GE Healthcare

Instructions 71-5000-15 AD **Pre-activated media**

CNBr-activated Sepharose 4 Fast Flow

Introduction

The preparation and use of affinity chromatography media by coupling biospecific ligands to CNBr-activated matrices is a widely used, successful and well-documented technique.

CNBr-activated Sepharose™ 4 Fast Flow is a pre-activated affinity matrix that combines the advantages of CNBr coupling with the high flow and stability characteristics of Sepharose 4 Fast Flow. In our experience, the CNBr coupling technique has a well-proven track record for the purification of therapeutic proteins. This, plus the performance of the matrix at large scale, makes the use of CNBr-activated Sepharose 4 Fast Flow particularly attractive for manufacturing applications in the biopharmaceutical industry. Furthermore, the medium is a member of the BioProcess™ media. BioProcess media are developed and supported for production scale chromatography. All BioProcess media are produced with validated methods and are tested to meet manufacturing requirements. Secure ordering and delivery routines give a reliable supply of media for production scale. Regulatory Support Files (RSF) are available to assist process validation and submissions to regulatory authorities.

To ensure best performance and trouble-free operation, please read these instructions before using CNBr-activated Sepharose 4 Fast Flow.

Sepharose™

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1. Product description

CNBr-activated Sepharose 4 Fast Flow is a bead-formed, highly cross-linked pre-activated matrix produced by reacting Sepharose 4 Fast Flow with cyanogen bromide (CNBr). This coupling makes the medium more rigid which in turn improves the pressure/flow characteristics. Proteins and other molecules containing primary amino groups can be coupled directly to the pre-activated medium. Multi-point attachment of proteins provides the immobilized product with good chemical stability. The resulting affinity medium can isolate a specific substance from a complex mixture, often achieving very high yield and purity in a single step. Many references demonstrate that binding affinity is frequently well maintained after CNBr coupling.

A typical application of pre-activated affinity media like CNBr-activated Sepharose 4 Fast Flow is based on antigen-antibody reactions with immobilized monoclonal antibodies as ligands. In such cases, purification factors of 2 000–20 000 can be obtained.

Table 1 summarizes the main characteristics of CNBr-activated Sepharose 4 Fast Flow.

Table 1. Medium characteristics.

Mean particle size	90 µm
Particle size range	45–165 µm
Bead structure	Highly cross-linked 4% agarose, spherical
Linear flowrate	
Base matrix 150–250 cm/h, 0.1 MPa (1 bar), XK 50/60 column, bed height 25 cm	
Swelling factor	4–5 ml drained medium/g
Coupling capacity	13–26 mg α-chymotrypsinogen/ml
pH stability*	
long term	3–11
short term (CIP)	3–11

* Refers to stability of coupling between ligand abd base matrix. Ligands can be less stable.

Sepharose 4 Fast Flow matrix

Sepharose 4 Fast Flow is a highly cross-linked agarose matrix. In its pre-activated CNBr form, it offers much improved performance when compared with the well established CNBr-activated Sepharose 4B. The Sepharose 4 Fast Flow matrix has higher rigidity and can thus be run at high flow rates (see Table 1).

The higher mechanical strength of the cross-linked matrix makes it well-suited for use in large columns. Scaling up a purification developed on CNBr-activated Sepharose 4 Fast Flow is therefore simple and more predictable. The coupled product is stable at low pH, which is often required for elution from some immunoadsorbents.

For applications that require operation at high pH, note that the amide bond formed when using the companion product NHS-activated Sepharose 4 Fast Flow is stable up to pH 13 for normal use.

2. Coupling

CNBr-activated Sepharose 4 Fast Flow is supplied freeze-dried in the presence of additives. These additives need to be washed away at low pH (pH-2–3) before coupling the desired ligand. The use of low pH (pH2–3) preserves the activity of the reactive groups, which otherwise hydrolyse at high pH.

In order to retain maximum binding capacity of CNBr-activated Sepharose 4 Fast Flow prior to coupling the ligand, use cold (0–4 °C) solutions. The time interval between washing and coupling must be minimised; therefore preparations of all required solutions prior to coupling is recommended.

1. Prepare the coupling solution, i.e. dissolve the ligand to be coupled in a suitable coupling buffer, e.g. 0.1 M $NaHCO_3$ pH 8.3 containing 0.5 M NaCl. For good coupling efficiency avoid unnecessarily dilute solutions (Recommended ratio of volumes, coupling solution/medium is 0.5:1). The coupling pH depends on the ligand. Normally pH in the range 7–9 is used.

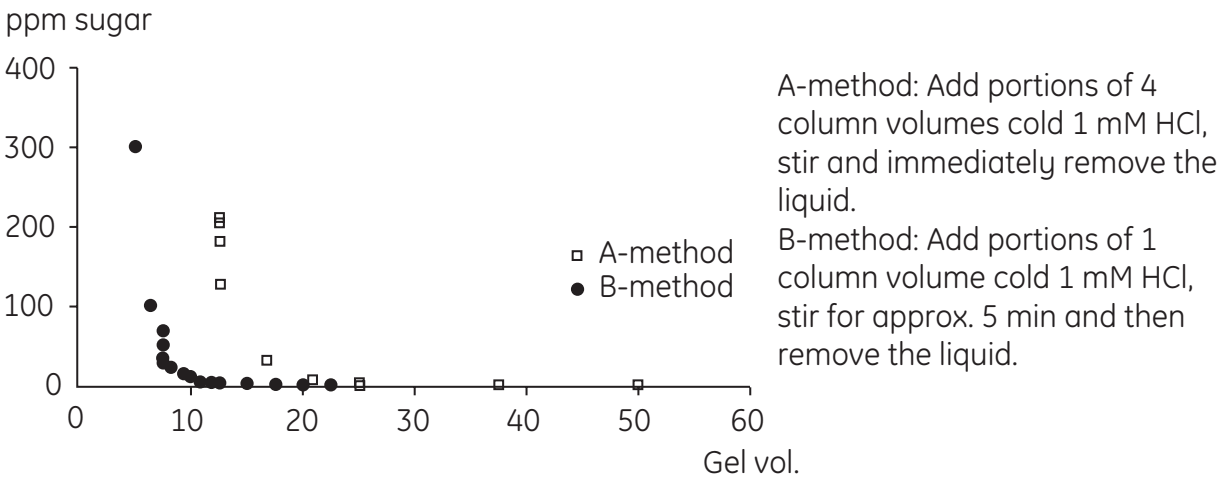

Fig 1. The content of sugar in the filtrate after washing with different medium volumes of cold 1 mM HCl.

2. CNBr-activated Sepharose 4 Fast Flow is supplied freeze-dried with sugar additives and is washed initially with 10–15 medium volumes of cold 1 mM HCl, see Fig 1. Use small wash portions (e.g. 1 medium volume) and let the mixture equilibrate a few minutes during each washing step. After washing, determine the exact medium volume obtained using e.g. centrifugation or PD-10 column (the medium volume may vary between experiments).
3. Mix the washed medium and coupling solution. Adjust pH to the desired value. To obtain good reproducibility it is wise to adjust total reaction volume to a fixed value with coupling buffer.
4. Coupling is normally very fast. At room temperature the reaction is usually completed after 2–4 hours. If coupling is performed at 4 °C, it can be performed overnight. It may be practical to follow the reaction using UV-absorbance measurements.

5. Wash away exess ligand with at least 5 medium volumes of coupling buffer.
6. After coupling, non-reacted groups on the medium should be blocked. Transfer the medium to 0.1 M Tris-HCl buffer pH 8.0 or 1 M ethanolamine pH 8.0. Let it stand for 2 hours.
7. Wash the coupled medium using alternate low and high pH. Recommended buffers are 0.1M acetate buffer pH 3-4 containing 0.5 M NaCl and 0.1 M Tris-HCl buffer pH 8–9 containing 0.5 M NaCl. A suitable procedure could be 3x1 medium volume Tris HCl buffer followed by 3×1 volumes acetate buffer. Repeat this cycle 3–6 times.
8. The coupled medium is now ready for use. To prevent microbial growth, store in 20% ethanol for example.

3. Column packing guidelines

General column packing guidelines for Sepharose Fast Flow based media.

3.1 Recommended columns

Lab-scale columns

- Tricorn™ 5/20 (5 mm i.d.) for bed volumes up to 0.55 ml at bed heights up to 2.8 cm
- Tricorn 5/50 (5 mm i.d.) for bed volumes up to 1.1 ml at bed heights up to 5.8 cm
- Tricorn 10/20 (10 mm i.d.) for bed volumes up to 2.2 ml at bed heights up to 2.8 cm
- Tricorn 10/50 (10 mm i.d.) for bed volumes up to 4.5 ml at bed heights up to 5.8 cm
- Tricorn 10/100 (10 mm i.d.) for bed volumes up to 8.5 ml at bed heights up to 10.8 cm
- XK 16/20 (16 mm i.d.) for bed volumes up to 30 ml at bed heights up to 15 cm.
- XK 26/20 (26 mm i.d.) for bed volumes up to 80 ml at bed heights up to 15 cm.

Large scale columns

- BPG™ variable bed, glass columns. Inner diameters from 100–450 mm, bed volumes from 2.4–131 litres; bed height max 83 cm.
- CHROMAFLOW™ variable bed columns. Inner diameters from 400–600 mm.

3.2 Packing lab-scale columns

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. Make sure no air has been trapped under the column bed support. Close the column outlet leaving the bed support covered with packing buffer.
3. Re-suspend 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 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 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 based media are 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 (15 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 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 form 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.

3.3 Packing large scale columns

General packing recommendations

Columns can be packed in different ways depending on the type of column and equipment used. Always read and follow the relevant column instruction manual carefully. For general process-scale column packing instructions, please visit support section at:
www.gehealthcare.com/protein-purification

4. 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, 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 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 As

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

Ve 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:

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 2 shows a UV trace for acetone in a typical test chromatogram in which the HETP and As values are calculated.

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% acetone)
Flow velocity: 19 cm/h
W_e=18.7
W_h=0.9
HETP =0.024 cm
a: 0.90
b: 0.85
A_s: 0.94

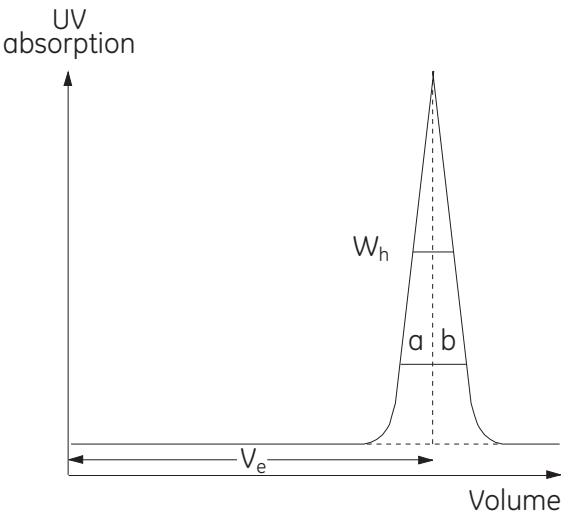

Fig 2. UV trace for acetone in a typical test chromatogram showing the HETP AND A_S value calculations.

5. Cleaning, Sanitization and Storage

For best performance of coupled CNBr-activated Sepharose 4 Fast Flow over a long working life, follow the general procedures described below. In all cases, we recommend testing the procedures at small scale first.

Equilibration

After packing, and before a chromatographic run, equilibrate with working buffer by washing with at least 5 bed volumes.

Cleaning-In-Place

Cleaning-in-place, (CIP), is a cleaning procedure which 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 prevents the build-up of these contaminants in the packed bed, and helps to maintain the capacity, flow properties and general performance of the medium.

A specific CIP protocol should be designed for each process according to the type of contaminants present and stability of coupled ligand. The frequency of CIP depends on the nature and the condition of the starting material and other process requirements, but one CIP cycle is generally recommended every 1–5 separation cycles. Following are generally recommended procedures.

CIP protocol

Precipitated or denatured substances	Wash with 2 column volumes of 6 M guanidine hydrochloride. Wash substances immediately with at least 5 column volumes of sterile filtered binding buffer.
Hydrophobically bound substances	Wash the column with 2 column volumes of a non-ionic detergent (conc. 0.1–0.5%). Wash immediately with at least 5 column volumes of sterile filtered binding buffer.

or

Wash the column with 3– column volumes of 70% ethanol. Wash immediately with at least 5 column volumes of sterile filtered binding buffer.

Sanitization

Sanitization inactivates microbial contaminants in the packed column and related equipment. A specific sanitization protocol should be designed for each process according to the type of contaminants present and stability of coupled ligand. Following are generally recommended procedures.

Equilibrate with a buffer consisting of 2% hibitane digluconate and 20% ethanol. Allow to stand for 6 hours, then wash with at least 5 column volumes of sterile binding buffer.

or

Equilibrate with 70% ethanol. Allow to stand for 12 hours, then wash with at least 5 column volumes of sterile binding buffer.

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

Storage

CNBr-activated Sepharose 4 Fast Flow is supplied freeze dried. Additives are included to preserve the bead form of the medium. When stored below 8 °C, the shelf life is at least 18 months. Packed columns should preferebly be equilibrated in binding buffer containing 20% ethanol (ligand dependent) to prevent microbial growth.

6. Ordering information

Product	Pack size	Code No
CNBr-activated Sepharose 4 Fast Flow	10 g	17-0981-01
	250 g	17-0981-03
	2 kg	17-0981-05

All products are supplied freese dried in the precence of additives.

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