cytiva

Chelating **Sepharose** Fast Flow

Affinity chromatography

Instructions for Use

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 can 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.

BioProcess™ chromatography resins are developed and supported for production-scale chromatography. BioProcess resins are produced with validated methods and are tested to meet manufacturing requirements. Secure ordering and delivery routines give a reliable supply of resin for production-scale. Regulatory Support Files (RSF) are available to assist process validation and submissions to regulatory authorities. BioProcess resins cover all purification steps from capture to polishing.

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Read these instructions carefully before using the products.

Safety

For use and handling of the products in a safe way, refer to the Safety Data Sheets.

1 Product Description

Table 1. Characteristics of Chelating Sepharose Fast Flow

Matrix	Cross-linked agarose, 6%, spherical
Particle size, d_{50v} [1]	~ 90 µm
Ionic capacity	30 to 37 µmol Cu^{2+}/mL resin
Pressure/flow characteristics	≥ 300 cm/h at 0.1 MPa in an XK 50/30 column with 5 cm diameter and 15 cm bed height (at 25°C using buffers with the same viscosity as water)[2]
Recommended maximum operating pressure	0.1 MPa (1.0 bar, 14.5 psi)[3]
pH stability, operational [4]	3 to 13
pH stability, CIP [5]	2 to 14
Functional pH [6]	4 to 8.5
Chemical stability	Stable to commonly used aqueous buffers, 0.01 M HCl 6 M guanidine hydrochloride 8 M urea 1.0 M NaOH[7] 20% ethanol
Physical stability	Negligible volume variation due to changes in pH or ionic strength
Operating temperature	4°C to 40°C
Storage	20% ethanol, 4°C to 30°C

1 Median particle size of the cumulative volume distribution

2 The pressure/flow characteristics describes the relationship between pressure and flow under the set circumstances. The pressure given must not be taken as the maximum pressure of the resin.

3 30 cm diameter, bed height 20 cm

4 pH range where resin can be operated without significant change in function

5 pH range where resin can be subjected to cleaning- or sanitization-in-place without significant change in function

6 Complex with metal ions

7 1.0 M NaOH must only be used for cleaning purposes.

2 Column packing

Chelating Sepharose Fast Flow is supplied preswollen in 20% ethanol. Prepare a slurry by decanting the 20% ethanol solution and replacing it with ultra pure water in a ratio of 75% settled resin to 25% ultra pure water.

Table 2. Recommended lab-scale columns for Chelating Sepharose Fast Flow

Empty column[1]	Packing flow rate (mL/min)		Max. recommended flow rate for chromatography (mL/min)
	first step	second step	
HiScale™ 16/20	2.5	8.7	5
HiScale 16/40	2.5	8.7	5
HiScale 26/20	6.6	23	13
HiScale 26/40	6.6	23	13
HiScale 50/20	24.5	85	49
HiScale 50/40	24.5	85	49
Tricorn™ 10/20	0.9	4.7	2
Tricorn 10/50	0.9	4.7	2
Tricorn 10/100	0.9	4.7	2
Tricorn 10/150	0.9	4.7	2
Tricorn 10/200	0.9	4.7	2
Tricorn 10/300	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

[1] For inner diameter and maximum bed volumes and bed heights, see *Chapter 7 Ordering information, on page 19*

Table 3. Recommended process-scale columns for Chelating Sepharose Fast Flow

Column	Inner diameter (mm)	Bed volume (L)	Bed height, max (cm)
AxiChrom	50 to 200	up to 16.7	50
AxiChrom	300 to 1600	up to 1005	50
BPG 100/500	100	up to 2.0	26
BPG 140/500	140	up to 4.0	26
BPG 200/500	200	up to 8.2	26
BPG 300/500	300	up to 18.0	26
BPG 450/500	450	36.0	23
Chromaflow™ 400/100-300	400	13 to 37	30
Chromaflow 600/100-300	600	28 to 85	30

Packing lab-scale columns

Step	Action
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 resin 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.

Step	Action
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 resins are packed in XK or Tricorn columns in a two-step procedure: Do not exceed 0.05 MPa (0.5 bar, 7.3 psi) in the first step and 0.15 MPa (1.5 bar, 21.8 psi) in the second step.
6	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, on page 5* 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 usually also gives a wellpacked bed. ***Note:*** *For subsequent chromatography procedures, do not exceed 75% of the packing flow rate. See Table 3, on page 5 for flow rates for chromatography.*

Step	Action
7	Maintain packing flow rate for at least 3 bed volumes after a constant bed height is reached. Mark the bed height on the column.
8	Stop the pump and close the column outlet.
9	If using a packing reservoir, disconnect the reservoir and fit the adapter to the column.
10	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.
11	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, visit support section at *cytiva.com*

3 Evaluation of packed column

Introduction

The packing quality needs to be checked by column efficiency testing. The test must be done after the packing, and at regular intervals during the working life of the column, and also when the separation performance is deteriorated.

Column efficiency testing

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). The values are easily determined by applying a test sample such as 1% acetone solution or sodium chloride to the column.

Note: *Use a concentration of 0.8 M NaCl in water as sample and 0.4 M NaCl in water as eluent.*

The calculated plate number depends on the test conditions and must only be used as a reference value. It is important that the test conditions and the equipment are the same so that the results are comparable.

Note: *Changing the solute, solvent, eluent, sample volume, flow velocity, liquid pathway, temperature, chromatography system, etc., influence the results.*

For more information about column efficiency testing, consult the *application note (28937207)*.

For optimal column efficiency results, the sample volume must be approximately 1% of the column volume and the flow velocity 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 the column use.

Method for measuring HETP and A_S

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

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

L = bed height (cm)

N = number of theoretical plates

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

V_R = 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_R and W_h are in the same units.

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

The reduced plate height, h, is calculated as follows:

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

d_{50V} = Median particle size of the cumulative volume distribution (cm)

As a guideline, a value of < 3 is very good.

The peak must be symmetrical, and the asymmetry factor as close to 1 as possible. A typical acceptable range could be 0.8 < A_S < 1.5.

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

Peak asymmetry factor calculation:

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

a = ascending part of the peak width at 10% of peak height

b = descending part of the peak width at 10% of peak height

The Figure below shows a UV trace for acetone in a typical test chromatogram from which the HETP and A_S values are calculated.

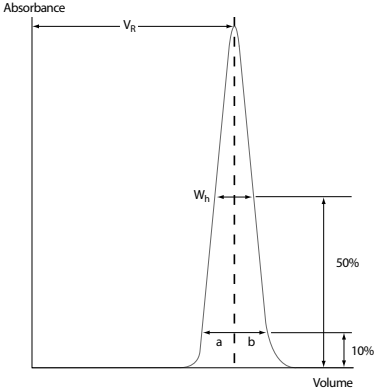

Fig 1. A typical test chromatogram showing the parameters used for HETP and A_S calculations.

4 Immobilized Metal Ion Affinity Chromatography (IMAC)

Charging the columns with metal ions

Step	Action
1	Prepare a 0.2 M solution of the desired metal ion (Cu^{2+}, Zn^{2+}, Ni^{2+}, Co^{2+}, $Fe^{3+,}$ etc.) in ultra pure water. Solutions of Zn^{2+} ions must have a pH of approximately 5.5 or lower to avoid solubillity problems that arise at pH 6 or higher. Fe^{3+} ions must be immobilized at low pH, approximately pH 3.0, to avoid formation of insoluble ferric compounds.

Step	Action
2	Wash the column with at least 2 column volumes (CV) of ultra pure water.
3	Apply approximately 0.2 CV of the metal ion solution to the column.
4	Wash the column with at least 5 CV ultra pure 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 to 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 resin 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 to 8.5. The binding is often strongest at the upper end of this interval.

Care must be taken in preparing the sample for IMAC. The sample must 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 to 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 to 0.05 M sodium phosphate buffer, containing 0.5 to 1.0 M NaCl, pH 6.8, or higher
- 0.05 M Tris-HCl, containing 0.5 to 1.0 M NaCl, pH 7.0, or higher

The well-prepared sample is applied to the equilibrated column at a flow velocity of 150 cm/h, or higher. This is followed by washing out of unbound solutes with at least 5 CV equilibration buffer or until the A_{280} of the solutes 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* *Chapter 5 Choice of metal ions, on page 14**).*

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 stepwise 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 to 4.0 If the target protein is strongly bound, it is advisable to check its stability in an acidic environment. The following buffers can be used:

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

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

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

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

   Elution buffer: 0.02 M sodium phosphate, 0.5 to 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 resin 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 to 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 histidinetagged 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+}, for example, 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 Cytiva and this product is precharged with nickel and is designed for purification of histidine-tagged proteins (see *Chapter 7 Ordering information, on page 19*).

Another IMAC resin in the Cytiva offering is IMAC Sepharose 6 Fast Flow. They are uncharged resins, allowing the user to customize the charge to the target protein. These resins are commonly used to purify his-tagged proteins, but also untagged recombinant or native proteins.

6 Regeneration, Cleaning, Sanitization, and Storage

Regenerating the resin

Before the resin is immobilized with a new metal ion, the resin must be stripped or regenerated. To make sure that the resin 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 to 3 column volumes of 0.5 M NaCl.

Re-immobilization of the resin is performed according to the method previously described (see *Charging the columns with metal ions, on page 10*).

Strongly bound ferric ions and ferric compounds can be removed by leaving the resin 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 to 15 minutes, reversed flow direction.

Remove precipitated proteins, hydrophobically bound proteins and lipoproteins by washing the column with 1.0 M NaOH solution at a flow velocity of approximately 40 cm/h, contact time 1 to 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, reversed flow direction. Apply increasing gradients to avoid air bubble formation when using high concentrations of organic solvents.

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

Alternatively, wash the column with 2 bed volumes of detergent in a basic or acidic solution. Use, for example, 0.1% to 0.5% nonionic detergent in 0.1 M acetic acid. Wash at a low flow velocity of approximately 40 cm/h, contact time 1 to 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 resin to a minimum.

Wash the column with 0.5 to 1.0 M NaOH at a flow velocity of approximately 40 cm/h, contact time 30 to 60 minutes, reversed flow direction.

Re-equilibrate the column with 3 to 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 resin for longer periods of time in 20% ethanol or in 0.01 M NaOH[1]

[1] In most cases, no long term stability data has been generated by Cytiva in 0.01 M NaOH. In some cases, accelerated studies at elevated temperature indicate that storage in 0.01 M NaOH can be a viable option but no guarantees can be made regarding retained function of the product.

7 Ordering information

Product	Quantity	Product code
Chelating Sepharose Fast Flow	50 mL	17057501
Chelating Sepharose Fast Flow	500 mL	17057502
Chelating Sepharose Fast Flow	5 L	17057504

Related products	Quantity	Product code
Ni Sepharose 6 Fast Flow	25 mL	17531801
Ni Sepharose 6 Fast Flow	100 mL	17531802
Ni Sepharose 6 Fast Flow	500 mL	17531803
Ni Sepharose 6 Fast Flow	1 L	17531804
Ni Sepharose 6 Fast Flow	5 L	17531805
HisTrap FF	5 x 1 mL	17531901
HisTrap FF	5 x 5 mL	17525501
HisTrap FF	1 x 20 mL	28936551
HisTrap HP	5 x 1 mL	17524701
HisTrap HP	5 x 5 mL	17524802
HiTrap Chelating HP	5 x 1 mL	17040801
HiTrap Chelating HP	5 x 5 mL	17040903
HiScale 16/20 column, 16 mm i.d.	1	28964441
max 40 mL bed volume, bed height up to 20 cm		
HiScale 16/40 column, 16 mm i.d.	1	28964424
bed flow 16 to 80 mL, bed height 8 to 40 cm		
HiScale 26/20 26 mm i.d.	1	28964514
max bed volume 106 mL, bed height 20 cm		
Hiscale 26/40 column, 26 mm i.d.	1	28964513
bed volume 69 to 212 mL, bed height max 20cm		
HiScale 50/20 column, 50 mm i.d.	1	28964445
max bed volume 393 mL, bed heights up to 20 cm		

Related products	Quantity	Product code
HiScale 50/40 column, 50 mm i.d.	1	28964444
bed volume 274 to 785 mL, bed heights 14 to 40 cm		
HiScreen IMAC FF	4.7 mL	28950517
HiTrap IMAC FF	1 mL	17092101
HiTrap IMAC FF	5 mL	17092103
IMAC Sepharose 6 Fast Flow	25 mL	17092107
IMAC Sepharose 6 Fast Flow	100 mL	17092108
IMAC Sepharose 6 Fast Flow	5 L	17092110
IMAC Sepharose 6 Fast Flow	1 L	17092109
Tricorn 10/20 column, 10 mm i.d.	1	28406413
max 2.2 mLbed volume or 2.8 cm bed height		
Tricorn 10/50 column, 10 mm i.d.	1	28406414
max 4.5 mLbed volume or 5.8 cm bed height		
Tricorn 10/100 column, 10 mm i.d.	1	28406415
max 8.5 mL bed volume or 10.8 cm bed height		
Tricorn 10/150, 10 mm i.d.	1	28406416
7.6 to 12.3 mL bed volume, 9.6 to 15.6 cm bed height		
Tricorn 10/200, 10 mm i.d.	1	28406417
11.5 to 16.2 mL bed volume, 14.6 to 20.6 cm bed height		
Tricorn 10/300, 10 mm i.d.	1	28406418
19.4 to 24.1 mL bed volume, 24.6 to 30.6 cm bed height		
XK 16/20 column, 16 mm i.d.	1	18877301
max 30 mL bed volume or 15 cm bed height		
XK 26/20 column, 26 mm i.d.	1	18100072
max 65 mL bed volume or 12.5 cm bed height		

Related products	Quantity	Product code
XK 50/20 column, 50 mm i.d.	1	18100071
max 270 mL bed volume or 14 cm bed height		
XK 50/30 column, 50 mm i.d.	1	18875101
max 550 mL bed volume or 28.5 cm bed height		

Literature		
Recombinant Protein Handbook, Protein Amplification and Simple Purification	1	18114275
Affinity Chromatography Handbook, Principles and Methods	1	18102229
Affinity Chromatography Columns and Media Product Profile	1	18112186
Datafile BPG columns		18111523
Datafile BPG 450 columns		18116059
Datafile Chromaflow columns		18113892

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