GE Healthcare
Life Sciences

Instructions 18-1060-29 AE **Gel filtration chromatography**

Superdex™ prep grade

Superdex prep grade (pg) is a preparative gel filtration chromatography medium; part of BioProcess™ media. BioProcess media are developed and supported for production scale chromatography. All BioProcess media are produced with validated methods and are tested to meet manufacturing requirements. Secure ordering and delivery routines give a reliable supply of media for production scale. Regulatory Support Files (RSF) are available to assist process validation and submissions to regulatory authorities.

These instructions contain information about Superdex pg characteristics, process operation (including column packing) and optimization, maintenance of the media, compatible equipment and troubleshooting.

To ensure best performance and trouble-free operation, please read these instructions carefully before using Superdex pg.

Table of contents

1 Media characteristics 3
2 Packing columns 6
3 Evaluation of column packing 11
4 Maintenance 14
5 Equipment 15
6 Method design and optimization 16
7 Troubleshooting guide 20
8 Ordering information 22

1 Media characteristics

Superdex pg is a preparative gel filtration medium with a composite matrix that combines the excellent gel filtration properties of cross-linked dextran (Sephadex™) with the physical and chemical stabilities of highly cross-linked agarose. Superdex pg is a separation medium with a mean particle size of 34 µm that yields steep selectivity curves and high resolution (see Fig 1).

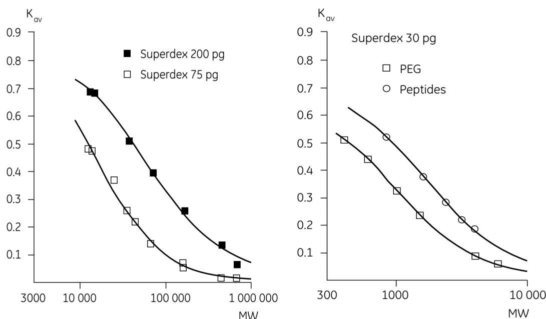

Fig 1. Selectivity curves for Superdex pg.

Typical flow velocity for Superdex pg is 10 to 50 cm/h. However, flow velocity and sample feed must be optimized for each separation method to ensure maximum productivity (see Section 6.) The pressure/flow velocity curves are shown in Figure 2.

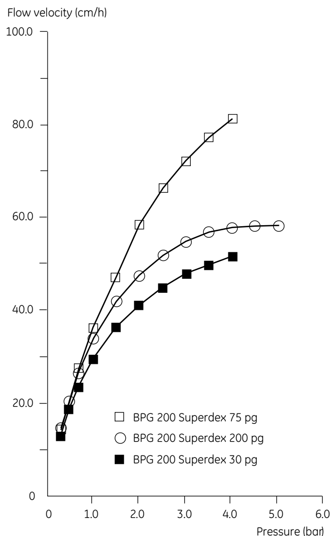

Fig 2. Pressure/flow velocity curves in BPG™ 200 with a bed height of 60 cm.

Stability

Superdex pg may be used in aqueous solutions with a pH range of 3 to12 for continuous operation, and pH 1 to 14 for cleaning-in-place (CIP). To prevent any non-specific interactions with the media, it is recommended to use an eluent with an ionic strength of at least 0.15 M. Chaotropic agents, detergents and polar organic solvents can also be used, but strong oxidizing agents should be avoided (see Table 1).

Table 1. Media characteristics

Separation range (MW):	
Superdex 30 pg	up to 10 000
Superdex 75 pg	3 000–70 000
Superdex 200 pg	10 000–600 000
Mean particle size	34 µm
Bead size range	24 to 44 µm >75%
Bead structure	composite of cross-linked agarose and dextran, spherical
Solutions in which the media is stable	all commonly used buffers 8 M urea 6 M guanidine hydrochloride
Solutions that can be used for cleaning	30% acetonitrile 30% isopropanol 1 M NaOH 1 M acetic acid 70% ethanol (Superdex 30 pg) 24% ethanol (Superdex 75 pg, Superdex 200 pg) 0.1 M hydrochloric acid (Superdex 30 pg)
pH stability:	
Working range	3 to 12
Cleaning-in-place	1 to 14
Autoclavable	at 121°C, 0.5 M NaCl pH 7 for 30 min

2 Packing columns

Superdex pg media are supplied pre-swollen. Replace the storage solution with packing solution before use.

Recommended columns

Lab-scale columns

- XK 16/20–XK 16/100 (16 mm i.d.), for bed volumes up to 190 ml and bed heights up to 95 cm.
- XK 26/20–XK 26/100 (26 mm i.d.), for bed volumes up to 510 ml and bed heights up to 95 cm.
- XK 50/20–XK 50/100 (50 mm i.d.), for bed volumes up to 1880 ml and bed heights up to 95 cm.

Large scale columns

- BPG variable bed, glass columns with 100–450 mm i.d., bed volumes from 2.4–131 L and bed heights up to 83 cm.

Process scale-up, while maintaining the linear flow velocity, can be easily achieved by increasing the column width, as long as the bed height is constant.

When packing Superdex pg media use:

- 10 µm nets in the column
- a pulse damper if a pulsating pump is used (e.g., a diaphragm pump). An air trap may be used as a pulse damper if it is not completely filled with liquid.

Packing recommendations

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

Column efficiency depends on the quality of the column packing. It is therefore, important to pack and test the column according to the following instructions.

Slurry preparation

Table 2 describes the packing parameters for a bed height of about 100 cm.

This is calculated as:

$$\frac{\text{Volume of the sedimented media}}{\text{Total volume of the media and the buffer}}$$

Calculate the exact amount of media needed using the above equation. Stir the media gently to make a homogenous slurry and pour the slurry into the column. The amount of media per liter of packed volume is 1.1 to 1.15 L sedimented media, depending on the column size.

Note: *Never use a motorized stirrer to make a homogenous slurry.*

. **Table 2** Packing instructions for Superdex pg

Column	Slurry concentration (%)	Slurry volume (ml)	Compression (%)	Packed bed height (ml)	Flow rate Step 1 (ml/min)	Time for packing Step 1 (min)
XK 16/100	52±2	430	15	93–94	3±0.5	90
XK 26/100	60±2	1 000	10	93–94	4±0.5	90
XK 50/100	65–70	3 000	10	94–95	10±2	120

Column	Pressure Step 2 (MPa)	Flow rate Step 2 (ml/min)	Time for packing Step 2 (min)	Maximum flow rate of the packed column (ml/min)	Maximum pressure of the packed column (MPa)
XK 16/100	0.45±0.05	430	30	3	0.35
XK 26/100	0.35±0.05	1 000	30	6	0.35
XK 50/100		20 + 5 (never <20)	30	16	0.30

Column preparation

Pump water into the column through the bottom inlet to remove any air trapped under the net. Sometimes, it might be necessary to suck the trapped air through the net with a tube connected to a pump. Close the bottom valve before switching off the pump.

Note: *Take care not to damage the net.*

Washing procedure

Superdex pg is supplied in a storage solution of 20% ethanol (Superdex 200 pg) or in 0.2 M sodium acetate in 20% ethanol (Superdex 30 pg and Superdex 75 pg). Ethanol affects the sedimentation properties of the media and hence, it must be washed off completely before packing the column.

A simple and convenient way to wash the media in the column is to attach the top adapter and wash the sedimented bed with two column volumes of water at a back-pressure of approximately 2 bar. Make sure that ethanol is completely washed out before starting to pack the column.

Note: *The media must be re-suspended after this washing step for efficient packing in the column.*

Packing the column

These instructions are for packing Superdex pg media in XK 16/100, XK 26/100 or XK 50/100 columns.

1 Pour the medium slurry into the column in one continuous motion along a glass rod held against the wall of the column. This prevents the introduction of air bubbles into the packed bed. Fill the remainder of the column and the reservoir with distilled water immediately. Mount the lid on the packing reservoir and connect it to the pump.

2 Open the column outlet and start step 1 of packing by pumping distilled water through the column according to the flow rate and time in Table 2. Start step 2 by choosing a flow rate or a pressure according to recommended values in Table 2. Adjust the flow rate to maintain a constant pressure as mentioned in step 2 of Table 2. Maintain the same flow rate for thirty minutes, which is the time taken for the media to settle and stabilize in

the column. Close the column outlet and, switch off and disconnect the pump.

3 Dismantle the column from the stand and remove the packing reservoir over a sink. Remount the column and fill with distilled water.

4 Wet the column adapter by submerging the plunger end in 20% ethanol, and drawing with a syringe. Ensure that all air bubbles have been removed. Insert the adapter at the top of the column, taking care not to trap air under the net.

5 Open the adapter outlet, push the adapter into the column and down onto the medium bed, allowing the distilled water to displace any trapped air in the tubing.

6 Lock the adapter in position, connect it to the pump, open the column outlet and continue packing at the maximum flow rate for twenty minutes.

7 Mark the position of the bed surface on the column. Close the column outlet and stop the pump. Reposition the adapter to approximately 3 mm below the marked position.

The column is now ready for equilibration.

If required, the quality of packing can be checked using the testing procedure described in Section 3.

3 Evaluation of column packing

Intervals

Test the column efficiency to check the quality of packing. Testing should be done after packing, at regular intervals during the working life of the column or 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 with 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 number of plates will vary according to the test conditions and 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, etc. will influence the results.*

Sample volume and flow velocity

For optimal results, the sample volume should be at maximum 2.5% 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:

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

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

L = bed height (cm)

N = number of theoretical plates

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 $0.7 < A_S < 1.3$).

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

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

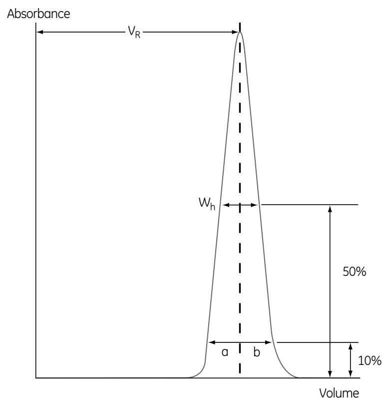

Fig 3. A typical test chromatogram showing the parameters used for HETP and A_s calculations.

4 Maintenance

This section describes maintenance procedures of Superdex pg media for a longer working life.

Cleaning-In-Place

Cleaning-in-place (CIP) is a cleaning procedure that removes contaminants such as lipids, precipitates or denatured proteins that might be trapped in the packed column after a number of separations. Regular CIP prevents the build-up of these contaminants in the media bed and also helps maintain the flow properties and general performance of the media.

The CIP protocol should be designed according to the type of contaminants present. Though the frequency of CIP depends on the nature and the condition of the starting material, one CIP cycle is generally recommended after every 5 separation cycles.

Sanitizing

Sanitizing the packed column with chemical agents inactivates microbial contaminants, such as vegetative cells. It also helps to maintain a high level of process hygiene.

For example, to sanitize the packed media against contaminants, wash the column with 0.5 M NaOH at a reversed flow velocity of 10 cm/h for an hour.

Remove NaOH after sanitizing

Before applying the sample, remove any traces of NaOH, by washing the column with at least two column volumes of buffer, or until pH is stable, at 20 cm/h with normal flow direction.

Sterilizing

Equilibrate the medium with 0.5 M NaCl, pH 7. Dismantle the column and autoclave the medium at 121°C for thirty minutes.

Sterilize the column parts according to instructions in the column manual. Re-assemble the column. Pack and test as described in Section 2 and Section 3.

Storing

The medium should be stored in 20% ethanol (Superdex 200 pg) or in 0.2M sodium acetate in 20% ethanol (Superdex 30 pg and Superdex 75 pg) at 2°C to 30°C for longer periods of time. However, the packed columns should be sanitized and equilibrated in working buffer containing 20% ethanol to prevent microbial growth before storage.

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

5 Equipment

Successful separations with Superdex pg media require suitable equipment:

- See Section 2 for a list of recommended columns.
- The buffer delivery system (pump, gradient mixers, tubing and valves) should be compatible and able to withstand the high flow velocities and pressures needed for packing and operating the column. They should also be resistant to the chemical agents used in the cleaning and sanitizing procedures.
- Equipment for monitoring the pressure, flow and UV absorption of the effluent should be connected to a recorder or a computer for accurate fractionation of the separated substances.

Contact your local GE Healthcare representative for more information about systems and equipment.

6 Method design and optimization

Gel filtration is widely used in process chromatography, particularly for polishing the final product, that is, removal of product aggregates, transfer of product to correct formulation buffer or desalting. Since molecules are separated according to differences in their size, gel filtration medium is selected on the basis of its separation range and resolution with respect to the molecular weight of the molecule of interest (see Fig 1).

Three factors contribute to the maximum productivity and maximum purity of a large scale gel filtration process:

- optimizing the method to ensure best resolution
- optimizing the process for high productivity
- scaling up

Optimizing 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 Fig 4).

Resolution is affected by flow velocity, column efficiency and bed height. The higher the flow velocity, the lower the resolution. The flow velocity at which optimal efficiency is obtained depends on the molecular weight of the molecule of interest. As a rule-of-thumb, larger molecules require lower flow velocity, while smaller molecules require higher flow velocity.

Column efficiency depends on the quality of the packed column. This can be calculated by determining HETP, see Section 3. The number of theoretical plates obtained (N) should be as high as possible. Typical values of >10 000 m^{-1} are observed for Superdex pg.

$$N = L/(HETP)$$

A poorly packed column will exhibit uneven flow, zone broadening and loss of resolution.

Bed height also affects resolution. A higher bed height improves the resolution. Typical bed height for Superdex pg is 60 cm.

Column size and sample volume are interdependent. Recommended sample volumes for Superdex pg lie between 0.5% and 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.

Process optimization

GE Healthcare offers a range of columns suitable for method development or small scale production, such as XK columns or BPG columns. All these columns have compatible bed heights and are suitable for process scale-up.

Note: *It is advisable to optimize the procedure at laboratory scale to save both time and material.*

GE Healthcare also offers a range of prepacked XK columns, known as HiLoad™ columns, as a convenient alternative for method development (see Section 8). Optimizing a gel filtration step for maximum productivity involves the following parameters:

- feed concentration
- flow velocity
- feed volume

Conditions which lead to maximum resolution are often in conflict with other experimental objectives, for example, some of the parameters optimized for maximum productivity also influence the resolution of separation. Therefore, any gel filtration step involves compromising either the resolution or the productivity.

Feed concentration should be as high as possible while keeping a check on the viscosity. Also, high sample concentrations can decrease resolution.

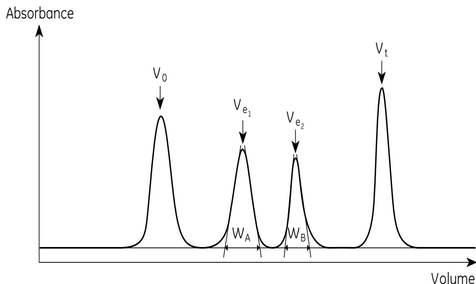

Fig 4. A typical gel filtration chromatogram showing substances eluting at different elution volumes.

$$K_{av} = (V_e - V_o)/(V_c - V_o)$$

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

where

V_e = elution volume

V_t = total liquid volume

V_c = geometric column volume

W_b = peak width at base

V_o = void volume

Flow velocity influences resolution. Increasing flow velocity will generally decrease resolution. The optimal flow velocity range varies with the gel filtration medium and the sample. As a rule of-thumb, smaller molecules can be separated at a 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 adsorption technique that gives a highly concentrated feed (for example, ion exchange chromatography).

For a test run, the following conditions are appropriate:

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 then adjust feed volume accordingly.

Scaling up

Process scale-up, usually in the order of 100-fold is done after optimizing the gel filtration step at laboratory scale. Simple scale up involves increasing the diameter of the column, while keeping the bed height constant. When scaling-up, some parameters remain constant while others increase.

Maintain:

- bed height
- flow velocity (cm/h)
- sample concentration and volume (in relation to bed volume)
- efficiency in terms of N

Increase:

- flow rate (ml/min)
- column diameter

Some deviations from the results at small scale may be observed due to the larger equipment employed during process scale-up. Check the buffer delivery system and the monitoring system for time delays or volume changes. Different lengths and diameters of outlet pipes can cause zone spreading on larger systems.

7 Troubleshooting guide

High back pressure

1. Check that all valves between the pump and the collection vessel are completely open.
2. Check that all valves are clean and free from blockage.
3. Check if equipment in use is generating backpressure, for example, due to 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 adapter and the media bed, or back-mixing of the sample before application.
5. Check the efficiency of the packed column (see Section 3).
6. Check if there has been any change in the sample pre-treatment method.

Contaminants

1. Check the connections and pre-filters.
2. Check the in-going components such as buffers, sample, etc.
3. Check if 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 normally has to be repacked.

However, if only a small amount of air has been trapped on top of the bed, or between the adapter net and head, it can be removed by pumping eluent in the opposite direction. After pumping with the reversed flow, check the efficiency of the packed bed (see Section 3) and compare with the original efficiency values.

8 Ordering information

Product	Pack size	Code No.
Superdex 30 pg	150 ml	17-0905-01
Superdex 30 pg	1 l	17-0905-03
Superdex 30 pg	5 l	17-0905-04
Superdex 75 pg	150 ml	17-1044-01
Superdex 75 pg	1 l	17-1044-02
Superdex 75 pg	5 l	17-1044-04
Superdex 200 pg	150 ml	17-1043-01
Superdex 200 pg	1 l	17-1043-02
Superdex 200 pg	5 l	17-1043-04
Superdex 200 pg	10 l	17-1043-05
Prepacked columns		
HiLoad 16/600 Superdex 30 pg	1 × 120 ml	28-9893-31
HiLoad 26/600 Superdex 30 pg	1 × 320 ml	28-9893-32
HiLoad 16/600 Superdex 75 pg	1 × 120 ml	28-9893-33
HiLoad 26/600 Superdex 75 pg	1 × 320 ml	28-9893-34
HiLoad 16/600 Superdex 200 pg	1 × 120 ml	28-9893-35
HiLoad 26/600 Superdex 200 pg	1 × 320 ml	28-9893-36

Superdex 200 pg is supplied as a suspension in 20% ethanol. Superdex 30 pg and Superdex 75 pg are supplied as a suspension in 0.2 M sodium acetate in 20% ethanol.

Related literature

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

Handbook	Code No
Gel Filtration: Principles and Methods	18-1022-18

Empty columns

For information about process scale columns, please ask for the following Data Files:

Data File	Code No
BPG 100, 140, 200, 300, 450	18-1115-23
XK empty columns	28-9976-59

For additional information, including data files, application references and regulatory support files, please contact your local GE Healthcare representative.

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First published Jun. 1994

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18-1060-29 AE 11/2011