Chelating Sepharose® Fast Flow INSTRUCTIONS

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I 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 IV contains specific methods for purification of histidine-tagged fusion proteins using gravity columns or centrifugaion.

Chelating Sepharose Fast Flow belongs to the BioProcess™ Media family. BioProcess Media are separation media develo - ped, 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.

II Product description

Table 1. Gel characteristics

Total capacity:	24-30 µmole Zn^{2+}/ml drained gel
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 gel is stable over a long period of time without adverse effects on its subsequent chromatographic performance.

pH stability, short term refers to the pH interval for regeneration, cleaning-in place and sanitization procedures.

Preparing the gel

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 gel to 25% distilled water.

III Immobilized Metal Ion Affinity Chromatography (IMAC)

Packing Sepharose Fast Flow gels

1.Equilibrate all material to the temperature at which the chromatography will be performed.

2. De-gas the gel slurry.

3. Eliminate air from the column dead spaces by flushing the end pieces with distilled water. Make sure no air has been trapped under the column net. Close the column outlet with a few centimeters of distilled water remaining in the column.

4. Pour the slurry into the column in one continuous motion. Pouring the slurry down a glass rod held against the wall of the column will minimize the introduction of air bubbles.

5. Immediately fill the remainder of the column with distilled water, mount the column top piece onto the column and connect the column to a pump.

6. Open the bottom outlet of the column and set the pump to run at the desired flow rate. Ideally, Fast Flow gels are packed at a constant pressure not exceeding 3 bar (0.3 MPa) in XK columns. If the packing equipment does not include a pressure gauge, use a packing flow rate of at least 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 gel.

Note: Do not exceed 75% of the packing flow rate in subsequent chromatographic procedures.

7. Maintain the packing flow rate for 3 bed volumes after a constant bed height is reached.

Using an adaptor

Adaptors should be fitted as follows:

1. After the gel has been packed as described above, close the column outlet and remove the top piece from the column. Carefully fill the rest of the column with distilled water to form an upward meniscus at the top.

2. Insert the adaptor at an angle into the column, ensuring that no air is trapped under the net.

3. Make all tubing connections at this stage. There must be a bubble-free liquid connection between the column and the pump and column and the sample application system.

4. Slide the plunger slowly down the column so that the air above the net and in the capillary tubings is displaced by eluent. Valves on the inlet side of the column should be turned in all directions during this procedure to ensure that air is removed.

5. Lock the adaptor in position, open the column outlet and start the eluent flow. Pass eluent through the column at the packing flow rate until the gel bed is stable. Re-position the adaptor on the gel surface as necessary.

Immobilizing metal ions

The gel is immobilized with metal ions in distilled water to avoid precipitating metal salts on the gel.

1. Make sure that the column is equilibrated in distilled water. If necessary, wash with at least 2 column volumes of distilled water.

2.Choose the metal ion (Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, Fe^{3+} etc) and make a 0.1-0.3 M neutral or weakly acid soluion. Fe^{3+} should be immobilized in low pH, approximately pH 3, to avoid precipitating of insoluble ferric compounds.

3. Apply approximately 1 column volume of the metal ion solution.

4. Wash the gel with at least 5 column volumes of distilled water.

5. Equilibrate the gel with at least 2 column volumes of chosen start buffer, see recommendations below, before applying the sample.

Note: When working with Fe^{3+} extra precautions have to be made. In neutral aqueous solutions, Fe^{3+} is easily reduced and forms compounds that can be hard to dissolve. Gels loaded with Fe^{3+} should not be left for a longer period of time in neutral solutions. It is advisable to strip the gel after each run and reload it.

Strongly bound ferric ions and ferric compounds can be removed by leaving the gel in 0.05 M EDTA over night.

Binding

Binding usually occurs in the pH range 5.5-8.5. Binding is often strongest at the upper end of this interval.

The choice of starting buffer depends on the properties of the chelated metal ion and the binding properties of the sample molecules. Sodium acetate and sodium phosphate are recommended buffers.

Chelating agents such as EDTA or citrate should not be included in the buffer.

The most commonly used buffers are:

- 0.01-0.2 M Sodium phosphate
- 0.05 M Sodium acetate

It is common to include salt, e.g. 0.15-0.5 M NaCl, in the buffers to eliminate any ion exchange effect.

As a general rule, for the binding of a protein with unknown binding properties to Chelating Sepharose Fast Flow it is advisable to use Zn^{2+} and a neutral phosphate or acetate buffer containing 0.15-0.5 M NaCl for initial experiments.

The presence of detergents in the buffers does not normally affect the adsorption of proteins.

A partial displacement of chelated metal ions is often noted as the protein is adsorbed. This is visible, especially when using metal ion solutions that are coloured, such as Cu^{2+}, as a downward extension of the zone of chelated ions.

Eluting

Proteins may be desorbed from the column by:

- Reducing pH (linear or step). Most proteins elute between pH 6 and 4. A final pH of 3-4 is often suitable. Sodium acetate, citrate or phosphate can be used.
- Competitive elution with a gradient or step-wise increasing concentration of imidazole (0-0.5 M), histidine (0-0.05 M), ammonium chloride (0-2 M) or other substance with affinity for the chelated metal ion. The gradient is best run in the starting buffer at a constant pH.
- Chelating agents such as EDTA or EGTA (0.05 M solutions) will strip the metal ions from the gel and cause elution of the proteins. This method does not resolve different proteins.

In all cases, 0.15-0.5 M NaCl should be included in the buffers to avoid ionic interactions.

As a general method when Cu^{2+} is used, we recommend following running conditions:

Reducing pH:

Binding buffer: 0.02 M Na_2HPO_4, 0.5 M NaCl, pH 7.2

Elution buffer: 0.02 M Na_2HPO_4, 0.5 M NaCl, pH 3.5

Competitive elution:

Binding buffer: 0.02 M Na_2HPO_4, 1 M NaCl, pH 7.2

Elution buffer: 0.02 M Na_2HPO_4, 1 M NH^4Cl, pH 7.2

Stripping elution:

Binding buffer: 0.02 M Na_2HPO_4, 0.5 M NaCl, pH 7.2

Elution buffer:
0.02 M Na_2HPO_4, 0.5 M NaCl, 0.05 M EDTA, pH 7.2

IV Purification of Histidine-tagged Fusion Proteins using Centrifugation or Gravity Flow.

Chelating Sepharose Fast Flow charged with nickel ions 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 and elution has to be determined. The optimal concentration 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 phosphate 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 Pre-packed PD-10 column (Sephadex® G-25).

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 nickel.

Wash the gel:

1. Gently shake the container of Chelating Sepharose Fast Flow to re-suspend the gel.

2. Use a pipet to 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 gel.

3. Sediment the gel by centrifugation at 500 x g for 2-5 minutes.

4. Carefully decant the supernatant and dispose of it.

5. Add five gel volumes of distilled water and shake until the gel is fully re-suspended, for example, end-over-end rotation for 5 minutes. Do not use magnetic stirrers.

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

7. Carefully decant the supernatant and dispose of it.

Charge the gel:

1. Add 0.5 gel volume of 0.1 M $NiSO_4$ solution and shake until the gel is fully re-suspended, for example, end-over-end rotation for 5 minutes.

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

3. Carefully decant the supernatant and dispose of it.

Wash the gel:

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

2. Re-sediment the gel 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 gel volumes of distilled water).

5. Re-suspend the gel in one gel volume of Chelating Sepharose Fast Flow Start Buffer, e.g. 20 mM Na_2HPO_4, 0.5 M NaCl, 10 mM imidazole pH 7.4.

Purification of histidine-tagged proteins using centrifugation.

Binding of sample:

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

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 gel 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 gel:

1. Add five gel volumes of Start Buffer and mix, e.g. end-over-end rotation for 5 minutes.

2. Re-sediment the gel 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 gel volumes of Start Buffer). Save the wash solutions in different tubes for analysis.

Elution:

1. Add two gel 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 gel by centrifugation at 500 x g for 2-5 minutes.

3. Repeat the elution steps four more times (total elution 5 x 2 gel 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.

Purification of histidine-tagged protein using a gravity flow column.

Prepare, for example, an empty PD-10 column (Code No. 17-0435-01).

1. Put a filter in the bottom of the column.

2. Purge air from the filter by adding some water to the column and let the flow start. (It might be necessary to apply vaccum from the bottom of the column so as to wet the filter).

3. Put the bottom cap in place and discard the water left in the column.(The volume of a PD-10 column is 13 ml. Recommended gel volume is max. 2 ml which facilitates easy handling).

Binding of sample:

Pour the gel into a prepared PD-10 column (with a filter in the bottom and the bottom cap on).

Add the sample, mix gently with a spatula and incubate for 5-30 minutes at room temperature (stir gently now and then) or seal the column with the top cap and incubate with gentle agitation, e.g. end-over-end rotation for 5-30 minutes at room temperature. The binding kinetics depend on the protein and the sample concentration.

2. Open the bottom cap and collect the flow-through material in a tube for further analysis, e.g. SDS-PAGE.

Wash the gel:

1. Add carefully five gel volumes of Start Buffer. Collect the wash material in a tube for further analysis, e.g. SDS-PAGE.

2. Repeat the washing step two more times (total wash 3 x 5 gel volumes of Start Buffer). Save the wash solutions in different tubes for analysis.

Elution:

1. Put the bottom cap in place. Add two gel volumes of Elution buffer e.g. 20 mM Na_2HPO_4, 0.5 M NaCl, 0.5 M imidazole pH 7.4, mix gently and incubate for ≈5 minutes. Open the bottom cap and collect the eluate in a tube.

2. Repeat the elution step four more times (total elution 5 x 2 gel 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 contens of interest.

Note: 500 mM imidazole has A_{280} ≈0.5 (5 mm cell). Use the Elution Buffer as blank. If imidazole needs to be removed use HiTrap Desalting or Pre-packed PD-10 column (Sephadex G-25).

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 prefered 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 gel in the purification. If the capacity of the gel 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 gel (His_6-tagged protein with mw: 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 hydrocloride must be buffer exchanged (with e.g. HiTrap Desalting or Pre-packed PD-10 column (Sephadex G-25)) 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 column. 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 gel and is found in the flow-through material. The concentration of imidazole is too high in the Start Buffer. Decrease the imidazole concentration in the Start Buffer.

Multiple Bands are Detected on Coomassie-stained/Silver-stai - ned 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 ß-mercaptoethanol (up to 20 mM) to the Start 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 use another purification method. Use a second purification step such as Glutathione Sepharose 4B (if the fusion protein also contains a GST-tag), ion exchange (HiTrap Q or HiTrap SP) or gel filtration (Superdex® Peptide, Superdex 75 or Superdex 200) maybe useful.

V Regeneration, Cleaning, Sanitization and Storage.

Regenerating the gel

Before the gel is immobilized with a new metal ion, the gel must be stripped or regenerated. To ensure that the gel is totally free from metal ions wash with 10 column volumes of 0.05 M EDTA, 0.5 M NaCl. Remove residual EDTA by washing with 2-3 column volumes of 0.5 M NaCl.

Re-immobilization of the gel is performed according to the method described above.

Strongly bound ferric ions and ferric compounds can be removed by leaving the gel in 0.05 M EDTA over night.

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 starting buffer.

Sanitization

Sanitization reduces microbial contamination of the gel bed 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 starting buffer.

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

Storage

Store the gel for longer periods of time in 20% ethanol or in 0.01 M NaOH at 4°C.

VI Ordering information

Product	Quantity	Code No.
Chelating Sepharose Fast Flow	50 ml	17-0575-01
Chelating Sepharose Fast Flow	500 ml	17-0575-02
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
Empty Disposible Columns PD-10	50	17-0435-01
HiTrap Chelating	5 x 1 ml	17-0408-01
HiTrap Chelating	1 x 5 ml	17-0409-01
HisTrap	kit	17-1880-01
HiTrap Q	5 x 1 ml	17-1153-01
HiTrap SP	5 x 1 ml	17-1151-01
HiTrap Desalting	5 x 5 ml	17-1408-01
Pre-packed Disposible Columns PD-10 (Sephadex G-25)	30	17-0851-01
Superdex Peptide HR 10/30	1	17-1453-01
HiLoad 16/60 Superdex 30 pg		17-1139-01
Superdex 75 HR 10/30	1	17-1047-01
HiLoad 16/60 Superdex 75 pg	1	17-1068-01
Superdex 200 HR 10/30	1	17-1088-01
HiLoad 16/60 Superdex 200 pg	1	17-1069-01
Glutathione Sepharose 4B	10 ml	17-0756-01
Glutathione Sepharose 4B	100 ml	27-4574-01
Pre-packed Glutathione Sepharose 4B	2	17-0757-01

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