GE Healthcare

Instructions 71–5001–87 AE **Affinity chromatography**

Chelating Sepharose Fast Flow

Immobilized metal ion affinity chromatography (IMAC) exploits a molecule's affinity for chelated metal ions. The amino acid histidine present in many proteins forms complexes with transition metal ions such as Cu^{2+}, Zn^{2+}, Ni^{2+} and Fe^{3+}. Chelating Sepharose™ Fast Flow with a suitable immobilized metal ion will therefore selectively retain proteins with exposed histidine. Exposed cysteine and tryptophane residues may also be involved in the binding to an immobilized metal ion but their contribution to the binding is much lower than the contribution from exposed histidine residues.

The strength of binding is affected by the buffer pH and the metal ion selected.

Chelating Sepharose Fast Flow consists of iminodiacetic acid groups coupled to Sepharose 6 Fast Flow by stable ether linkages via a 7-atom spacer.

Chelating Sepharose Fast Flow belongs to the BioProcess™ Media family.

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.

Sepharose

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1. Product description

Table 1. Medium characteristics

Total capacity:	30–37 µmol Cu^{2+}/ml drained medium
Bead structure:	6% highly cross-linked agarose
Bead size range:	45–165 µm
Mean particle size:	approx. 90 µm
Linear flow velocity:	>300 cm/h at 25 °C, 0.1 MPa (1 bar, 14.5 psi), XK50/30 column, 15 cm bed height
Max. operating pressure:	0.3 MPa (3 bar, 42 psi)
pH stability*	
Long term:	3–13
Short term:	2–14
Chemical stability:	All commonly used aqueous buffers, 0.01 M HCl, 1.0 M NaOH, 20% ethanol (tested at 40 °C for 7 days)
Physical stability:	Negligible volume variation due to changes in pH or ionic strength
Autoclavable:	In 0.1 M sodium acetate at 121 °C for 30 min

* 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

Chelating Sepharose Fast Flow is supplied pre-swollen in 20% ethanol. Prepare a slurry by decanting the 20% ethanol solution and replacing it with distilled water in a ratio of 75% settled medium to 25% distilled water.

Table 2. Recommended lab-scale columns for Chelating Sepharose 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 Chelating Sepharose 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 3 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 3 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, A_s. 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 4 shows a UV trace for acetone in a typical test chromatogram in which the HETP and A_s values are calculated.

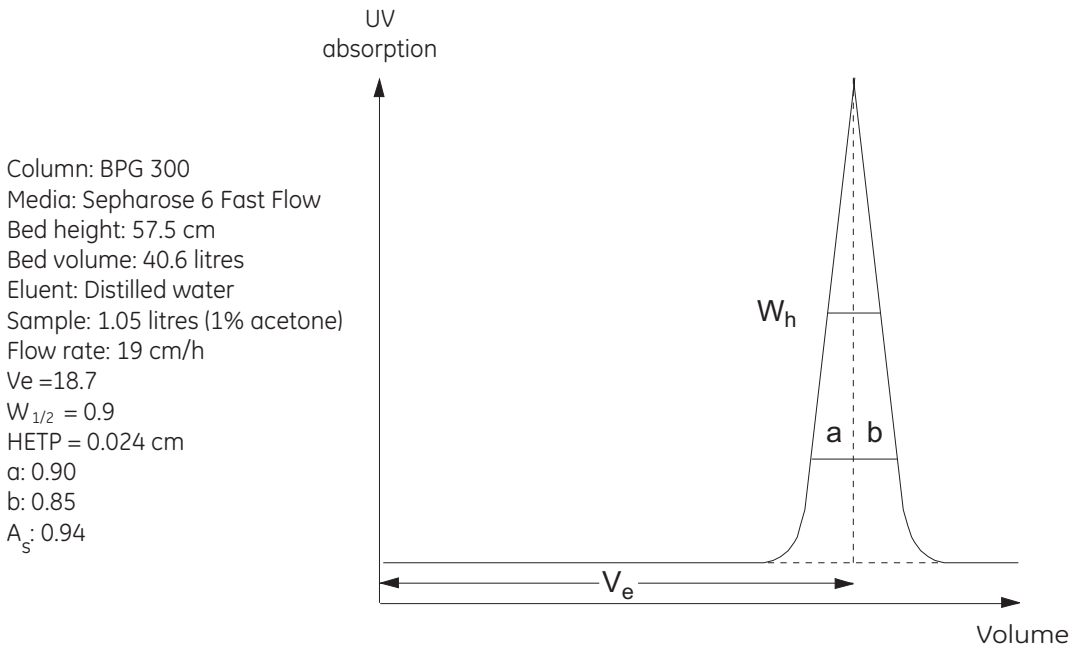

Fig 4. UV trace for acetone in a typical test chromatogram showing the HETP and A_s value calculations.

4 Immobilized Metal Ion Affinity Chromatography (IMAC)

Charging the columns with metal ions

1. Prepare a 0.2 M solution of the desired metal ion (Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+} etc.) in distilled water. Solutions of Zn^{2+} ions should have a pH of approximately 5.5 or lower to avoid solubillity problems that arise at pH 6 or higher. Fe^{3+} ions should be immobilized at low pH, approximately pH 3.0, to avoid formation of insoluble ferric compounds.
2. Wash the column with at least 2 column volumes (CV) of distilled water.
3. Apply approximately 0.2 CV of the metal ion solution to the column.
4. Wash the column with at least 5 CV of distilled water to remove excess of metal ions.

5. Continue washing the column with at least 5 CV of an acidic buffer [0.02 M sodium acetate, 0.5–1.0 M NaCl, pH 4.0] or until the pH of the effluent is 4.0. This will elute loosely bound ions that might otherwise leak out during adsorption/desorption phase of the actual chromatographic step.
6. Equilibrate the column with at least 2 CV of the chosen binding buffer (see recommedations below). The column is now ready for chromatographic seperation of the sample components.

Note: In neutral aqueous solutions, Fe^{3+} ions are easily reduced to form insoluble compounds that can be hard to remove. Columns loaded with Fe^{3+} should therefore not be left for a longer period of time in neutral solutions. It is also advisable to strip off the immobilized Fe^{3+} ions after each run and recharge the column as required. Strongly bound Fe^{3+} ions and ferric compounds can be removed by leaving the medium in 0.05 M EDTA overnight.

Binding

The binding of target solute(s) from a complex biological sample onto an IMAC adsorbent charged with transition metal ions (Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}) is usually carried out in the pH interval of 5.5–8.5. The binding is often strongest at the upper end of this interval.

Care should be taken in preparing the sample for IMAC. The sample should not contain chelating agents such as EDTA or citrate and higher concentrations of competing ions such as primary amines or imidazole/histidine. It is advisable that the sample (and buffers used for the chromatographic step) contains 0.5–1.0 M of a neutral salt (NaCl, KCl, etc) to counteract possible ionic interactions.

The choice of binding buffer depends on the properties of the chelated metal ion and the binding characteristics of the solute. For immobilized transition metal ions, the following equilibration and binding buffers are recommeded:

- 0.02–0.05 M Sodium phosphate buffer, containing 0.5–1.0 M NaCl, pH 6.8 or higher
- 0.05 M Tris-HCl, containing 0.5–1.0 M NaCl, pH 7.0 or higher.

The well-prepared sample is applied to the equilibrated column at a linear flow rate of 150 cm/h or higher. This is followed by washing out of unbound solutes with at least 5 CV of the equilibration buffer or until the A_{280} of the effluent is at or near the base line. The bound solutes are then eluted as described below.

Note: 1. For screening experimets (and especially when the binding characteristics of the target solute is unknown), it is advisable to use Cu^{2+} ions immobilized to Chelating Sepharose Fast Flow since Cu^{2+} ions have the broadest adsorbtion specificity for proteins and peptides (see also Choice of metal ions).
2. The presence of detergents in low concentration in the binding buffers does not normally affect the adsorption of proteins.
3. A partial displacement of immobilized metal ions can sometimes occur as the protein is bound to the adsorbent.

Elution

Elution of solutes bound to the column is achieved by one of three alternative procedures.

1. **Reducing pH** (linear or step-wise decrease in pH). Weakly bound proteins are eluted already at pH 6.0 while strongly bound proteins are eluted successively when pH is lowered from 6.0–4.0 If the target protein is strongly bound, it is advisable to check its stability in an acidic milieu.

The following buffers can be used:

Binding buffer: 0.02 M sodium phosphate, 0.5–1.0 M NaCl, pH 7.0

Elution buffer: 0.02 M sodium phosphate (alternatively sodium acetate), 0.5–1.0 M NaCl, pH 4.0

2. **Competitive elution** (linear or step-wise increase in the concentration of a competing ion, at constant pH). The most commonly used competing ions are: imidazole (0–0.05 M); histidine (0–0.05 M) or ammonium chloride (0–2 M). The following buffers can be used:

Binding buffer: 0.02 M sodium phosphate, 0.5–1.0 M NaCl, pH 7.0

Elution buffer: 0.02 M sodium phosphate, 0.5–1.0 M NaCl, 1 M NH_4Cl (or 0.025 M imidazole), pH 7.0

3. **Stripping of the immobilized metal ions.** This procedure will strip the metal ions from the medium and cause elution of bound proteins. This procedure is not often recommended. It can also be used to wash out denatured or precipitated protein. For this purpose, a 0.05 M solution of EDTA or EGTA, containing 0.5–1.0 M NaCl, (pH adjusted to 7.0), is used.

5 Choice of metal ions

When choosing the desired metal ion, consider the structural requirements underlying the basis of metal chelate-protein recognition.

Ni^{2+} is usually the the first choice metal ion when purifying most histidine-tagged recombinant proteins from cellular contaminants. The strength of binding between a protein and a metal ion is affected by several factors, including the length and position of the affinity tag on the protein, the type of ion used, and the pH of buffers. Some histidine-tagged proteins might therefore be easier to purify with ions other than Ni^{2+}, e.g. Zn^{2+} and Co^{2+}.

In other cases, i.e. for non-tagged proteins, Cu^{2+} and Zn^{2+} metal ions are the most frequently used. Cu^{2+} ions bind strongly to a wide range of proteins and some proteins will only bind to them. For screening experiments (and especially when the binding characteristics of the target protein is unknown), it is advisable to use Cu^{2+} ions immobilized to Chelating Sepharose Fast Flow. Zn^{2+} ions generally give a weaker binding and in some cases this can be exploited to achieve selective elution of a target protein. In some applications, Co^{2+}, Fe^{3+} and Ca^{2+} have also been used.

Ni Sepharose 6 Fast Flow is an additional IMAC product offering from GE Healthcare and this product is pre-charged with nickel and is designed for purification of histidine-tagged proteins (see Ordering information)

6. Regeneration, Cleaning, Sanitization and Storage.

Regenerating the medium

Before the medium is immobilized with a new metal ion, the medium must be stripped or regenerated. To ensure that the medium is totally free from metal ions wash with 0.5 column volumes of a 0.2 M solution of EDTA, 0.5 M NaCl. Remove residual EDTA by washing with 2–3 column volumes of 0.5 M NaCl.

Re-immobilization of the medium is performed according to the method previously described (see Charging the columns with metal ions).

Strongly bound ferric ions and ferric compounds can be removed by leaving the medium in 0.05 M EDTA overnight.

In some applications, substances such as denatured proteins or lipids do not elute in the regeneration procedures. These can be removed by cleaning-in-place procedures.

Cleaning-in-place (CIP)

Remove ionically bound proteins by washing the column with at least 0.5 column volumes of a 2 M NaCl solution, contact time 10–15 minutes, reversed flow direction.

Remove precipitated proteins, hydrophobically bound proteins and lipoproteins by washing the column with 1 M NaOH solution at a linear flow rate of approximately 40 cm/h, contact time 1–2 hours, reversed flow direction.

In both cases, wash with at least 3 bed volumes of starting buffer.

Remove strongly hydrophobically bound proteins, lipoproteins and lipids by washing the column with 4 bed volumes of up to 70% ethanol or 30% isopropanol, reversed flow direction. 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. Use, for example, 0.1–0.5% nonionic detergent in 0.1 M acetic acid. Wash at a low linear flow rate of approximately 40 cm/h, contact time

1–2 hours, reversed flow direction. After treatment with detergent always remove residual detergent by washing with 5 bed volumes of 70% ethanol.

In both cases, wash with at least 3 bed volumes of binding buffer.

Sanitization

Sanitization reduces microbial contamination of the medium to a minimum.

Wash the column with 0.5–1 M NaOH at a flow rate of approximately 40 cm/h, contact time 30–60 minutes, reversed flow direction.

Re-equilibrate the column with 3–5 bed volumes of sterile binding buffer.

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

Storage

Store the medium for longer periods of time in 20% ethanol or in 0.01 M NaOH.

7. Ordering information

Product	Quantity	Code No.
Chelating Sepharose Fast Flow	50 ml	17-0575-01
Chelating Sepharose Fast Flow	500 ml	17-0575-02
Chelating Sepharose Fast Flow	5L	17-0575-04

Prepacked columns		
HiTrap Chelating HP	5 x 1 ml	17-0408-01
HiTrap Chelating HP	5 x 5 ml	17-0409-03

Related products		
Ni Sepharose 6 Fast Flow	25 ml	17-5318-01
Ni Sepharose 6 Fast Flow	100 ml	17-5318-02
Ni Sepharose 6 Fast Flow	500 ml	17-5318-03
Ni Sepharose 6 Fast Flow	1 L	17-5318-04
Ni Sepharose 6 Fast Flow	5 L	17-5318-05
HisTrap FF	5x1 ml	17-5319-01
HisTrap FF	5x5 ml	17-5255-01
HisPrep FF	1x20 ml	17-5256-01

Empty 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

Literature

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
Datafile BPG columns		18-1115-23
Datafile BPG 450 columns		18-1160-59
Datafile Chromaflow columns		18-1138-92

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Licensing information

US pat 5,284,933, US pat 5,310,663, and their equivalents in other countries (assignee: Hoffmann-La Roche, Inc.) relate to the purification and preparation of fusion proteins and affinity peptides comprising at least two adjacent histidine residues (commonly known as the histidine-tag technology).

Any customer that wishes to use Chelating Sepharose Fast Flow or Ni Sepharose 6 Fast Flow for non-research/ commercial applications under these patents is requested to contact F. Hoffmann-La Roche AG, Corporate licensing, attn. Dr Andreas Maurer, CH-4070 Basel, Switzerland, tel +41 61 687 2548, fax +41 61 687 2113, for the purpose of obtaining a license.

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