

● Chelating Sepharose Fast Flow

Table of contents

1. Introduction	3
2. Product description	4
3. Packing columns	5
3.1 Recommended columns	5
3.2 Packing lab-scale columns	6
3.3 Packing large scale columns	7
4. Evaluation of packing	17
5. Immobilized Metal Ion Affinity Chromatography (IMAC)	20
6. Purification of Histidine-tagged Fusion Proteins	24
7. Regeneration, Cleaning, Sanitization and Storage	31
8. Ordering information	33

1 Introduction

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 Fast Flow by stable ether linkages via a 7-atom spacer.

Chapter 6 contains specific methods for purification of histidine-tagged fusion proteins.

Chelating 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 health care 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.

2 Product description

Table 1. Medium characteristics

Total capacity:	30-37 $\mu\text{mol Cu}^{2+}/\text{ml}$ drained medium
Bead structure:	6% highly cross-linked agarose
Bead size range:	45-165 μm
Mean particle size:	approx. 90 μm
Linear flow rate:	>700 cm/h at 25°C, 0.1 MPa (1 bar, 14.5 psi), K50/30 column, 5 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.

3 Packing columns

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.

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 4.5ml 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.
- XK 50/20 (50 mm i.d.) for bed volumes up to 275 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.
- BioProcess™ Stainless Steel (BPSS) fixed bed columns. Inner diameters from 400–1400 mm; bed volumes from 12–1500 litres, bed height 10–100 cm.
- INdEX™ variable bed columns. Inner diameters from 70–200 mm; bed volumes up to 24.8 litres; bed heights of max 79 cm.
- CHROMAFLOW™ variable bed columns. Inner diameters from 280–2000 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 6 Fast Flow based media are packed at a constant pressure of approximately 1.5 bar (0.15 MPa). If the packing equipment does not include a pressure gauge, use a packing flow velocity of approximately 500 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 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.

Sepharose 6 Fast Flow based medium are easy to pack since their rigidity allows the use of high flow rates, see Figure 1. Four suitable types of packing methods are given:

- CHROMAFLOW packing.
- Pressure packing (for columns with adaptors).
- Suction packing (for large columns with fixed bed heights).
- Hydraulic pressure packing.

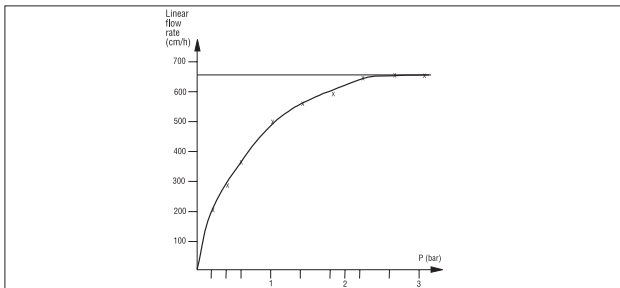


Figure 1. Pressure/flow rate curve for Sepharose 6 Fast Flow media.

How well the column is packed will have a major effect on the result of the separation. It is therefore very important to pack and test the column according to the following recommendations.

Begin the packing procedure by determining the optimal packing flow rate. Guidelines are given below for determining the optimal packing flow rates for columns with adaptors and fixed bed heights.

Determining optimal packing flow rate

The optimal packing flow rate is dependent on column size and type, bed height, packing solution and temperature. The optimal packing flow rate must therefore be determined empirically for each individual system.

To determine the optimal packing flow rate, proceed as follows:

1. Calculate the exact amount of medium needed for the slurry (this is especially important for columns with fixed bed

heights). The quantity of medium required per litre packed bed is approximately 1.15 litre sedimented medium.

2. Prepare the column exactly as for column packing.
3. Begin running the column at a low flow rate (e.g. 30% of the expected maximum flow rate) and record the flow rate and back pressure when the bed is packed and the pressure has stabilized.
4. Increase the flow rate in small steps and record the flow rate and pressure at each step after the pressure has stabilized.
5. Continue recording flow and pressure until the maximum flow rate has been reached, i.e. when the flow rate levels off at a plateau indicating bed compression or when the pressure reaches the pressure specification of the column used.
6. Plot pressure against flow rate as indicated in Figure 1. The optimal packing flow rate/pressure is 70–100% of the maximum flow rate/pressure.

The operational flow rate/pressure should be <70% of the packing flow rate/pressure.

Note: For BPSS columns, first pack the column by suction packing at a low flow rate. Then determine the flow/pressure characteristics as above by pumping buffer downwards through the column.

Packing CHROMAFLOW columns

Procedure

Prepare the column for packing as described in the User Manual.

Packing from the top

1. Set the top nozzle to the pack position (mid-position).

2. Fully retract the bottom nozzle (run position).
3. Ensure that the top mobile phase is closed.
4. Open the bottom mobile phase.
5. Open inlet (C) and start the packing pump. Adjust the flow to achieve the required packing conditions for the selected medium. Monitor column pressure and the outlet flow rate in order to record column packing parameters. (Remember to stir the medium slurry during packing to prevent it from settling.)
6. Continue pumping until the column is fully packed and the pump stalls due to build-up of medium in its pipelines. Turn off the packing pump.
7. Fully retract the top nozzle to its run position. Close outlet (C). Open inlet (B) from the water/buffer tank and open outlet (D). The pump should now be restarted to rinse the top slurry lines. (If the nozzle is full of liquid when in the packing position, make sure that the waste slurry outlet is open before retracting the nozzle.)
8. To clean-in-place, exchange the buffer tank for wash/buffer tank containing cleaning solution.

Packing from below

To pack from the bottom, carry out the same procedure for the connections and flow path via the bottom nozzle. The column is now ready to equilibrate and test.

Note: It is also possible to use a slightly different packing method where the amount of medium is predetermined. In this case the complete amount of medium is packed into the column causing compression of the bed. When all medium has entered the column the pump is stopped, the top nozzle is retracted, the bottom mobile phase valve closed and the medium is allowed to decompress within the column.

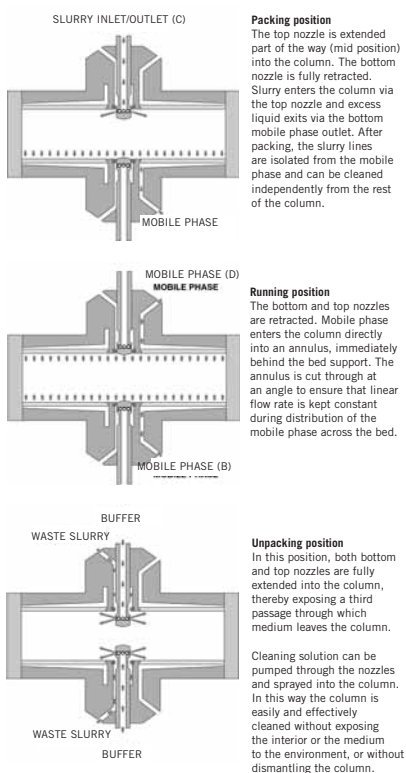


Figure 2. Principle of operation – CHROMAFLOW columns.

Pressure packing (BPG Columns)

BPG Columns are supplied with a movable adaptor. They are packed by conventional pressure packing by pumping the packing solution through the chromatographic bed at a constant flow rate (or back pressure).

1. Pour some water (or packing buffer) into the column. Make sure that there is no air trapped under the bottom bed support. Leave about 2 cm of liquid in the column.
2. Mix the packing buffer with the medium to form a 50–70% slurry (sedimented bed volume/slurry volume = 0.5–0.7). Pour the slurry into the column. Insert the adaptor and lower it to the surface of the slurry, making sure no air is trapped under the adaptor. Secure the adaptor in place.
3. Seal the adaptor O-ring and lower the adaptor a little into the slurry, enough to fill the adaptor inlet with packing solution.
4. Connect a pump and a pressure meter and start packing at the predetermined packing flow rate (or pressure). Keep the flow rate (or pressure) constant during packing and check the pressure at the column inlet. Never exceed the pressure limit for column or medium.
5. When the bed has stabilized, close the bottom valve and stop the pump. The bed starts rising in the column. Loosen the O-ring and lower the adaptor to 0.5–1.0 cm above the bed surface.
6. Seal the O-ring, start the pump and continue packing. Repeat steps 5 and 6 until there is a maximum of 1 cm between bed surface and adaptor when the bed has stabilized. Mark the bed height on the column tube.
7. Close the bottom valve, stop the pump, disconnect the column inlet and, without loosening the adaptor O-ring, push the adaptor down to approximately 3 mm below the

mark on the column tube. The packing solution will flush the adaptor inlet. Remove any trapped air by pumping liquid from the bottom (after the inlet tubing and the bottom valve have been properly filled).

Suction packing (BPSS Columns)

BioProcess Stainless Steel Columns are supplied with fixed end pieces. They are packed by suction, i.e. by sucking packing solution through the chromatographic bed at a constant flow rate.

1. Fit a packing device on top of the column tube.
2. Pour some water (or packing buffer) into the column. Make sure that there is no air trapped under the bottom bed support. Leave about 2–3 cm of liquid in the column.
3. Mix the packing buffer with the medium to form a 50% slurry (sedimented bed volume/slurry = 0.5). Pour the slurry into the column. Stir gently to make sure it is homogeneous.
4. Connect the column outlet valve to the suction side of a pump and start packing the bed by suction through the bed at the predetermined flow rate. Keep the flow rate constant during packing.
5. When the bed has stabilized, the top of the bed should be just below the junction between the column and the packing device.

If, when stabilized, the level of the bed is incorrect, add or remove slurry. Always stir the slurry thoroughly before packing.

6. Just before the last of the solution has entered the packed bed (before the surface starts to dry), close the valve at the column outlet, stop the pump, quickly remove the packing device and replace it with the lid.

Note: This final operation should be completed as quickly as possible because the bed will expand when the flow stops.

7. Start pumping buffer with upward flow through the column to remove any air bubbles trapped under the lid.

Hydraulic packing (INdEX Columns)

INdEX Columns are supplied with a hydraulic function which allows an extremely simple, rapid and reproducible packing procedure. The medium is packed at the same time as the adaptor is lowered into position at the correct pressure.

The adaptor is pushed down by a constant hydraulic pressure, forcing water or packing buffer through the slurry and compressing it so that a packed bed is gradually built up. The hydraulic pressure can be generated using a pump and a pressure relief valve.

When the adaptor reaches the surface of the settled medium, it continues downwards under hydraulic pressure compressing the medium. The extent to which the medium is compressed depends upon the pressure from the adaptor and the elasticity of the medium. The quantity of medium required when packing Sepharose 6 Fast Flow based media by hydraulic pressure is approximately 1.2 litre sedimented medium per litre packed bed.

1. Pour some water (or packing solution) into the column. Make sure that there is no air trapped under the bottom bed support. Leave about 2 cm of liquid in the column.
2. Pour the 75% slurry into the column. Fill the column with packing solution up to the top of the glass tube and mix the slurry. Allow the medium to sediment to just below the bevel of the glass tube (G), see Figure 3.

- Put the adaptor in a resting position against the bevel of the glass tube. Avoid trapping air bubbles under the adaptor by slightly tilting the adaptor while mounting.
- Lower the lid and secure it in place.
- Connect a pump to the inlet of the hydraulic chamber (A), with a manometer and a pressure relief valve in-line between the pump and the hydraulic chamber. The manometer should be placed after the valve in the direction of the flow.
- Open the hydraulic inlet (A), and the hydraulic outlet (C). Start the pump and flush the hydraulic chamber (E) free of air and any residual medium.
- Close (C) and open the elution inlet/outlet (B) to allow trapped air in the adaptor bed support to escape.
- Close (B) and open the elution inlet/outlet (D) to start the packing, applying a predefined constant hydraulic packing pressure. When packing Sepharose 6 Fast Flow based media in an INdEX column to a bed height of 15 cm, the recommended hydraulic packing pressure is 1.5 bar for INdEX 100 and 0.8 bar for INdEX 200.
- When the adaptor has reached the surface of the settled bed, continue to run the pump until the adaptor has been lowered 5 mm into the packed bed.
- Close (A) and (D) and stop the pump.
- Run the column with upward flow for a few minutes to remove residual air trapped in the adaptor. The column is now ready for use.
- To unpack the column, connect the outlet from the pump to (B) and open (C) while keeping (D) closed. This will cause the adaptor to rise from the bed surface.

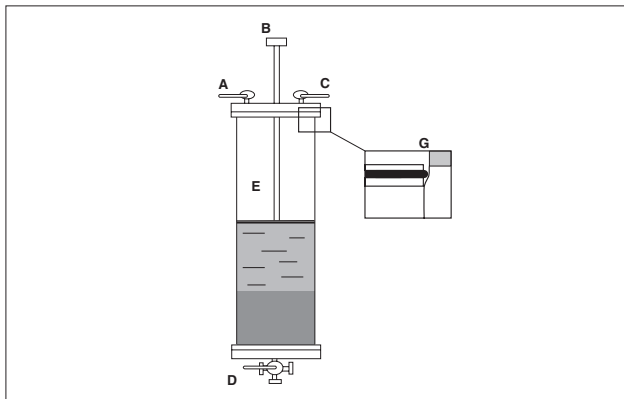


Figure 3. Schematic representation of INdEX column with a 4-port (2-way) valve mounted at the bottom outlet.

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% (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:

$$\begin{aligned} \text{HETP} &= L/N \\ \text{where } N &= 5.54(V_e/W_h)^2 \\ L &= \text{Bed height (cm)} \\ N &= \text{number of theoretical plates} \\ V_e &= \text{Peak elution distance} \\ W_h &= \text{Peak width at half peak height} \end{aligned}$$

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:

$$\text{HETP}/d$$

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.

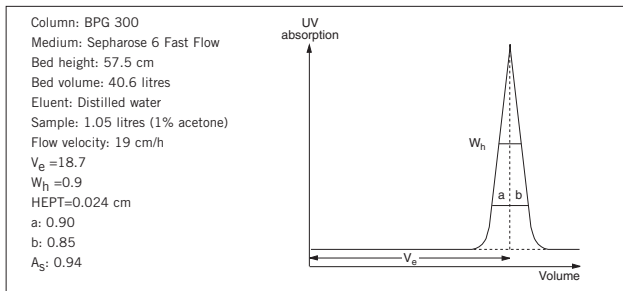


Figure 4. UV trace for acetone in a typical test chromatogram showing the HETP and A_S value calculations.

5 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 solubility 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 3.0] or until the pH of the effluent is 3.0. 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 recommendations below). The column is now ready for chromatographic separation 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 recommended:

- 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 overleaf.

Note:

1. For screening experiments (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 adsorption specificity for proteins and peptides.
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 3.0

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.

6 Purification of Histidine-tagged Fusion Proteins

Chelating Sepharose Fast Flow charged with different type of metal ions such as nickel, zinc, cobalt etc. will selectively retain proteins containing a histidine-tag. Histidine-tagged protein is then eluted using buffers containing imidazole.

The following protocols are designed to maximize the binding of histidine-tagged fusion proteins to Chelating Sepharose Fast Flow and to ensure complete elution. They are useful when the exact conditions required for binding and elution are not known.

To obtain highest purity the optimal concentration of imidazole during sample loading, washing and elution has to be determined. The optimal concentrations can be determined by eluting the fusion protein with a stepwise gradient of imidazole from 10 mM to 500 mM and testing each fraction for the presence of fusion protein and impurities by SDS-PAGE.

For purification of insoluble histidine-tagged proteins (fusion proteins expressed as inclusion bodies) requiring denaturing conditions, up to 8 M urea or 6 M guanidine hydrochloride can be used in the buffers to solubilise insoluble proteins.

Sample preparation

For optimal conditions for growth, induction and cell lysis conditions of recombinant histidine-tagged clones, please refer to manufacturer's protocols.

The sample should be fully dissolved. In order to avoid clogging we recommend filtration through a 0.45 μm filter to remove cell debris or other particulate material.

If the sample is dissolved in a buffer other than 20 mM phos-

phate buffer including 0.5 M NaCl pH 7.4, the sample should be adjusted to pH 7-8. This can be achieved by buffer exchange on a HiTrap™ Desalting column or PD-10 column or HiPrep™ 26/10 Desalting column depending on the sample volume.

Preparation of Chelating Sepharose Fast Flow

Chelating Sepharose Fast Flow as supplied is approximately a 75% slurry in 20% ethanol. The following procedure results in a 50% slurry of Chelating Sepharose Fast Flow charged with Ni²⁺.

Wash the medium:

1. Gently shake the container of Chelating Sepharose Fast Flow to re-suspend the medium.
2. Remove enough slurry to bind the histidine-tagged protein and transfer it to an appropriate container/tube. The binding capacity of Chelating Sepharose Fast Flow is ≈ 5 mg histidine-tagged protein/ml medium.
3. Sediment the medium by centrifugation at 500 x g for 2-5 minutes.
4. Carefully decant the supernatant and dispose of it.
5. Add five medium volumes of distilled water and shake until the medium is fully re-suspended, for example, end-over-end rotation for 5 minutes. Do not use magnetic stirrers.
6. Re-sediment the medium by centrifugation at 500 x g for 2-5 minutes.
7. Carefully decant the supernatant and dispose of it.

Charge the medium:

1. Add 0.5 medium volume of 0.1 M NiSO₄ solution (or other metal of choice) and shake until the medium is fully re-suspended.

ded, for example, end-over-end rotation for 5 minutes.

2. Re-sediment the medium by centrifugation at 500 x g for 2-5 minutes.

3. Carefully decant the supernatant and dispose of it.

Wash the medium:

1. Add five medium volumes of distilled water and mix, for example, end-over-end rotation for 5 minutes.

2. Re-sediment the medium by centrifugation at 500 x g for 2-5 minutes.

3. Carefully decant the supernatant and dispose of it.

4. Repeat the washing steps two more times (total wash 3 x 5 medium volumes of distilled water).

5. Re-suspend the medium in one medium volume of Chelating Sepharose Fast Flow binding buffer, e.g. 20 mM Na₂HPO₄, 0.5 M NaCl, 10 mM imidazole pH 7.4.

Note: Chelating Sepharose Fast Flow may also be charged with metal ions when packed in a column. Wash the column with 5-10 column volumes (CV) of distilled water. Load 0.5 CV of 0.1 M metal solution in distilled water. Wash with 5-10 CV distilled water. Equilibrate the column with 5 CV binding buffer. The column is now ready for loading the sample.

Purification of histidine-tagged proteins using centrifugation.

Binding of sample:

1. Add an appropriate amount of sample (in binding buffer) to the 50% slurry of Chelating Sepharose Fast Flow equilibrated in binding buffer. The binding capacity of Chelating Sepharose Fast Flow is \approx 5 mg histidine-tagged protein/ml medium.

2. Incubate with gentle agitation, end-over-end rotation for 5-30 minutes at room temperature. The binding kinetics depend on the protein and the sample concentration.
3. Sediment the medium by centrifugation at 500 x g for 2-5 minutes.
4. Carefully decant the supernatant and save it for analysis, e.g. SDS-PAGE.

Wash the medium:

1. Add five medium volumes of binding buffer and mix, e.g. end-over-end rotation for 5 minutes.
2. Re-sediment the medium by centrifugation at 500 x g for 2-5 minutes.
3. Carefully decant the supernatant and save it for analysis, e.g. SDS-PAGE.
4. Repeat the washing steps two more times (total wash 3 x 5 medium volumes of binding bufer). Save the wash solutions in different tubes for analysis, e.g. SDS-PAGE.

Elution:

1. Add two medium volumes of elution buffer, e.g. 20 mM Na_2HPO_4 , 0.5 M. NaCl, 0.5 M imidazole; pH 7.4. and mix, e.g. end-over-end rotation for 5 minutes.
2. Re-sediment the medium by centrifugation at 500 x g for 2-5 minutes.
3. Repeat the elution steps four more times (total elution 5 x 2 medium volumes of elution buffer). Save the eluates in different tubes for analysis, e.g. absorbance measurement at 280 nm, SDS-PAGE, ELISA, Western Blotting.

Pool the tubes with contents of interest.

Trouble-shooting guide when purifying histidine-tagged fusion proteins.

The sample is too viscous.

If the lysate is very viscous due to the presence of a high concentration of host nucleic acid, continue sonication until viscosity is reduced, add RNase A to a final concentration of 10 g/ml and/or DNase I to 5 µg/ml, and incubate on ice for 10-15 minutes. Alternatively, draw the lysate through a syringe needle several times.

A batch/centrifugation purification method is preferred to use in this case to prevent too low flow rate and clogging of the column.

The histidine-tagged fusion protein elutes during the wash.

- Check the pH and composition of buffers. If buffer compositions are incorrect, or chelating (e.g. EDTA) or strong reducing agents are present in the sample, the fusion protein may elute unexpectedly during the wash steps.
- Increase the amount of medium in the purification. If the capacity of the medium is exceeded, fusion protein may elute during the wash as well as during the elution. The binding capacity for Chelating Sepharose Fast Flow is ≈5 mg protein/ml medium (His₆-tagged protein with M_r: 27 600 Da).
- Prepare fresh Chelating Sepharose Fast Flow. If the matrix is incorrectly charged, the fusion protein will not bind effectively.

No histidine-tagged protein in the eluted fractions.

SDS-PAGE analysis of samples collected during the preparation of the bacterial sonicate may indicate that the majority of the fusion protein is located in the post-sonicate pellet. Possible causes and solutions are:

- Sonication may be insufficient. Cell disruption may be checked by microscopic examination or monitored by measuring the release of nucleic acids at A_{260} . Addition of lysozyme (0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl, pH 6.0-8.0) prior to sonication may improve results. Avoid frothing as this may denature the fusion protein.
- The protein may be insoluble (inclusion bodies) and found in the pellet. The protein can usually be solubilised from inclusion bodies using common denaturants such as 4-6 M guanidine hydrochloride, 4-8 M urea or detergents. Samples containing 8 M urea can be analysed using SDS-PAGE electrophoresis directly, but samples containing 6 M guanidine hydrochloride must be buffer exchanged with, for example HiTrap Desalting or PD-10 column or HiPrep 26/10 Desalting column against buffer containing 8 M urea before loading on a gel.
- The elution is too mild and the histidine-tagged protein is still bound to the medium. Elute with a higher concentration of imidazole or decrease pH to determine the optimal elution conditions.
- The protein of interest does not bind to the medium and is found in the flow-through material. The concentration of imidazole is too high in the binding buffer. Decrease the imidazole concentration in the binding buffer.

Multiple bands are detected on CoomassieTM-stained/Silver-stained SDS-PAGE.

- Add a protease inhibitor. Multiple bands may be a result of partial degradation of fusion proteins by proteases. Addition of 1 mM PMSF or other protease inhibitor to the lysis buffer may reduce the number of additional bands. Serine protease inhibitors must be removed prior to cleavage by thrombin.

- Elute with a stepwise imidazole gradient to determine the optimal imidazole concentration for elution of the histidine-tagged protein. Specific contaminants may be washed away using an imidazole concentration in the wash buffer that is higher than 10 mM and not high enough to elute the fusion protein. Other contaminants may be retained on the Chelating Sepharose Fast Flow by using an elution buffer which has a lower imidazole concentration than 500 mM.
- Add detergent (e.g. up to 2% Triton™ X-100 or 2% Tween™ 20), glycerol (up to 50%), or β-mercaptoethanol (up to 20 mM) to the binding buffer used during the wash steps. Contaminants may be non-specifically associating with the fusion protein and the association may be disrupted by altering the buffer used for washing.
- If the histidine-tagged fusion protein and a contaminant have similar affinities for Chelating Sepharose Fast Flow, then it will not be possible to optimize wash or elution steps to improve purity. In this case, it is necessary to add a second purification method ion exchange or gel filtration may be useful.

Note: Purification and preparations of fusion proteins and affinity peptides comprising at least two adjacent histidine residues may require a license under US pat 5,284,933 and US pat 5,310,663, including corresponding foreign patents (assigne: Hoffman La Roche, Inc).

7 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 described in chapter 5.

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 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% non-ionic 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.

8 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	1 x 5 ml	17-0409-01
HisTrap kit	kit	17-1880-01
Lab-scales columns		
Tricorn 5/20 column	1	18-1163-08
Tricorn 5/50 column	1	18-1163-09
Tricorn 10/20 column	1	18-1163-13
Tricorn 10/50 column	1	18-1163-14
Tricorn 10/100 column	1	18-1163-15
XK 16/20 column	1	18-8773-01
XK 16/40 column	1	18-8774-01
XK 26/20 column	1	18-1000-72
XK 26/40 column	1	18-8768-01
XK 50/20 column	1	18-1000-71
Data files		
BPG columns		18-1115-23
BPG 450 column		18-1160-59
BioProcess Stainless Steel columns		18-1121-08
IndEX columns		18-1115-61
CHROMAFLOW columns		18-1138-92

Further information.

Please read these instructions carefully before using Chelating Sepharose Fast Flow Medium. For further information visit www.amershambiosciences.com or contact your local Amersham Biosciences representative.

Important Information

BioProcess, BPG, CHROMAFLOW, Drop Design, HiPrep, HisTrap, HiTrap INdEX, Sepharose, Tricorn are trademarks of Amersham Biosciences Limited. Amersham and Amersham Biosciences are trademarks of Amersham plc. Coomassie is a trademark of Union Carbide Chemicals and Plastic Company Inc. Tween is a trademark of ICI Americas Inc.

© Amersham Biosciences AB 2002 - All rights reserved.

All goods and services are sold subject to the Conditions of Sale of the company within the Amersham Biosciences group which supplies them, save where otherwise agreed in writing. Under the terms of such Conditions of Sale, Amersham Biosciences warrants that the Goods (as defined) meet written specifications at the time of shipment and that Equipment (as defined) shall be free of defects in workmanship or materials under normal usage for a period of one year, but such warranty will be rendered void in the case of abnormal working conditions, failure to follow Amersham Biosciences instructions and/or other misuse. AMERSHAM BIOSCIENCES EXPRESSLY EXCLUDES ALL OTHER WARRANTIES, REPRESENTATIONS, TERMS AND CONDITIONS (STATUTORY, EXPRESS IMPLIED OR OTHERWISE) AS TO QUALITY, CONDITION, DESCRIPTION, MERCHANTABILITY OR FITNESS FOR PURPOSE (EXCEPT FOR THE IMPLIED WARRANTY OF TITLE). AMERSHAM BIOSCIENCES SHALL HAVE NO LIABILITY FOR ANY INDIRECT CONSEQUENTIAL OR PUNITIVE DAMAGE OF ANY KIND FROM ANY CAUSE ARISING OUT OF THE SALE, INSTALLATION, USE OR INABILITY TO USE ANY PRODUCT OR SERVICE, INCLUDING WITHOUT LIMITATION, LOSS OF PROFITS, OR GOODWILL OR BUSINESS INTERRUPTION. A copy of the Amersham Biosciences Conditions of Sale is available on request.

Amersham Biosciences AB

Björkgatan 30, SE-751 84 Uppsala
Sweden

Amersham Biosciences UK Limited

Amersham Place
Little Chalfont Bucks, Buckinghamshire HP7 9NA
England

Amersham Biosciences Corp

800 Centennial Avenue,
Piscataway, New Jersey 08855
USA

Amersham Biosciences Europe GmbH

Munzinger Strasse 9
D-79111 Freiburg
Germany

Amersham Biosciences K. K.

Sanken Building, 3-25-1
Shinjuku-ku, Tokyo 169-0073
Japan

 Amersham
Biosciences

