Butyl-S **Sepharose** 6 Fast Flow

HIC resins

Instructions for Use

Butyl-S Sepharose™ 6 Fast Flow Flow is a separation resin for hydrophobic interaction chromatography (HIC). Substances are separated on the basis of their different hydrophobicity. Butyl-S Sepharose 6 Fast Flow belongs to Cytiva's range of BioProcess™ resins. 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.

This instruction contains information about resin characteristics as well as column packing, testing and maintenance.

To ensure best performance and trouble-free operation, read these instructions carefully before using Butyl-S Sepharose 6 Fast Flow.

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Read these instructions carefully before using the products.

Safety

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

1 Characteristics

Introduction

The base matrix, Sepharose 6 Fast Flow, is a crosslinked, 6% agarose derivative with excellent flow characteristics, making it ideal for process-scale applications, particularly the initial capture and intermediate purification stages of a separation when high flow rates are required.

The high physical and chemical stabilities of the matrix prevent bed compression and the formation of fines, and allow efficient maintenance procedures for increased lifelength. Figure 1 shows a typical pressure/flow curve for Butyl-S Sepharose 6 Fast Flow. Table 1 lists its main characteristics.

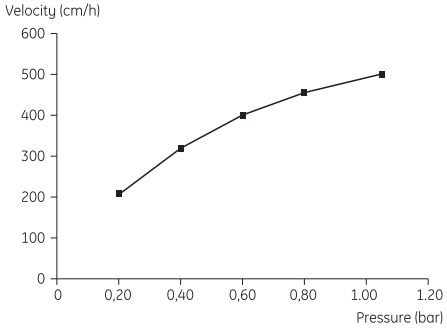


Fig 1. Typical pressure/flow curve for Butyl-S Sepharose 6 Fast Flow in an XK 50/30 column, bed height 15 cm; mobile phase 0.1 M NaCl

Table 1. Characteristics of Butyl-S Sepharose 6 Fast Flow

Matrix	Cross-linked agarose, 6%, spherical
Type of ligand	Butyl-S:-S-$(CH_2)_3$-CH_3 attached via a 12-atom spacer
Ligand concentration	~ 10 µmol ligand/mL resin
Particle size, d_{50V} [1]	~ 90 µm
Pressure/flow characteristics	250-400 cm/h at < 0.1 MPa in a XK 50/60 column with 5 cm diameter and 25 cm bed height (at 20°C using buffers with the same viscosity as water)[2],[3]
pH stability, operational[4]	3 to 13
pH stability, CIP[5]	2 to 14
Chemical stability	Stable to commonly used aqueous buffers, 1 mM HCl, 1.0 M NaOH[6], 30% isopropanol, 6 M guanidine hydrochloride, 8 M urea
Autoclavability	20 min at 121°C in 0.05 M sodium phosphate pH 7, 1 cycle
Operating temperature	4°C to 40°C
Delivery conditions	20% ethanol

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 Pressure/flow test performed on the base matrix.

4 pH range where resin can be operated without significant change in function.

5 pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function.

6 1.0 M NaOH should only be used for cleaning purposes

Method design and optimization

The main purpose of optimizing a chromatographic step is to reach the predefined purity level with the highest possible product recovery by choosing the most suitable combination of critical chromatographic parameters. In process chromatography, in contrast to analytical or small-scale preparative chromatography, this has to be accomplished as quickly and economically as possible, i.e., finding the conditions that give the highest possible productivity and process economy.

Recommendations for optimizing the critical operational parameters that maximize the use of a HIC step can be found in our handbook 11001269, or in related literature available from your local Cytiva office.

2 Column packing

General guidelines

Purifying biological macromolecules by HIC is a typical high selectivity technique where the difference in retention for the molecules to be separated can be substantial at any specific ionic strength.

Therefore, relatively short columns can be used if the selectivity of the adsorbent is exploited in an optimal way. Typical bed heights range from 3 to 15 cm, which also minimizes back-pressure and allows high throughput.

Recommended columns

Laboratory-scale columns

- Tricorn™, i.d. 5-10 mm, bed volumes up to 24 mL, bed heights up to 30 cm
- HiScale™, i.d. 16-50 mm, bed volumes up to 785 mL, bed heights up to 40 cm
- XK, i.d. 16-50 mm, bed volumes up to 559 mL, bed heights up to 28 cm

Large-scale columns

- BPG, i.d. 100-450 mm, bed volumes up to 130 L, bed heights up to 58 cm
- CHROMAFLOW™, i.d. 280-2000 mm, bed volumes up to 1570 L, bed heights up to 50 cm
- AxiChrom™, i.d. 50-200 mm, bed volumes up to 16.7 L, bed heights up to 50 cm
- AxiChrom, i.d. 300-1600 mm, bed volumes up to 1005 L, bed heights up to 50 cm

Packing laboratory-scale columns

Step	Action
1	Assemble the column (and packing reservoir if necessary).
2	Remove air from the column dead spaces by flushing the end-piece and adapter with packing buffer. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with packing buffer.

Step	Action
3	Re-suspend resin stored in its container by shaking (do not stir sedimented resin). Mix the packing buffer with the resin to form a 50% to 70% slurry (sedimented bed volume/slurry volume = 0.5–0.7).
4	Pour the slurry into the column in a single continuous motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
5	If using a packing reservoir, immediately fill the remainder of the column and reservoir with packing buffer. Mount the adaptor (or lid of the packing reservoir) and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.

Step	Action
6	Open the bottom outlet of the column and set the pump to run at the desired flow rate. Ideally, Sepharose 6 Fast Flow based resins are packed at a constant pressure of approximately 1.5 bar (0.15 MPa). If the packing equipment does not include a pressure gauge, use a packing flow velocity of approximately 500 cm/h (10 cm bed height, 25 °C, low viscosity buffer). If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate the pump can deliver. This should also give a reasonably well-packed bed. ***Note:*** *Do not exceed 75% of the packing flow velocity in subsequent chromatographic procedures using the same pump.*
7	When the bed has stabilized, close the bottom outlet and stop the pump.
8	If using a packing reservoir, disconnect the reservoir and fit the adapter to the column
9	With the adapter inlet disconnected, push the adapter down approximately 2 mm into the bed. Allow packing solution to flush the adapter inlet.
10	Connect the pump, open the bottom outlet and continue packing. The bed will compress further and a space will form between the bed surface and the adapter.

Step	Action
11	Close the bottom outlet. Disconnect the column inlet and lower the adapter approximately 2 mm into the bed. Connect the pump.

Equilibration

To equilibrate, pump approximately 100 mL of start buffer through the column at a flow rate of 3.5 mL/min. The column is fully equilibrated when the pH and/or conductivity of the effluent is the same as the start buffer.

Sample preparation

The amount of sample that can be applied to the column differs considerably, depending on the degree of substitution of the resin, the nature of the sample and on start buffer conditions.

High ligand density does not necessarily correspond to high capacity for adsorption of protein, but a high ligand density can encourage multi-point attachment of proteins which otherwise might have difficulty adsorbing to lower ligand densities. A moderate ligand density allows selective binding of the protein of interest by adjustment of the binding buffer concentration.

The sample should be dissolved in start buffer. Alternatively the sample may be transferred to start buffer by dialysis or by buffer exchange using a HiTrap™ Desalting or a PD-10 Desalting columns. The viscosity of the sample should not exceed that of the buffer. For normal aqueous buffer systems, this corresponds to a protein concentration of approximately 50 mg/ml.

Before application the sample should be centrifuged or filtered through a 0.45 µm filter to remove any particulate matter.

Operating flow rates

The flow rate used for sample binding and subsequent elution will depend on the degree of resolution required, but is normally within the range 5 to 10 mL/min for Sepharose 6 Fast Flow resins. The lower the flow rate, the better the resolution.

Binding

The binding of proteins to hydrophobic resin is influenced by:

- the structure of the ligand (e.g., carbon chain or an aromatic ligand)
- the ligand concentration
- the ionic strength of the buffer
- the salting-out effect (see The Hofmeister series below)
- the temperature

Those salts which cause salting-out (e.g., ammonium sulphate) also promote binding to hydrophobic ligands. The column is equilibrated and the sample is applied in a solution of high ionic strength. A typical starting buffer is 1.7 M $(NH_4)_2SO_4$, which is just below the concentration employed for salting-out proteins.

Hydrophobic interactions are weaker at lower temperatures. This must be taken into account if chromatography is done in a cold room.

Elution

Bound proteins are eluted by reducing the strength of the hydrophobic interaction. This can be done by:

- reducing the concentration of salting-out ions in the buffer with a decreasing salt gradient (linear or step)
- increasing the concentration of chaotropic ions in the buffer with an increasing gradient (linear or step)
- eluting with a polarity-reducing organic solvent (e.g., ethylene glycol) added to the buffer
- eluting with detergent added to the buffer

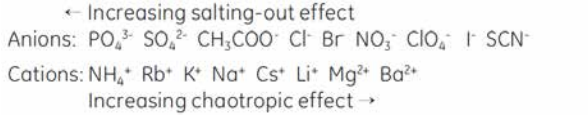


Fig 2. The Hofmeister series

Increasing the salting-out effect strengthens hydrophobic interactions; increasing the chaotropic effect weakens hydrophobic interactions.

A suggested starting gradient is a linear gradient from 0% to 100% B with:

Buffer A: 50 mM phosphate buffer, pH 7.0 + 1.7 M $(NH_4)_2SO_4$

Buffer B: 50 mM phosphate buffer, pH 7.0

Note: *When working with proteins which have a tendency to aggregate, start with a lower $(NH_4)_2SO_4$ concentration to avoid protein precipitation*

Packing process-scale columns

General packing procedures

Columns can be packed in different ways depending on the type of column and equipment used. Always read and follow the column instruction manual carefully. Sepharose 6 Fast Flow based resins are easy to pack since their rigidity allows the use of high flow rates (see *Figure 1, on page 3*).

The following parameters refers to large-scale packing.

Preferred packing solution: 10% to 20% ethanol

Resin slurry concentration: 50%

Packing pressure Sepharose 6 Fast Flow: 1.3 to 1.5 bar

Packing flow velocity Sepharose 6 Fast Flow: 400 to 600 cm/h

Two types of packing methods are described:

- Pressure packing (for columns with adapters)
- Chromaflow packing

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 rate. Guidelines are given below for determining the optimal packing flow rates for columns with adapters and fixed bed heights.

Determining optimal packing flow rates

The optimal packing flow rate depends column size and type, medium volume, packing solution, and temperature. The optimal packing flow rate must therefore be determined empirically for each individual system.

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

Step	Action
1	Calculate the amount of resin needed for the slurry (this is especially important for columns with fixed bed heights). The quantity of resin required per liter packed volume is approximately 1.15 L sedimented resin
2	Prepare the column as for column packing.
3	Begin packing the resinium at a low flow velocity (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 medium.
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% to 100% of the maximum flow rate/pressure.
6	Plot the pressure/flow rate curve as in Fig. 1 and determine the optimal packing flow rate.

The operational flow rate/pressure should be no greater than 70% of the packing flow rate/pressure.

Packing methods

Pressure packing

BPG columns

BPG glass columns are supplied with a movable adapter. They are packed by conventional pressure packing by pumping the packing solution through the chromatographic bed at a constant flow rate (or back pressure).

Step	Action
1	Pour some water (or packing solution) into the column. Make sure that no air is trapped under the bottom net. Leave about 2 cm of liquid in the column.
2	Mix the packing buffer with the resin to form a 50% to 70% slurry. (Sedimented bed volume/slurry volume = 0.5–0.7). Pour the slurry into the column. Insert the adapter and lower it to the surface of the slurry making sure no air is trapped under the adapter. Secure the adapter in place.
3	Seal the adapter O-ring and lower the adapter a little further into the slurry to fill the adapter inlet with packing solution.
4	Connect a pump and a pressure gauge and start packing at the predetermined packing flow rate (or pressure). Keep this flow rate (or pressure) constant during packing and check the pressure at the column inlet. Never exceed the pressure limit for column or resin.

Step	Action
5	When the bed has stabilized, 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 adapter to 0.5 to 1 cm above the bed 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 bed surface and adapter when the bed has stabilized.
7	Close the bottom valve, stop the pump, disconnect the column inlet and push the adapter down to approximately 3 mm below the mark on the column tube without loosening the adapter O-ring. The packing solution will flush the adapter inlet. Remove any trapped air by pumping liquid from the bottom (after the inlet tubing and the bottom valve have been properly filled).

Packing Chromaflow columns

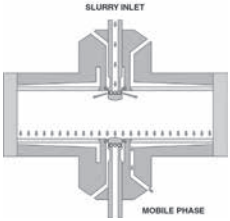
Prepare the column for packing as described in the User Manual.

Packing from the top

Step	Action
1	Set the top nozzle to the packing position (mid-position).
2	Fully retract the bottom nozzle (running position).

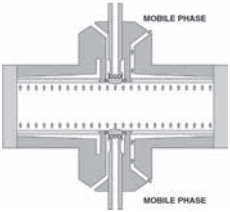
Step	Action
3	Make sure that the top mobile phase is closed.
4	Open the bottom mobile phase.
5	Open Inlet **C** and start the packing pump. Adjust the flow rate to achieve the required packing conditions for the selected resin. Monitor column pressure and the outlet flow rate to record column packing parameters. (Remember to stir the resin slurry during packing to prevent it from settling.)
6	Continue pumping until the column is fully packed and the pump stalls due to build-up of resin in its pipelines. Turn off the packing pump.
7	Fully retract the top nozzle to its run position. Close Outlet (**C**). Open Inlet (**B**) from the water/buffer tank and open Outlet (**D**). Restart the pump to rinse the top slurry lines. (If the nozzle is full of liquid when in the packing position, make sure that the waste slurry outlet is open before retracting the nozzle.)
8	To clean-in-place, exchange the buffer tank for a wash/ buffer tank containing cleaning solution.

Table 2. Principle of operation – Chromaflow columns



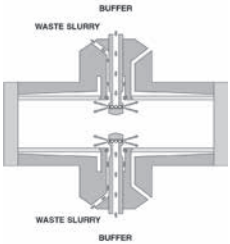
Packing position

The top nozzle is extended part of the way (mid position) into the column. The bottom nozzle is fully retracted. Slurry enters the column via the top nozzle and excess liquid exits via the bottom mobile phase outlet. After packing, the slurry lines are isolated from the mobile phase and can be cleaned independently from the rest of the column.



Running position

The bottom and top nozzles are retracted. Mobile phase enters the column directly into an annulus, immediately behind the bed support. The annulus is cut through at an angle to ensure that linear flow rate is kept constant during distribution of the mobile phase across the bed.



Unpacking position

In this position, both bottom and top nozzles are fully extended into the column, thereby exposing a third passage through which medium leaves the column.

Cleaning solution can be pumped through the nozzles and sprayed into the column. In this way the column is easily and effectively cleaned without exposing the interior or the resin to the environment, or without dismantling the column.

Packing AxiChrom columns

AxiChrom columns are packed using mechanical axial compression packing. For more information regarding the AxiChrom columns and packing procedures see below documentation:

- *AxiChrom Columns* 28929041
- *Operating Instructions* 28933108
- *Operating Instructions* 28943123
- *Operating Instructions* 29065430

3 Evaluation of packed column

Introduction

The packing quality needs to be checked by column efficiency testing. The test must be done after the packing, and at regular intervals during the working life of the column, and also when the separation performance is deteriorated.

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). The values are easily determined by applying a test sample such as 1% acetone solution or sodium chloride to the column.

Note: *Use a concentration of 0.8 M NaCl in water as sample and 0.4 M NaCl in water as eluent.*

The calculated plate number depends on the test conditions and must only be used as a reference value. It is important that the test conditions and the equipment are the same so that the results are comparable.

Note: *Changing the solute, solvent, eluent, sample volume, flow velocity, liquid pathway, temperature, chromatography system, etc., influence the results.*

For more information about column efficiency testing, consult the *application note 28937207*.

For optimal column efficiency results, the sample volume must be approximately 1% of the column volume and the flow velocity 30 cm/h. If an acceptance limit is defined in relation to column performance, the column plate number can be used as one of the acceptance criteria for the 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 $0.8 < A_S < 1.5$.

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

Peak asymmetry factor calculation:

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

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

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

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

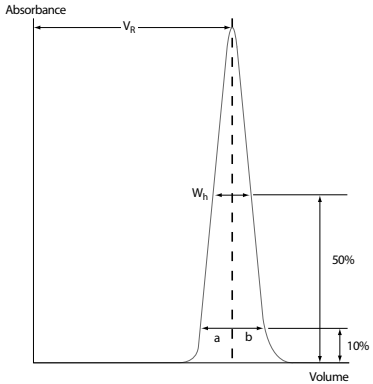


Fig 3. A typical test chromatogram showing the parameters used for HETP and A_S calculations.

4 Resin and column maintenance

Regeneration

For best performance from the resin, wash bound substances from the column after each chromatographic cycle.

Wash with 2 bed volumes of water, followed by 2 to 3 bed volumes of starting buffer.

To prevent a slow build up of contaminants on the column over time, you may have to apply more rigorous cleaning protocols on a regular basis.

Cleaning-in-place (CIP)

CIP removes very tightly bound, precipitated or denatured substances generated during previous production runs. In some applications, substances such as lipids or denatured proteins may remain in the column bed and not be eluted by regeneration. You should therefore develop CIP protocols for the types of contaminants known to be present in the feed. Recommended procedures for removing specific contaminants are described below. CIP procedures can normally be carried out for hundreds of cycles without affecting column performance.

Suggested protocol to remove precipitated proteins:

- Wash the column with 4 bed volumes of 0.5 to 1.0 M NaOH at 40 cm/h, followed by 2 to 3 bed volumes of water.

Suggested protocol to remove tightly bound hydrophobic proteins, lipoproteins and lipids:

- Wash the column with 4 to 10 bed volumes of up to 70% ethanol or 30% isopropanol followed by 3 to 4 bed volumes of water. (Apply gradients to avoid air bubbles forming when using high concentrations of organic solvents.)
- Alternatively, wash the column with detergent in a basic or acidic solution. Wash at a flow velocity of 40 cm/h. Remove residual detergent with 5 bed volumes of 70% ethanol followed by 3 to 4 bed volumes of water.

CAUTION
70% ethanol can require the use of explosion-proof areas and equipment.

To remove other contaminants, the following method is suggested:

- Wash the column with 4 bed volumes of 0.5 to 1.0 M NaOH at 40 cm/h, followed by 2 to 3 bed volumes of water.

The CIP protocols given above should be used as guidelines when formulating a cleaning protocol specific for the raw material used. The frequency of CIP will depend on the raw material applied to the column, but we recommend to use a CIP procedure at least every 5 cycles during normal use. Depending on the nature of the contaminants, different protocols may have to be used in combination. If fouling is severe, the protocols may have to be further optimized. During CIP, reverse the flow direction through the column.

Sanitization

Sanitization is the reduction microbial contamination in the column and related equipment to an acceptable minimum. A specific sanitization protocol should be designed for each process according to the type of contaminants present. The following is a recommended protocol.

Wash the column with 0.5 to 1.0 M NaOH at a flow velocity of approximately 40 cm/h, contact time 30 to 60 minutes.

Storage

For column storage, wash with 5 column volumes of distilled water followed by 5 column volumes of 20% ethanol. Degas the ethanol/water mixture thoroughly and apply at a low flow rate to avoid overpressuring the column. Make sure that the column is sealed well to avoid drying out. Whenever possible use a storage and shipping device, if supplied by the manufacturer. Store columns and unused resin at 4°C to 30°C in 20% ethanol.

5 Ordering information

Resin and prepacked columns

Product	Pack size	Product code
Butyl-S Sepharose 6 Fast Flow	25 mL	17097810
	200 mL	17097802
	1 L[1]	17097803
	5 L[1]	17097804
HiTrap Butyl-S FF	5 x 1 mL	17097813
HiTrap Butyl-S FF	5 x 5 mL	17097814

[1] Pack sizes available upon request

Empty laboratory-scale columns

Product	Quantity	Product code
Tricorn 5/20	1	28406408
Tricorn 5/50	1	28406409
Tricorn 5/100	1	28406410
Tricorn 5/150	1	28406411
Tricorn 5/200	1	28406412
Tricorn 10/20	1	28406413
Tricorn 10/50	1	28406414
Tricorn 10/100	1	28406415
Tricorn 10/150	1	28406416
Tricorn 10/200	1	28406417
Tricorn 10/300	1	28406418
Tricorn 10/600	1	28406419
XK 16/20	1	28988937
XK 16/40	1	28988938

Product	Quantity	Product code
XK 16/70	1	28988946
XK 16/100	1	28988947
XK 26/20	1	28988948
XK 26/40	1	28988949
XK 26/70	1	28988950
XK 26/100	1	28988951
XK 50/20	1	28988952
XK 50/30	1	28988953
XK 50/60	1	28988964
XK 50/100	1	28988965

Related literature

Type	Name	Product code
Data files	BPG 100, 140, 200, 300, 450	18111523
	Chromaflow	18113892
	AxiChrom columns	28929041
Handbook	Hydrophobic Interaction Chromatography and Reversed Phase Chromatography: Principles and Methods	11001269
Instructions	Predictable scale-up through column design and robust packing methodology	28949052
	Constant Flow Packing method	29001795
	Pack-in-place packing procedure	29001797

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