GE Healthcare
Life Sciences

Instructions 71-5000-14 AD **Pre-activated media**

NHS-activated Sepharose™ 4 Fast Flow

The preparation and use of affinity chromatography media by coupling biospecific ligands to pre-activated media is a widely used, successful and well-documented technique. NHS-activated Sepharose 4 Fast Flow is a pre-activated agarose matrix that increases the choice of coupling chemistries available.

NHS (N-hydroxysuccinimide) coupling forms a chemically stable amide bond with ligands containing primary amino groups. NHS-activated Sepharose 4 Fast Flow provides a spacer arm and is therefore particularly suitable for immobilising small protein and peptide ligands.

The advantages of high stability and a spacer arm combined with the high flow and stability characteristics of Sepharose 4 Fast Flow make this pre-activated medium attractive for pharmaceutical companies producing therapeutic products. The pre-activated medium can be used to prepare affinity adsorbents which can isolate specific substances from complex mixtures, often achieving very high purity in a single step. NHS-activated Sepharose 4 Fast Flow belongs to the BioProcess™ media family, and fulfills industrial demands for security of supply, robust performance and regulatory support.

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

Contents

1 Product description

NHS-activated Sepharose 4 Fast Flow is a bead-formed, highly cross-linked pre-activated matrix prepared by coupling Sepharose 4 Fast Flow with 6-aminohexanoic acid via a spacer arm. The terminal carboxyl group is activated by esterification with N-hydroxysuccinimide. Ligands containing primary amino groups couple directly to this active ester to form a chemically very stable amide linkage.

To maintain high activity, NHS-activated Sepharose 4 Fast Flow is supplied as a suspension in 100% isopropanol, which should be washed away before use. Specific regulations may apply when using this product since it can require the use of explosion-proof areas and equipment.

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

Table 1. Medium characteristics

Mean particle size	90 µm
Particle size range	45 to 165 µm
Bead structure	Highly cross-linked 4% agarose, spherical
Linear flow velocity[1]	150 cm/h at 100 kPa
Ligand density	16 to 23 µmol NHS/ml drained medium
pH stability[2]	
long term	3 to 13
short term (CIP)	2 to 13

[1] At 25°C in an XK 50/60 column, 25 cm bed height (base matrix). The flow velocity after coupling may differ depending on the ligand.

[2] Depends on the ligand. Tested with lysine as ligand with single-point attachment.

2 Coupling

NHS-activated Sepharose 4 Fast Flow is supplied as suspension in 100% isopropanol. Instructions for removal of isopropanol from the medium and preparation for coupling the ligand are given below.

In order to retain maximum binding capacity of the pre-activated medium prior to the coupling step, use cold (0°C to 4°C) solutions. The time interval for all washing steps must be minimized; we recommend preparing all required solutions prior to coupling the ligand.

1 Prepare the coupling solution, i.e. dissolve the ligand to be coupled in a suitable coupling buffer. For good coupling efficiency avoid unnecessarily dilute solutions (a suggested ratio of volumes, coupling solution/medium is 0.5:1). The coupling pH depends on the ligand to be coupled, normally pH 6 to 9 can be used. Be aware that the NHS-ester groups rapidly hydrolyse at higher pH. A standard buffer is 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3.

2 Wash NHS-activated Sepharose 4 Fast Flow with 10 to 15 medium volumes of cold 1 mM HCl immediate before use.

3 Mix the washed medium and the coupling solution then adjust the pH to the desired level. In order to obtain good reproducibility it is recommended to be very careful when determining the medium volume used in each experiment.

4 Coupling is normally very fast at room temperature. It is therefore important to optimize coupling time to maintain the biological activity of the ligand; at room temperature the reaction is usually completed after 2 to 4 hours. If the coupling is performed at 4°C it can be left overnight.

5 After the coupling is completed, any non-reacted groups on the medium should be blocked. Thus, the medium can be kept in 0.5 M ethanolamine, 0.5 M NaCl, pH 8.3 or 0.1 M Tris-HCl, pH 8.5 for a few hours.

6 To wash the medium after coupling, use a method which alternates two different buffers (high and low pH respectively). Buffers often used are 0.1 M Tris-HCl buffer pH 8 to 9 and 0.1 M acetate buffer, 0.5 M NaCl pH 4 to 5. A suitable procedure could be 3 x 1 medium volumes Tris buffer followed by 3 x 1 medium volumes acetate buffer. This cycle is repeated 3 to 6 times.

7 The coupled affinity medium is now ready for use. To prevent microbial contamination, store in 20% ethanol.

3 Column packing guidelines

Following are general guidelines for packing Sepharose 4 Fast Flow based media.

Table 2. Recommended lab-scale columns for NHS-activated Sepharose 4 Fast Flow

Empty column[1]	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	42	24
XK 50/30	24.5	42	24

[1] For inner diameter and maximum bed volumes and bed heights, see Ordering information.

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 operation. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
4. When 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 4 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.

   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. 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 well-packed bed.

   Note: *For subsequent chromatography procedures, do not exceed 75% of the packing flow rate. See Table 2 for chromatography flow rates.*
6. Maintain the 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 a packing reservoir is used, 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

Table 3 lists recommended process-scale columns for NHS-activated Sepharose 4 Fast Flow.

Table 3. Recommended process-scale columns for NHS-activated Sepharose 4 Fast Flow

Column	Inner diam (mm)	Bed volume (l)	Max. bed height (cm)
BPG™ 100/500	100	up to 2.0 l	26
BPG 140/500	140	up to 4.0 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 to 37 l	30
Chromaflow 600/100–300	600	28 to 85 l	30

For general process-scale column packing instructions, please visit the online technical support section at *www.gelifesciences.com/purification_techsupport*.

4 Evaluation of packing

Test column efficiency to check the quality of the packing. Test should be made directly after packing and at regular intervals during the working life of the column 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 (when monitoring at 280 nm). NaCl can also be used as a test substance (when monitoring conductivity). Use a concentration of 2.0 M NaCl in water with 0.5 M NaCl in water as eluent.

Note: *The calculated plate number will vary according to the test conditions and it should only be used as a reference value. Keep test conditions and equipment constant so that results are comparable. Changes of solute, solvent, eluent, sample volume, flow velocity, liquid pathway, temperature, etc. will influence the results.*

For optimal results, keep the sample volume at maximum 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 one of the acceptance criteria for column use.

Method for measuring HETP and A_s

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

$$HETP = \frac{L}{N}$$

$$N = 5.54 \times \left(\frac{V_e}{W_h}\right)^2$$

where:

L = Bed height (cm)

N = number of theoretical plates

V_e = 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_e and W_h are in the same units

The concept of reduced plate height is often used for comparing column performance.

Calculate reduced plate height, h, as follows:

$$h = \frac{HETP}{d_{50v}}$$

where:

d_{50V} 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 to 1 as possible (values between 0.8 to 1.5 are usually acceptable).

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

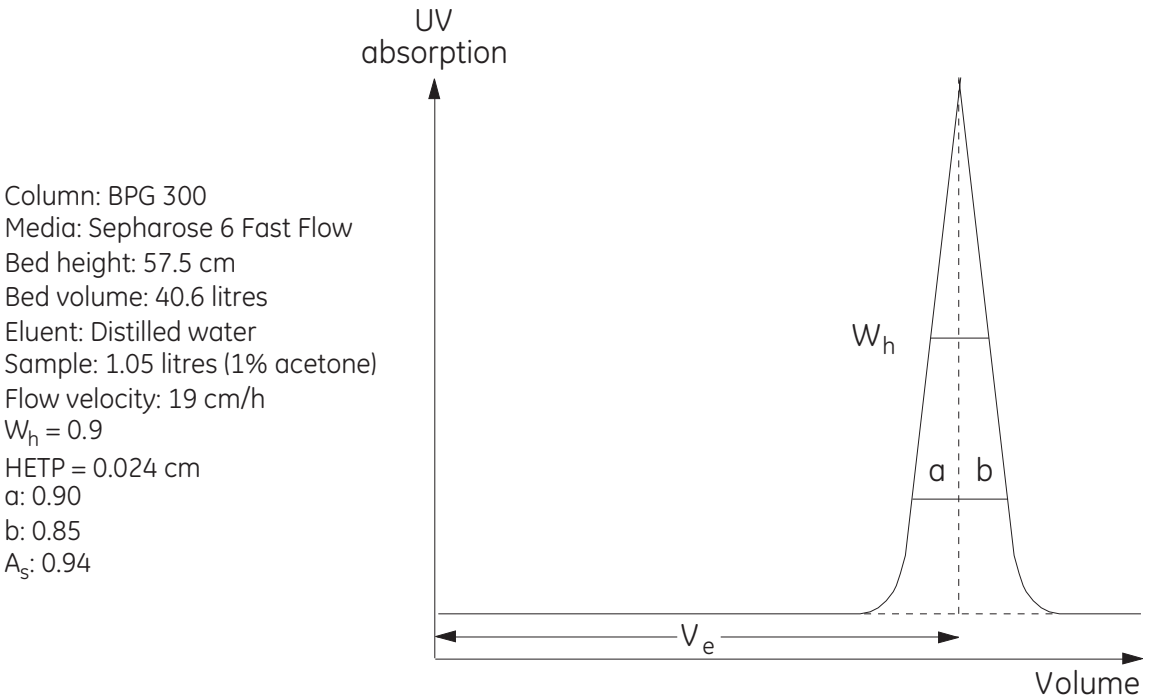

Fig 1. A typical test chromatogram showing the parameters used for HETP and A_s calculations.

Peak asymmetry factor calculation:

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

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 the HETP and A_s values are calculated.

5 Maintenance

For best performance of coupled NHS-activated Sepharose 4 Fast Flow over a long working life, follow the 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 to 5 separation cycles.

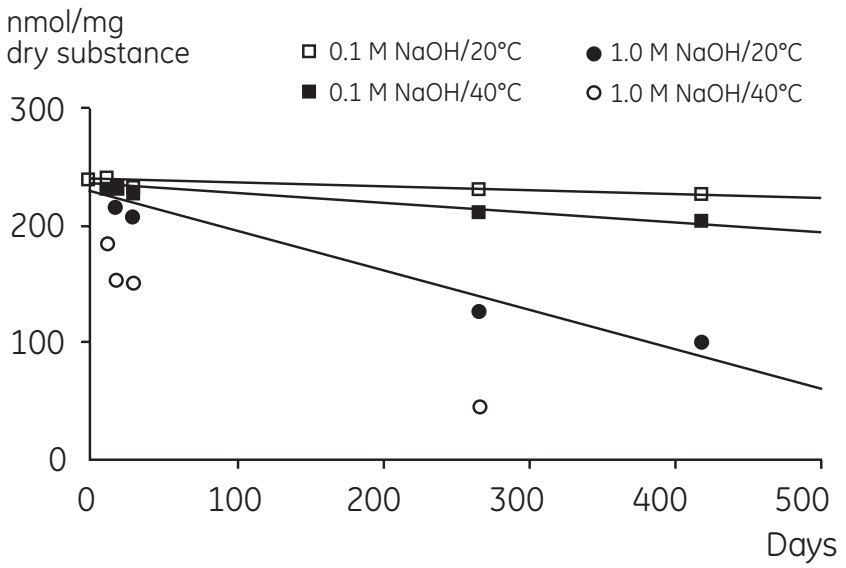

Fig 2. Residual content of lysine after storage of lysine coupled to NHS-activated Sepharose 4 Fast Flow in 0.1 and 1.0 M NaOH at 20°C and 40°C.

If ligand stability permits, coupled NHS-activated Sepharose 4 Fast Flow withstands exposure to 0.1 M NaOH for long periods of time. (Only a slight decrease in the lysine content of a coupled medium was noted after 500 days exposure to 0.1 M NaOH, see Fig 2).

The following are generally recommended procedures.

CIP protocol

Precipitated or denatured substances	Wash with 4 column volumes of 0.1 M NaOH 1 to 2 h contact time. Wash with at least 5 column volumes of sterile filtered binding buffer. or 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 to 0.5%). Wash immediately with at least 5 column volumes of sterile filtered binding buffer. or Wash the column with 3 to 4 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 the stability of the coupled ligand. The following are recommended procedures:

If ligand stability permits, a recommended sanitization procedure is to equilibrate the packed column with 0.1 M NaOH in 20% ethanol and allow to stand for 1 hour. Wash with at least 5 column volumes of sterile binding buffer.

or

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. Consult your local safety regulations for more information.*

Storage
NHS-activated Sepharose 4 Fast Flow is supplied as suspension in 100% isopropanol. Unused media can be stored in the container at 2°C to 8°C. The medium will hydrolyse and thus lose capacity if stored incorrectly at too high a temperature.

Ensure that the screw top is fully tightened. Note that the medium may have to be stored in an explosion-proof environment. Consult your local safety regulations for more information.

Packed columns should be equilibrated in binding buffer containing 20% ethanol to prevent microbial growth. Note that the stability of the coupled medium is dependent on the attached ligand.

6 Troubleshooting

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 adapter and the media bed, or back mixing of the sample before application.
5. Check the efficiency of the column packing.
6. Check if there have been any changes in the pre-treatment of the sample.

Infections

1. Check the connections and prefilters.
2. Check the in-going components 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 adapter net and head, it can be removed by pumping eluent in the opposite direction. After this, check the efficiency of the packed bed and compare the result with the original efficiency values.

7 Ordering information

Product	Pack size	Code No
NHS-activated Sepharose 4 Fast Flow	25 ml	17-0906-01
	500 ml	17-0906-02
	5 L	17-0906-04

This product is supplied as suspension in 100% isopropanol.

For additional information, including Data File, application references and Regulatory Support File, please contact your local GE Healthcare representative.

Empty lab-scale columns	Quantity	Code No.
Tricorn 10/20 column, 10 mm i.d., max 2.2 ml bed volume or 2.8 cm bed height	1	28-4064-13
Tricorn 10/50 column, 10 mm i.d., max 4.5 ml bed volume or 5.8 cm bed height	1	28-4064-14
Tricorn 10/100 column, 10 mm i.d., max 8.5 ml bed volume or 10.8 cm bed height	1	28-4064-15
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

Literature	Quantity	Code No.
Data File, BPG columns	1	18-1115-23
Data File, Chromaflow columns	1	18-1138-92
Datafile, NHS-activated Sepharose 4 Fast Flow	1	18-1113-53
The Recombinant Protein Handbook, Protein Amplification and Simple Purification	1	18-1142-75
Affinity Chromatography Handbook, Principles and Methods	1	18-1022-29
Affinity Chromatography Columns and Media Product Profile	1	18-1121-86

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