cytiva

Superdex prep grade

Size exclusion chromatography

Instructions for Use

Superdex™ prep grade (pg) is a BioProcess™ preparative size exclusion chromatography resin. 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.

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

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Important
Read these instructions carefully before using the product.

Safety
For use and handling of the product in a safe way, refer to the Safety Data Sheet.

1 Resin characteristics

Superdex pg is a preparative size exclusion chromatography resin with a composite matrix that combines the excellent size exclusion chromatography properties of dextran with the physical and chemical stabilities of cross-linked agarose.
Superdex pg is a separation resin with a particle size d_{50V}[1] of approximately 34 µm that yields steep selectivity curves and high resolution
(see Fig 1).

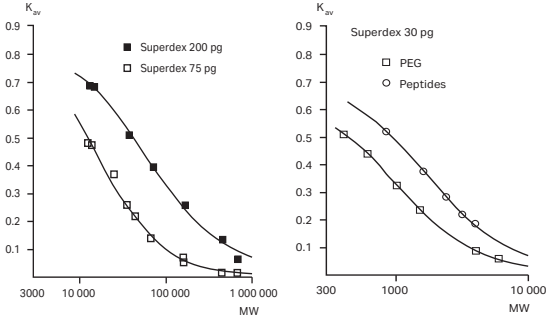


Fig 1. Selectivity curves for Superdex pg.

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 give maximum productivity
(see Section 6.) The pressure/flow velocity curve is shown in Figure 2.

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

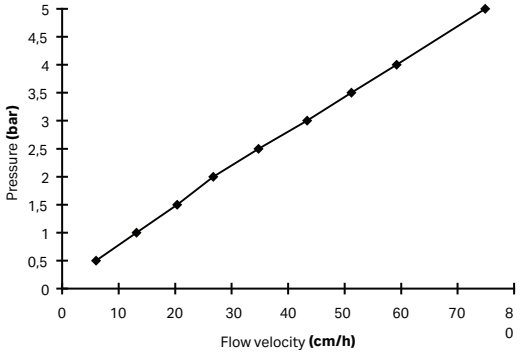


Fig 2. Pressure vs flow velocity for Superdex 200 prep grade in BPG 200 at 82.8 cm bed height.

Stability

Superdex pg can 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 nonspecific interactions with the resins, 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 avoid strong oxidizing agents (see Table 1).

Table 1. Resins characteristics

Fractionation range (M_r):	Dextrans	Globular proteins
Superdex 30 pg		up to ~ 10 000
Superdex 75 pg	~ 500 to 30 000	~ 3000 to 70 000
Superdex 200 pg	~ 1000 to 100 000	~ 10 000 to 600 000
Particle size, d_{50v}[1]	~ 34 µm	
Matrix	Cross-linked agarose, spherical	
Pressure/ flow characteristics	40 to 60 cm/h at <0.3 MPa in a BPG 200 column with 20 cm diameter and 83 cm bed height (at room temperature using buffers with the same viscosity as water).[2]	
Chemical stability	Stable to commonly used aqueous buffers: 8 M urea, 6 M guanidine hydrochloride, 30% acetonitril, 30% isopropanol, 0.01 M NaOH, 1 M acetic acid, 24% ethanol, 0.001 M hydrochloric acid, 1% SDS	
pH stability:		
Operational[3]	3 to 12	
CIP[4]	1 to 14	
Autoclavability	20 min at 121°C, 1 cycle	

[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] pH range where resin can be operated without significant change in function.
[4] pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.

2 Packing columns

Superdex pg resins are supplied preswollen. Replace the storage solution with packing solution before use.

Recommended columns

Lab-scale columns

- XK 16/20 to XK 16/100 (16 mm i.d.) for bed volumes up to 190 mL and bed heights up to 95 cm.
- XK 26/20 to XK 26/100 (26 mm i.d.) for bed volumes up to 510 mL and bed heights up to 95 cm.
- XK 50/20 to 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 to 450 mm i.d., bed volumes from 2.4 to 131 L and bed heights up to 83 cm.

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

When packing Superdex pg resins use:

- 10 µm nets in the column
- a pulse damper if a pulsating pump is used (e.g., a diaphragm pump). An air trap can 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.

The slurry concentration is calculated as:

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

Calculate the exact amount of resin needed using the above equation. Stir the resin gently to make a homogenous slurry and pour the slurry into the column. The amount of resin per liter of packed volume is 1.1 to 1.15 L sedimented resin, 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 to 4	2±1	90
XK 26/100	60±2	1000	10	93 to 94	3±1	90
XK 50/100	65 to 70	3000	10	94 to 95	10±2	120

Column	Pressure Step 2 (MPa)	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	0.4±0.1	30	3	0.35
XK 26/100	0.4±0.1	30	6	0.35
XK 50/100		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 0.2 M sodium acetate in 20% ethanol (Superdex 30 pg and Superdex 75 pg) or in 20% ethanol (Superdex 200 pg). Ethanol and especially sodium acetate affects the sedimentation properties of the resins and so it must be washed off completely before packing the column.

Superdex 30 pg and Superdex 75 pg

A simple and convenient way to wash the resin in the column is to attach the top adapter and wash the sedimented bed with at least four column volumes of 20% ethanol followed by at least four column volumes of packing solution (water, at a back pressure of approximately 2 bar). Make sure that the sodium acetate and ethanol is completely washed out before starting to pack the column.

Superdex 200 pg

Attach the top adapter and wash the sedimented bed with four column volumes of packing solution (water) at a back pressure of approximately 2 bar. Make sure that the ethanol is completely washed out before starting to pack the column.

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

Packing the column

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

1. Pour the resin 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 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 resin 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. Make sure 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 resin 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 must 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* (28937207).

Note: *The calculated number of plates will vary according to the test conditions and must 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 must 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} = Median particle size of the cumulative volume distribution (cm)

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

The peak must 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

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

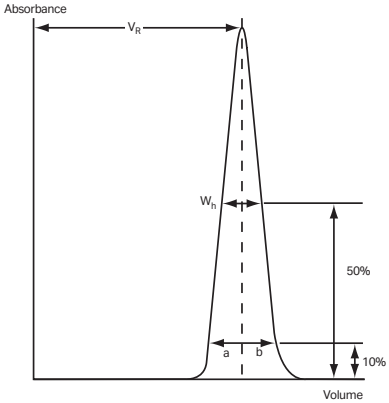


Fig 3. A typical test chromatogram showing the parameters used for HETP and A_s calculations.

4 Maintenance

This section describes maintenance procedures of Superdex pg resins 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 resin bed and also helps maintain the flow properties and general performance of the resin.

The CIP protocol must 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 resin 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 resin with 0.5 M NaCl, pH 7. Dismantle the column and autoclave the resin at 121°C for 20 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 resin must 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 4°C to 30°C for longer periods of time. However, the packed columns must 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 resins require suitable equipment:

- See Section 2 for a list of recommended columns.
- The buffer delivery system (pump, gradient mixers, tubing, and valves) must be compatible and able to withstand the high flow velocities and pressures needed for packing and operating the column. They must 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 must be connected to a recorder or a computer for accurate fractionation of the separated substances.

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

6 Method design and optimization

Size exclusion chromatography 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, size exclusion chromatography resin is selected on the basis of its fractionation 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 size exclusion chromatography process:

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

Optimizing for best resolution

The best resolution is obtained when the molecule of interest has an elution volume that 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 size exclusion chromatography 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

Cytiva 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.*

Cytiva also offers a range of prepacked XK columns, known as HiLoad™ columns, as a convenient alternative for method development (see Section 8). Optimizing a size exclusion chromatography 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 size exclusion chromatography 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.

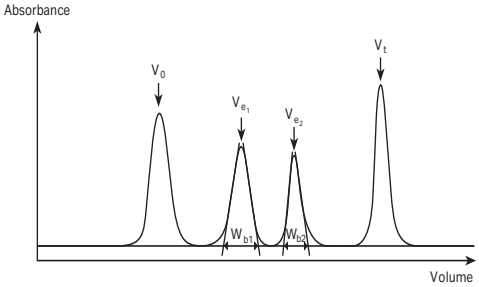


Fig 4. A typical size exclusion chromatography result showing substances eluting at different elution volumes.

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

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

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 size exclusion chromatography resin and the sample. As a rule of thumb, smaller molecules can be separated at a higher flow velocity.

Feed volume greatly influences resolution in size exclusion chromatography techniques and is thus, usually limited to approximately 4% of the total column volume.

It is often suitable to use size exclusion chromatography 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 size exclusion chromatography 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 can 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 resin.
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 resin 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 pretreatment 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	Product code
Superdex 30 pg	150 mL	17090501
Superdex 30 pg	1 L	17090503
Superdex 30 pg	5 L	17090504
Superdex 75 pg	150 mL	17104401
Superdex 75 pg	1 L	17104402
Superdex 75 pg	5 L	17104404
Superdex 200 pg	150 mL	17104301
Superdex 200 pg	1 L	17104302
Superdex 200 pg	5 L	17104304
Superdex 200 pg	10 L	17104305
Superdex 200 pg	60 L*	17104306
Prepacked columns		
HiLoad 16/600 Superdex 30 pg	1 × 120 mL	28989331
HiLoad 26/600 Superdex 30 pg	1 × 320 mL	28989332
HiLoad 16/600 Superdex 75 pg	1 × 120 mL	28989333
HiLoad 26/600 Superdex 75 pg	1 × 320 mL	28989334
HiLoad 16/600 Superdex 200 pg	1 × 120 mL	28989335
HiLoad 26/600 Superdex 200 pg	1 × 320 mL	28989336

*. Pack size available upon request

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	Product code
Size Exclusion Chromatography: Principles and Methods	18102218

Empty columns

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

Data File	Product code
BPG 100, 140, 200, 300, 450	18111523
XK empty columns	28997659

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

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