 ml	17-1196-01

All bulk media products are supplied in suspension in 20% ethanol.

Handbooks	
Gel Filtration: Principles and Methods	18-1022-18
Handbook Process Chromatography	18-1121-56

Columns

For information about BPG columns, please ask for the following Data Files.

Data File	Code No
BPG 100, 140, 200, 300	18-1115-23

For additional information, including Data File, application references and Regulatory Support File, please contact your local GE Healthcare representative.

9. Further information

Please read these instructions carefully before using Sephacryl High Resolution media.For further information visit www.gehealthcare.com or contact your local GE Healthcare representative.

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

GE Healthcare Bio-Sciences AB, a General Electric Company.

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