GE Healthcare

Instructions 71–5020–61 AC **Affinity media**

Benzamidine Sepharose 4 Fast Flow (high sub)

Benzamidine Sepharose™ 4 Fast Flow (high sub) is an excellent tool for removal and/or purification of trypsin and trypsin-like serine proteases, as well as, zymogens including urokinase and prekallikrein. Removal of serine proteases is easily done directly from serum, monoclonal cell supernatants, and bacterial lysates.

Sepharose™

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1. Characteristics

Benzamidine Sepharose 4 Fast Flow (high sub) is based on highly cross-linked 4% agarose, which enables rapid processing of large sample volumes. The ligand, p-aminobenzamidine, is covalently coupled via an amide bond to a long spacer arm attached to Sepharose 4 Fast Flow via a stable ether linkage. Coupling is optimized to give high binding capacity. Total binding capacity is ≥ 35 mg trypsin per ml medium.

Table 1. Characteristics of Benzamidine Sepharose 4 Fast Flow (high sub)

Ligand	p-aminobenzamidine (pABA)
Spacer arm	14 atoms
Ligand coupling method	Amide
Ligand concentration	≥ 12 (mol benzamidine/ml medium
Binding capacity	≥ 35 mg trypsin/ml medium
Average particle size	90 µm (60–165 µm)
Matrix	highly cross-linked 4% agarose
Recommended working flow velocity	30–300 cm/h
Recommended pH*	
long term	2–8
short term	1–9
Chemical stability	Stable in all commonly used aqueous buffers
Storage temperature	+4 to +8 °C
Delivery and storage buffer	Supplied in 0.05 M acetate buffer, pH 4 containing 20% ethanol

* 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 gel 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. Packing columns

Benzamidine Sepharose 4 Fast Flow (high sub) is supplied in 0.05 M acetate buffer pH 4 containing 20% ethanol as a bacteriostat. Decant the solution and replace it with binding buffer before use. In general, we recommend a bed height of 5–15 cm to allow high flow rates to be used.

Recommended columns

Lab-scale columns

- Tricorn™ 5/50 column (5 mm i.d.) for bed volumes of 0.6–1.1 ml and bed heights of 3–6 cm
- Tricorn 10/100 column (10 mm i.d.) for bed volumes of 6.3–8.5 ml and bed heights of 8–11 cm
- XK 16/20 (16 mm i.d.) for bed volumes of 2–34 ml and bed heights of 1–17 cm.
- XK 26/20 (26 mm i.d.) for bed volumes of 0–80 ml and bed heights of 0–15 cm

Packing lab-scale columns (Tricorn 10/100, XK 16/20, and XK 26/20)

1. Assemble the column (and packing reservoir if necessary).
2. Remove air from the column dead spaces by flushing the end-piece and adaptor with packing buffer. Ensure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with binding buffer.
3. Resuspend medium stored in its container by shaking (avoid stirring the sedimented medium). Mix the packing buffer with the medium to form 50–70% 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 air bubble formation.
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 adaptor or in the inlet tubing.

6. Open the bottom outlet of the column and set the pump to run at the desired flow rate. Ideally, Sepharose 4 Fast Flow is packed at a constant pressure of approximately 1 bar (0.1 MPa). If the packing equipment does not include a pressure gauge, use a packing flow velocity of approximately 400 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 allowed by the pump. This should also give a sufficiently 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 adaptor to the column.
9. With the adaptor inlet disconnected, push down the adaptor approximately 2 mm into the bed, allowing the packing solution to flush the adaptor inlet.
10. Connect the pump, open the bottom outlet, and continue packing. The bed will be further compressed at this point and a space will be formed between the bed surface and the adaptor.
11. Close the bottom outlet. Disconnect the column inlet, and lower the adaptor approximately 2 mm into the bed. Connect the pump. The column is now ready to use.

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% acetone solution to the column.

Calculated plate number will vary depending on the test conditions and 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, 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, 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:

$$HETP = L/N$$

where

L = Bed height (cm)

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

N = Number of theoretical plates

V_e = Peak elution distance

W_h = Peak width at half peak height

V_e and W_h are expressed in the same units.

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

Reduced plate height is calculated as follows:

HETP/d

where

d = 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 1 shows a UV trace for acetone in a typical test chromatogram in which HETP and As values are calculated.

Column:	BPG 300
Media:	Sepharose 6 Fast Flow
Bed height:	57.5 cm
Bed volume:	40.6 l
Eluent:	Distilled water
Sample:	1.05 l (1% 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

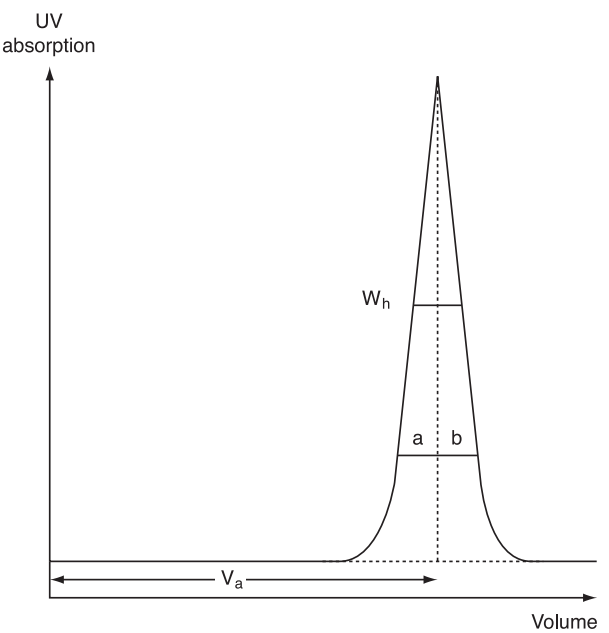

Fig 1. UV trace for acetone in a typical test chromatogram showing HETP and As value calculations.

4. Recommended buffers

Binding buffer
0.05 M Tris-HCl, 0.5 M NaCl, pH 7.4.
After the sample has been loaded, wash the gel with binding buffer until the baseline is stable.

Elution buffer
0.05 M glycin, pH 3.0 or
10 mM HCl, 0.5 M NaCl, pH 2.0

Note: Add 60–200 µl 1 M Tris-HCl, pH 9 per ml of fraction to be collected to each collection tube; this will prevent denaturation of eluted protein as a result of low pH.

Competitive elution buffer
20 mM p-aminobenzamidine in binding buffer.

Bound substances can be eluted specifically or non-specifically. Use of a competing agent for the target molecule, inhibitor p-aminobenzamidine, elutes specifically bound substances.

5. Stability

The amide bound between the ligand and spacer is stable over almost the whole pH range. However, the ligand itself is sensitive to pH below 2 and above 8. At these pH values, the ligand is first hydrolyzed to p-aminobenzamide and then further to p-aminobenzoic acid without being detached from the matrix. Only the benzamidine group has affinity for trypsin, and a decrease in trypsin capacity is therefore observed, see Figure 2. Benzamidine Sepharose 4 Fast Flow has successfully been stored in 8 M urea and 8 M guanidine hydrochloride for one week without reduction in trypsin capacity and ligand density.

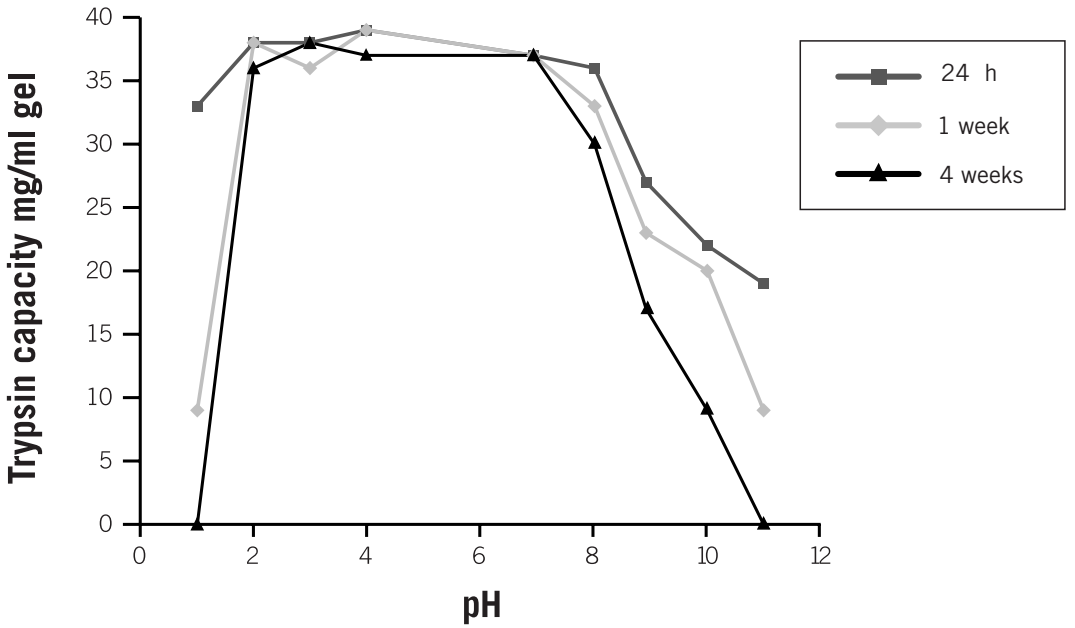

Fig 2. Benzamidine Sepharose 4 Fast Flow (high sub) has been tested for trypsin capacity after being stored at pH 1–11 at ambient temperature for 24 h, 1 week, and 4 weeks.

6. Cleaning in place (CIP) and sanitization

A cleaning and sanitization protocol is required for each application. Short-term use of pH 1–9 is possible, for example, more than half of the trypsin capacity is available after 1 week (168 h) storage at pH 9 see Figure 2. However, prolonged exposure to pH greater than 8 and lower than 2 should be avoided due to hydrolysis of the ligand at high pH, and decomposition of the matrix at low pH. The general recommendation is to use a solution of guanidine hydrochloride to remove precipitated or denatured substances. For hydrophobically bound substances, a solution of non-ionic detergent or ethanol is recommended. For sanitization of Benzamidine Sepharose 4 Fast Flow, we recommend storage in a solution containing 0.1 M acetic acid/20% ethanol. [3]

7. Storage

Store Benzamidine Sepharose 4 Fast Flow (high sub) in an acetate buffer, pH 4 containing 20 % ethanol as a preservative. Storage temperature +4 to +8 oC

8. Scaling up

Before scaling up, parameters such as flow rate, bed height, capacity etc, needs to be optimized in small-scale experiments. During optimization, a bed height of 5–15 cm is recommended, which allows the use of high flow rates. Maximum flow rate is approximately inversely proportional to the bed height. When scaling up, some parameters will change while othersremain constant.

- Keep the bed height constant or the chromatogram pattern will change.
- Select column according to bed volume, and required binding capacity
- Define linear flow rate during sample application to ensure that residence time is not shorter than that established in the small-scale experiments. Residence time is equal to the bed height (cm) divided by the linear flow velocity (cm/h) applied during sample loading.
- Keep sample concentration and gradient slope constant.

The larger equipment needed when scaling up may cause some deviations from the optimized method at small scale. In such cases, check the buffer delivery system and monitoring system for time delays or volume changes. Different lengths and diameters of outlet pipes can cause zone spreading on larger systems.

9. References

1. Preparation of high-capacity affinity adsorbent using formyl carriers and their use for low-and high-performance liquid affinity chromatography of trypsin-family proteases. *J of Chromatogr.* **363,** *231–242, (1986).* Kanamori, A. et al.
2. Optimisation of conditions for the affinity chromatography of human enterokinase on immobilised p-aminobenzamidine. *Eur. J. Biochem.* **88,** *183–189, (1978).* Grant, D. et al.

3. Cleaning, sanitization and storage, in Handbook of Process Chromatography: A Guide to optimization, scale-up and validation. *Academic Press, pp 188–214 (1997)*. Sofer G. and Hagel, L.

10. More information

Please visit *www.gehealthcare.com/protein-purification* for more detailed information. Also refer to the handbooks (see ordering information or the web).

11. Ordering information

Product	Quantity	Code No
Benzamidine Sepharose 4 Fast Flow (high sub) Larger quantities are available as Custom Design Media	25 ml	17-5123-10
Related Products		
Glutathione Sepharose 4 Fast Flow	25 ml	17-5132-01
Lab scale columns:		
Tricorn™ 5/50 (5 mm i.d.)	1	18-1163-09
Tricorn 10/100 (10 mm i.d.)	1	18-1163-15
XK 16/20 (16 mm i.d.)	1	18-8773-01
XK 26/20 (26 mm i.d.)	1	18-1000-84
HiTrap™ column family:		
HiTrap Benzamidine FF (high sub)	2 x 1ml	17-5143-02
HiTrap Benzamidine FF (high sub)	5 x 1 ml	17-5143-01
HiTrap Benzamidine FF (high sub)	1 x 5 ml	17-5144-01
HiTrap Desalting	5 x 5 ml	17-1408-01
GSTrap™ FF	5 x 1 ml	17-5030-01
GSTrap FF	2 x 1 ml	17-5030-02
GSTrap FF	1 x 5 ml	17-5031-01
Affinity Chromatography Handbook Principles and methods	1	18-1022-29
Affinity Chromatography, Column and Media Guide	1	18-1121-86

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