GE Healthcare

Instructions 71-7055-00 AG Affinity chromatography

Blue Sepharose 6 Fast Flow

Blue Sepharose™ 6 Fast Flow is Cibacron™ Blue 3G covalently attached to the Sepharose 6 Fast Flow matrix by the triazine coupling method. The blue dye binds many proteins, such as albumin, interferon, lipoproteins and blood coagulation factors. It also binds several enzymes including kinases, dehydrogenases, and most enzymes requiring adenyl-containing cofactors e.g. NAD^+.

The highly cross-linked matrix provides a stable, rigid medium.

Blue Sepharose Fast Flow belongs to the BioProcess™ media family. BioProcess media are separation media developed, made and supported for industrial scale – especially the manufacture of healthcare products. With their high physical and chemical stability, very high batch-to-batch reproducibility, and Regulatory Support File back-up, BioProcess media are ideal for all stages of an operation – from process development through scale-up and into production.

Large quantities can be delivered at short notice.

Sepharose™

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1. Product description

Table 1. Medium characteristics

Total binding capacity	>18 mg human serum albumin/ml drained medium
Ligand density medium	approx. 7 µmol Cibacron Blue 3G/ml drained
Average particle size	90 µm (45–165 µm)
Bead structure	6% highly cross-linked agarose
Linear flow velocity*	300 cm/h (25 °C, XK 50/30 column, 15 cm bed height)
pH stability**	
Long term	4–12
Short term	3–13
Chemical stability	7 days at 40 °C in: 70% ethanol 6M guanidine hydrochloride 8 M Urea
Temperature stability	4–40 °C
Autoclavable	121 °C for 15 min in distilled water
Storage	+4–8 °C in 0.1 M KH_2PO_4, pH 8.0 and 20% ethanol

* Linear flow rate = $\frac{\text{volumetric flow rate } (cm^3/h)}{\text{column cross-sectional area } (cm^2)}$

** The ranges given are estimates based on our knowledge and experience. Please note the following:
pH stability, long term refers to the pH interval where the medium is stable over a long period of time without adverse effects on its subsequent chromatographic performance.
pH stability, short term refers to the pH interval for regeneration, cleaning-in-place and sanitization procedures.

2. Column packing

Blue Sepharose 6 Fast Flow is supplied preswollen in 20% ethanol, 0.1 M KH_2PO_4, pH 8.0. Decant the 20% ethanol solution and replace it with binding buffer. The binding buffer should not contain agents which significantly increase the viscosity, but the column may be equilibrated with viscous buffers at reduced flow rates after packing is completed.

Table 2. Recommended lab-scale columns for Blue Sepharose 6 Fast Flow

Empty Column*	Packing flow rate (ml/min) first step	second step	Max. recommended flow rate for chromatography (ml/min)
Tricorn™ 10/20	0.9	4.7	2
Tricorn 10/50	0.9	4.7	2
Tricorn 10/100	0.9	4.7	2
XK 16/20	2.5	8.7	5
XK 26/20	6.6	23	13
XK 50/20	24.5	85	49
XK 50/30	24.5	85	49

* For inner diameter and maximum bed volumes and bed heights, see Ordering information.

Table 3. Recommended process-scale columns for Blue Sepharose 6 Fast Flow

Column	Inner diam (mm)	Bed volume (L)	Bed height (max (cm)
BPG™ 100/500	100	up to 2.0 L	26
BPG 140/500	140	up to 4.0 L	26
BPG 200/500	200	up to 8.2 L	26
BPG 300/500	300	up to 18.0 L	26
BPG 450/500	450	up to 36.0 L	23
Chromaflow™ 400/100-300	400	13–37 L	30
Chromaflow 600/100-300	600	28–85 L	30

Packing lab-scale columns

1. Assemble the column (and packing reservoir if necessary).
2. Remove air from the end-piece and adapter by flushing with water. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with water.
3. Resuspend the medium and 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.
4. If using a packing reservoir, immediately fill the remainder of the column and reservoir with water. Mount the adapter 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.
5. Open the bottom outlet of the column and set the pump to run at the desired flow rate. Ideally, Sepharose 6 Fast Flow media are packed in XK or Tricorn columns in a two-step procedure: Do not exceed 0.5 bar (0.05 MPa) in the first step and 1.5 bar (0.15 MPa) in the second step. If the packing equipment does not include a pressure gauge, use a packing flow rate of 2.5 ml/min (XK 16/20 column) or 0.9 ml/min (Tricorn 10/100 column) in the first step, and 8.7 ml/min (XK 16/20 column) or 4.7 ml/min (Tricorn 10/100 column) in the second step. See Table 2 for packing flow rates for other columns. If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate your pump can deliver. This should also give a wellpacked bed.

   Note: For subsequent chromatography procedures, do not exceed 75% of the packing flow rate. See Table 2 for flow rates for chromatography.
6. Maintain packing flow rate for at least 3 bed volumes after a constant bed height is reached. Mark the bed height on the column.
7. Stop the pump and close the column outlet.
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 into the column until it reaches the mark. Allow the packing solution to flush the adapter inlet. Lock the adapter in position.
10. Connect the column to a pump or a chromatography system and start equilibration. Re-adjust the adapter if necessary.

Packing large scale columns

For general process-scale column packing instructions, please visit support section at *www.gehealthcare.com/protein-purification*.

3. Evaluation of packing

To check the quality of the packing and to monitor this during the working life of the column, column efficiency should be tested directly after packing, prior to re-use, and when separation performance is seen to deteriorate.

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, As. These values are easily determined by applying a sample such as 1% (v/v) acetone solution to the column. Sodium chloride can also be used as a test substance. Use a concentration of 2.0 M NaCl in water with 0.5 M NaCl in water as eluent.

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 max. 2.5% of the column volume and the flow velocity between 15 and 30 cm/h.

If an acceptance limit is defined in relation to column performance, the column plate number can be used as part of the acceptance criteria for column use.

Method for measuring HETP and A_s

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

Conditions

Sample volume: 2.5% of the bed volume
Sample conc.: 1.0% (v/v) acetone
Flow velocity: 15 cm/h
UV: 280 nm, 1 cm, 0.1 AU

Calculate HETP and A_s from the UV curve (or conductivity curve if NaCl is used as sample) as follows:

$$HETP = L/N$$

$$N = 5.54(V_e/W_h)^2$$

where L = Bed height (cm)
N = number of theoretical plates
V_e = Peak elution distance
W_h = Peak width at half peak height

V_e and W_h are in the same units.

To facilitate comparison of column performance the concept of reduced plate height is often used.

The reduced plate height is calculated:

$$HETP/d$$

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

The peak should be symmetrical, and the asymmetry factor as close as possible to 1 (values between 0.8–1.5 are usually acceptable). A change in the shape of the peak is usually the first indication of bed deterioration due to use.

Peak asymmetry factor calculation:

$$A_s = b/a$$

where

a = 1st half peak width at 10% of peak height
b = 2nd half peak width at 10% of peak height.

Figure 5 shows a UV trace for acetone in a typical test chromato-gram in which the HETP and A_s values are

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

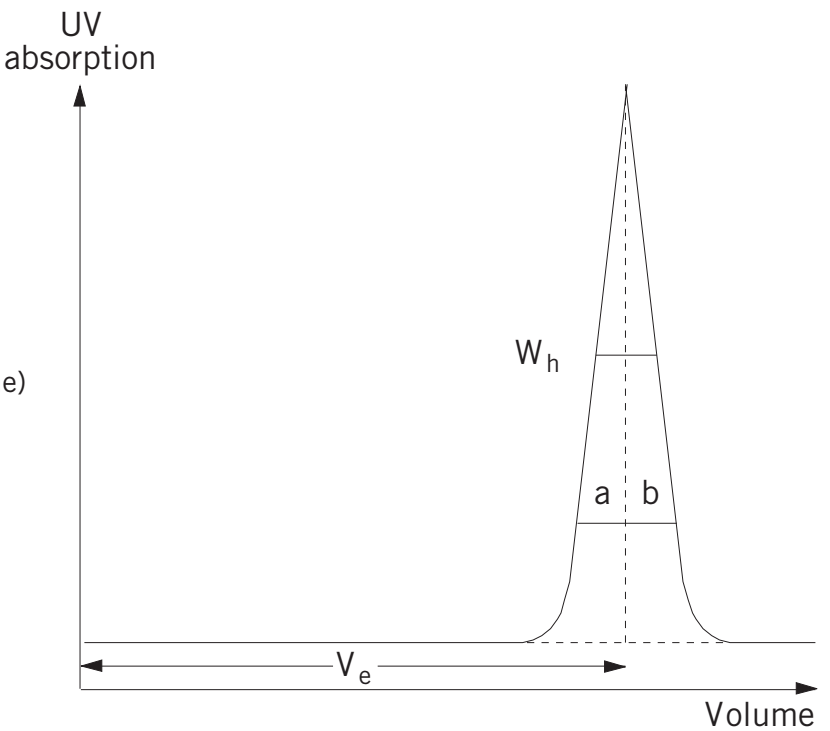

Figure 4. UV trace for acetone in a typical test chromatogram showing the HETP and A_s value calculations.

4. Operation

Binding

Different substances differ in their affinity for Blue Sepharose 6 Fast Flow. The available capacity will depend upon parameters such as flow rate, pH, buffer composition and temperature.

1. Sample pH should be the same as that of the binding buffer. Filter the sample through a 0.22 µm or 0.45 µm filter to prolong the working life of the medium.
2. After the sample has been loaded, wash the medium with binding buffer until the base line is stable.

Elution

1. Elution conditions vary with the sample. Elution may be accomplished by a change in pH, polarity (e.g. ethylene glycol) or ionic strength of the buffer. Enzymes can often be eluted at less than 1 M NaCl.
2. Competitive elution with low concentrations of the cofactor is required for very specifically bound proteins. Either step or continuous gradients may be used.

5. Regeneration, Cleaning, Sanitization and Storage

Regeneration

Depending on the nature of the sample, reversibly bound material can be eluted with 4–5 washing cycles of alternate high pH (0.1 M Tris-HCl, 0.5 M NaCl, pH 8.5) and low pH (0.1 M sodium acetate, 0.5 M NaCl, pH 4.5) buffers, followed by re-equilibration in binding buffer.

In some applications, substances like denaturated proteins or lipids do not elute in the regeneration procedure. These can be removed by cleaning-in-place.

Cleaning-in-place (CIP)

Remove precipitated proteins by washing the column with 4 bed volumes of 0.1 M NaOH solution at a low linear flow rate (40 cm/h), followed by washing the column with 3–4 bed volumes of 70% ethanol or 2 M potassium thiocyanate.

Alternatively, wash the column with 2 bed volumes of 6 M guanidine hydrochloride.

In both cases wash immediately with at least 5 bed volumes of sterile filtered binding buffer at pH 8.0.

Remove strongly bound hydrophobic proteins, lipoproteins and lipids by washing the column with 3–4 bed volumes of up to 70% ethanol or 30% isopropanol. (Apply increasing gradients to avoid air bubble formation when using high concentrations of organic solvents.)

Alternatively, wash the column with 2 bed volumes of detergent in a basic or acidic solution, e.g. 0.1% non-ionic detergent in 1 M acetic acid. Wash at a flow rate of 40 cm/h. Remove residual detergent by washing with 5 bed volumes of 70% ethanol.

In both cases wash immediately with at least 5 bed volumes of sterile filtered binding buffer at pH 8.0.

Sanitization

Sanitizate the column by equilibrating it with a buffer consisting of 2% hibitane digluconate and 20% ethanol and allow to stand for 6 hours.

Alternatively, equilibrate the column with 70% ethanol and allow to stand for 12 hours.

In both cases wash with at least 5 bed volumes of sterile filtered binding buffer at pH 8.0.

Column performance is not significantly changed by the cleaning-in-place and sanitization procedures described above.

All of the above recommended procedures can be performed directly on the packed column.

Storage

For longer periods of storage, e.g. weeks, we recommend that the medium be stored at 4–8 °C in 20% ethanol, 0.1 M KH_2PO_4, pH 8.0.

The medium must not be frozen.

6. Ordering information

Product	Pack size	Code No.
Blue Sepharose 6 Fast Flow	50 ml	17-0948-01
Blue Sepharose 6 Fast Flow	500 ml	17-0948-02
Blue Sepharose 6 Fast Flow	1 L	17-0948-03
Blue Sepharose 6 Fast Flow	5 L	17-0948-04

Lab-scale columns

Tricorn 10/20 column, 10 mm i.d., max 2.2 ml bed volume or 2.8 cm bed height	1	18-1163-13
Tricorn 10/50 column, 10 mm i.d., max 4.5 ml bed volume or 5.8 cm bed height	1	18-1163-14
Tricorn 10/100 column, 10 mm i.d., max 8.5 ml bed volume or 10.8 cm bed height	1	18-1163-15
XK 16/20 column, 16 mm i.d., max 30 ml bed volume or 15 cm bed height	1	18-8773-01
XK 26/20 column, 26 mm i.d., max 65 ml bed volume or 12.5 cm bed height	1	18-1000-72
XK 50/20 column, 50 mm i.d., max 270 ml bed volume or 14 cm bed height	1	18-1000-71
XK 50/30 column, 50 mm i.d., max 550 ml bed volume or 28.5 cm bed height	1	18-8751-01

Datafiles	
BPG columns	18-1115-23
BPG 450 column	18-1160-59
CHROMAFLOW columns	18-1138-92
Blue Sepharose 6 Fast Flow	18-1060-75

Literature	
Affinity Chromatography Handbook,	18-1022-29
Handbook of Process Chromatography: A Guide to Optimization, Scale-Up, and Validation (Academic Press, 1997, Sofer, G. and Hagel, L.)	18-1121-56

7. Further information

Please read these instructions carefully before using Blue Sepharose 6 Fast Flow medium. For further information visit *www.gehealthcare.com/protein-purification* or contact your local GE Healthcare representative.

www.gehealthcare.com/protein-purification
www.gehealthcare.com

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