GE Healthcare

Instructions 71-5009-64 AE **Ion exchange chromatography**

CM Sepharose Fast Flow
DEAE Sepharose Fast Flow
Q Sepharose Fast Flow
SP Sepharose Fast Flow

CM, DEAE, Q and SP Sepharose™ Fast Flow ion exchangers are part of the range of separation media called BioProcess™ Media. BioProcess Media are developed and supported for process scale chromatography. All media are produced following validated methods and are tested to ensure they meet the performance requirements of industrial manufacturing. Regulatory Support Files contain information to assist process validation and submission to regulatory authorities. BioProcess Media are available for all purification steps from capture to polishing. To ensure best performance and trouble-free operation, please read these instructions before use.

Sepharose

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1. Characteristics of Sepharose Fast Flow ion exchangers

The base matrix of Sepharose Fast Flow ion exchangers is highly cross-linked agarose which gives the ion exchangers high chemical and physical stability. This means that characteristics such as capacity, elution behavior and pressure/ flow rate are unaffected by the solutions commonly used in process chromatography and cleaning procedures, for details see table under each respective ion exchanger. High physical stability gives good flow characteristics and low back pressures. Flow velocities ranging between 300 and 700 cm/h through a bed height of 15 cm at a pressure of 1 bar are typical for these media, see Fig 1. Furthermore, the high rigidity of the matrix minimizes volume variations during change of pH or ionic strength.

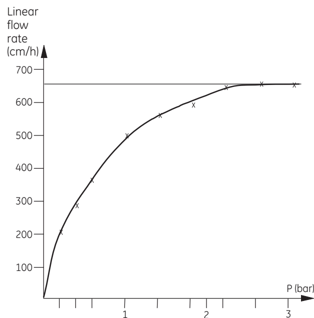


Fig 1. A typical pressure/flow rate curve for Sepharose Fast Flow ion exchangers.

Characteristics of CM Sepharose Fast Flow

CM Sepharose Fast Flow is a weak cation exchanger. The ion exchange group is a carboxy methyl group, see below.

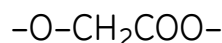


Table 1. Characteristics of CM Sepharose Fast Flow.

Property	Description
Ion exchange type	Weak cation
Total ionic capacity	0.09–0.13 mmol/ml media
Exclusion limit	4 x 106 (globular proteins)
Matrix	Cross-linked agarose, 6%
Bead form	Spherical, 45–165 µm
Flow rate	300–600 cm/h*
Working temperature	4–40°C
Working pH	See Figure 2.
pH stability	2–14 (short-term, CIP) 4–13 (long-term)
Chemical stability	All commonly used aqueous buffers 1 M NaOH 8 M urea 6 M guanidine hydrochloride 70% ethanol
The following should be avoided	Oxidizing agents Long exposures (1 week, 20°C) to pH <4

* 15 cm bed height, 1 bar, 25°C, XK 50/30 column.

15 cm bed height, 1 bar, 25°C, XK 50/30 column. The titration curve in Figure 2 shows the pH working range of CM Sepharose Fast Flow, i.e. the pH range in which the CM group is charged.

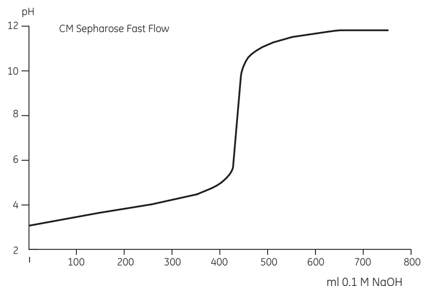
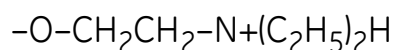


Fig 2. Titration curve of CM Sepharose Fast Flow.

Characteristics of DEAE Sepharose Fast Flow

DEAE Sepharose Fast Flow is a weak anion exchanger. The ion exchange group is a diethylaminoethyl group, see below.



$–O–CH_2CH_2–N^+(C_2H_5)_2H$

Table 2. Characteristics of DEAE Sepharose Fast Flow.

Property	**Description**
Ion exchange type	Weak anion
Total ionic capacity	0.11–0.16 mmol/ml media
Exclusion limit	4 × 106 (globular proteins)
Matrix	Cross-linked agarose, 6%
Bead form	Spherical, 45–165 µm

Flow rate	300–600 cm/h*
Working temperature	4–40°C
Working pH	See Figure 3.
pH stability	2–14 (short-term, CIP) 2–12 (long-term)
Chemical stability	All commonly used aqueous buffers 1 M NaOH 8 M urea 6 M guanidine hydrochloride 70% ethanol
The following should be avoided	Oxidizing agents Long exposures (1 week, 20°C) to pH <4

* 15 cm bed height, 1 bar, 25°C, XK 50/30 column.

The titration curve in Figure 3 shows the pH working range of DEAE Sepharose Fast Flow, i.e. the pH range in which the DEAE group is charged.

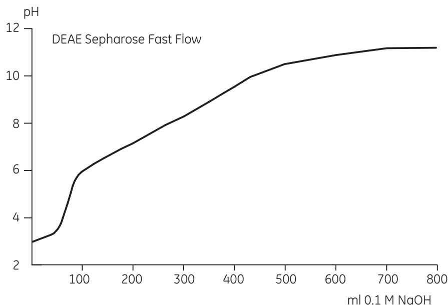
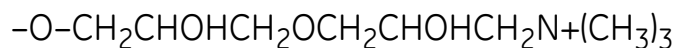


Fig 3. Titration curve of DEAE Sepharose Fast Flow.

Characteristics of Q Sepharose Fast Flow

Q Sepharose Fast Flow is a strong anion exchanger. The ion exchange group is a quaternary amine group, see below.



$-O-CH_2CHOHCH_2OCH_2CHOHCH_2N^+(CH_3)_3$

Table 3. Characteristics of Q Sepharose Fast Flow.

Property	Description
Ion exchange type	Strong anion
Total ionic capacity	0.18–0.25 mmol/ml media
Exclusion limit	4 x 106 (globular proteins)
Matrix	cross-linked agarose, 6%
Bead form	Spherical, 45–165 µm
Flow rate	400–700 cm/h*
Working temperature	4–40°C
Working pH	See Figure 4.
pH stability	2–14 (short-term, CIP) 2–12 (long-term)
Chemical stability	All commonly used aqueous buffers 1 M NaOH 8 M urea 6 M guanidine hydrochloride 70% ethanol
The following should be avoided	Oxidizing agents Long exposures (1 week, 20°C) to pH <4

*15 cm bed height, 1 bar, 25°C, XK 50/30 column.

The titration curve in Figure 4 shows the broad pH working range of Q Sepharose Fast Flow, i.e. the pH range in which the Q group is charged.

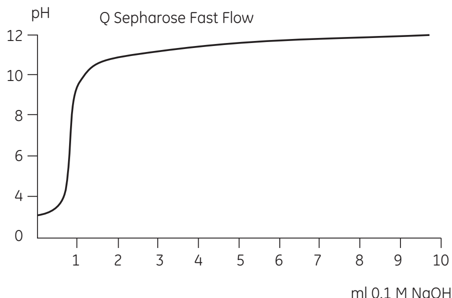
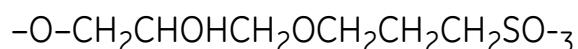


Fig 4. Titration curve of Q Sepharose Fast Flow.

Characteristics of SP Sepharose Fast Flow

SP Sepharose Fast Flow is a strong cation exchanger. The ion exchange group is a sulphopropyl group, see below.



$-O-CH_2CHOHCH_2OCH_2CH_2CH_2SO\text{-}_3$

Table 4. Characteristics of SP Sepharose Fast Flow.

Property	Description
Ion exchange type	Strong cation
Total ionic capacity	0.18–0.25 mmol/ml media
Exclusion limit	4 x 106 (globular proteins)
Matrix	Cross-linked agarose, 6%
Bead form	Spherical, 45–165 µm
Flow rate	400–700 cm/h*
Working temperature	4–40°C

Working pH	See Figure 5
pH stability	3–14 (short-term, CIP) 4–13 (long-term)
Chemical stability	All commonly used aqueous buffers 1 M NaOH 8 M urea 6 M guanidine hydrochloride 70% ethanol
The following should be avoided	Oxidizing agents Long exposures (1 week, 20°C) to pH <4

*15 cm bed height, 1 bar, 25°C, XK 50/30 column

The titration curve in Figure 5 shows the broad pH working range of SP Sepharose Fast Flow, i.e. the pH range in which the SP group is charged.

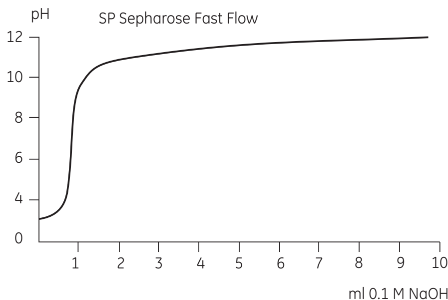


Fig 5. Titration curve of SP Sepharose Fast Flow.

2. Column packing guidelines

Sepharose Fast Flow ion exchangers CM, DEAE and Q are supplied in suspension in 20% ethanol as standard shipping solution. SP Sepharose Fast Flow is supplied in suspension in 20% ethanol and 0.2 M sodium acetate as standard. Large pack sizes of the media in 2% benzyl alcohol (2% benzyl alcohol and 0.2 M sodium acetate for SP Sepharose Fast Flow) are available on request.

Decant the shipping solution and replace with starting buffer before use.

Recommended columns

The following columns from GE Healthcare are recommended for use with Sepharose Fast Flow ion exchangers.

Laboratory scale columns:

Tricorn columns. Inner diameter 5 mm and 10 mm and bed volumes up to 12 ml at bed heights of 15 cm.

XK columns. Inner diameters of 16 mm and 26 mm and bed volumes up to 80 ml at bed heights of 15 cm.

A step-by-step demonstration of column packing can be seen in "Column Packing – The Movie" available in a CD format, see ordering information.

Pilot and Production scale columns:

As with all ion exchange separations, a short wide column is ideal. This shape minimizes back pressure and increases throughput.

GE Healthcare columns are developed with these considerations in mind. The design of our columns also facilitates quick and efficient distribution of the sample at the top of the media bed and collection of separated zones at the outlet.

Recommended columns are:

- BPG™ 100, 200 or 300 Columns (available for media volumes up to 45 liters). BPG columns are fitted with one adaptor.
- INdEX™ variable bed columns: inner diameters from 70 to 200 mm; bed volumes up to 25 liters; bed heights max 61 cm.
- Chromaflow™ variable and fixed bed columns. Inner diameters 280 to 2000 mm.

Packing recommendations

The packing method used depends on the type of chromatography media, the type of column, and the equipment in use. Always read and follow the relevant column instruction manual carefully.

Sepharose Fast Flow ion exchangers are easy to pack since their rigidity allows the use of high flow rates (Figure 1). Recommended bed heights are 10 to 15 cm. The different packing methods that are suitable for Sepharose Fast Flow ion exchangers are listed below.

- Pressure packing: for columns with adaptors, for example BPG columns.
- Hydraulic pressure packing, for example, INdEX columns.
- Chromaflow packing method for standard Chromaflow columns.

Packing guidelines

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. Always begin by determining the optimal packing flow rate. Guidelines for determining optimal packing flow rates for columns with adaptors and fixed bed heights are given below.

Determining optimal packing flow rates

The optimal packing flow rate is dependent on temperature, column size and type, media batch and volume. As a result, the optimal packing flow rate must

be determined empirically for each individual system. To determine the optimal packing flow rate, 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 liter packed volume is approximately 1.15 liters sedimented media.
2. Set up the column as for packing according to the instructions in the column manual.
3. Begin packing the media at a low flow rate (30 cm/h).
4. Increase the pressure in increments and record the flow rate when the pressure has stabilized. Do not exceed the maximum pressure of the column, or the maximum flow rate for the media.
5. The maximum flow rate 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 rate. The optimal packing flow rate/pressure is 70–100% of the maximum flow rate/pressure.
6. Plot the pressure/flow rate curve as in Figure 1 and determine the optimal packing flow rate. The operational flow rate/pressure should be <70% of the packing flow rate/pressure.

Packing your column

Follow the detailed instructions in the Column User Manual which is supplied with your column. Copies can be ordered from your local representative.

3. Evaluating the 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, A_S. These values are easily determined by applying a sample such as 1% acetone solution to the column. (Colored compounds and salt solutions should be avoided since they may interact with the media.)

It is important 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 rate, liquid pathway, temperature, etc. will influence the results. For optimal results, the sample volume should be at maximum 1.0% of the column volume, and the linear flow rate 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 AS

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

Conditions

Sample volume:	1.0–2.0% of bed volume
Sample conc:	1.0% (v/v) acetone in water, 0.8 M NaCl or 10x buffer
Eluent:	water, 0.5 M NaCl in water or dilute buffer
Flow rate:	20–30 cm/h
Detection:	
Acetone:	UV 280 nm;
NaCl, buffer:	Conductivity

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

$$HETP = L/N$$

and $$N = 5.54\ (VR/Wh)^2$$

where VR = Retention volume

Wh = Peak width at half peak height

L = Bed height

N = Number of theoretical plates

VR and Wh 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 thus:

$$HETP / d$$

where d is the diameter of the bead. As a guide, a value of <3 is normally acceptable.

The peak should be symmetrical, and the asymmetry factor as close to 1 as possible (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 6 shows a UV trace for acetone in a typical test chromatogram in which the HETP and A_S values are calculated.

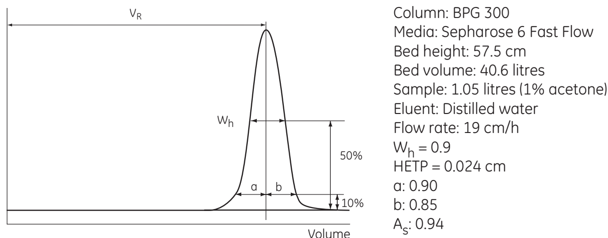


Fig 6. UV trace for acetone in a typical test chromatogram showing the HETP and A_S value calculations.

4. Maintenance

For best performance of Sepharose Fast Flow ion exchangers 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.

Standard CIP protocol

Ionically bound proteins	Wash with 0.5 column volumes of filtered 2 M NaCl. Contact time 10–15 min. Reversed flow direction.
Precipitated, hydrophobically	
bound proteins or lipoproteins	Wash with 1 M NaOH at 40 cm/h. Contact time 1–2 hours.
Lipids and very hydrophobic proteins	Wash with 2–4 column volumes of 0.5% non-ionic detergent (e.g. 1 M acetic acid). Contact time 1–2 hours. Reversed flow direction. Alternatively, wash with 2–4 column volumes of up to 70% ethanol* or 30% isopropanol. Contact time 1–2 hours. Reversed flow direction.

(* Specific regulations may apply when using 70% ethanol since it can require the use of explosion-proof areas and equipment.)

Sanitization

To reduce microbial contamination in the packed column, sanitization using 0.5–1.0 M NaOH with a contact time of 1 hour is recommended. The CIP protocols above will sanitize the media as well as remove bound contaminants.

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 to +30°C. Ensure that the screw-top is fully tightened. Packed columns should be equilibrated in working buffer containing 20% ethanol (+ 0.2 M sodium acetate for SP Sepharose Fast Flow) to prevent microbial growth.

5. Process optimization

For further information and details about process optimization and scale up, please consult the following handbooks produced by GE Healthcare:

Ion Exchange Chromatography and Chromatofocusing: Principles and

Methods, code number 11-0004-21.

Handbook of Process Chromatography, code number 18-1121-56.

6. Troubleshooting 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 rate.

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 13.

6 Check if there have been any changes in the pre-treatment of the sample.

Table 5. How the experimental conditions of an ion exchange cycle affect the main parameters of a separation step. Optimizing the key parameter helps to meet the goal of the step and contributes to an efficient and economic separation scheme.

	Main goal of a particular step			
Conditions	**Selectivity**	**Efficiency (Theoretical Plates)**	**Capacity**	
			Throughput (g/h)	Binding capacity (g/l media)
Adsorption pH	Great influence		Influence	Great influence
Desorption pH	Great influence			
Adsorption conductivity (ms-1)				Large increase
Gradient shape	Influence	Influence	Influence	
Increase flow rate (cm/h)		Decrease	Large increase	Decrease
Increase bed height (cm)	Increase	Increase	Decrease	
Increasing column diameter (cm)			Large increase	
Decreasing particle diameter (µm)		Large increase		Increase

Infections

1 Check the connections and prefilters.

2 Check the used fluids 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 13) and compare the result with the original efficiency values.

7. Ordering information

Product	Pack size	Code No
SP Sepharose Fast Flow Supplied in suspension in 20% ethanol and 0.2 M sodium acetate.	25 ml 300 ml 10 liters 60 liters	17-0729-10 17-0729-01 17-0729-05 17-0729-60
Q Sepharose Fast Flow Supplied in suspension in 20% ethanol.	25 ml 300 ml 10 liters 60 liters	17 0510-10 17 0510-01 17 0510-05 17 0510-60
DEAE Sepharose Fast Flow Supplied in suspension in 20% ethanol.	25 ml 500 ml 10 liters 60 liters	17 0709-10 17 0709-01 17 0709-05 17 0709-60
CM Sepharose Fast Flow Supplied in suspension in 20% ethanol.	25 ml 500 ml 10 liters 60 liters	17 0719-10 17 0719-01 17 0719-05 17 0719-60

Large pack sizes of the media in 2% benzyl alcohol (2% benzyl alcohol and 0.2 M sodium acetate for SP Sepharose Fast Flow) are available on request. Contact your local GE Healthcare representative for further information.

Handbooks

Ion Exchange Chromatography and Chromatofocusing:

Handbook	Code No
Principles and Methods	11-0004-21
Handbook of Process Chromatography	18-1121-56
Column Packing – The Movie (Tricorn and XK columns)	18-1165-33

Columns

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

Column	Code No
BPG 100, 140, 200, 300	18-1115-23
BPG 450	18-1060-59
INdEX	18-1115-61
Chromaflow	18-1118-84

For additional information, including Application Notes, References and Regulatory Support Files, please contact your local GE Healthcare representative.

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