IMAC **Sepharose** 6 Fast Flow

Affinity media

Instructions for Use

cytiva.com

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1 Introduction

Immobilized metal ion affinity chromatography (IMAC) is a widely used separation method for purifying a broad range of proteins and peptides. It is based on the specific interaction between certain amino acid side chains exposed on the surface of proteins (mainly His and to a lesser extent Cys and Trp) and transition metal ions, most often Zn^{2+}, Ni^{2+}, Cu^{2+} or Co^{2+}. The metal ions are bound to chelating ligands that are covalently linked to an insoluble polymer matrix, for example agarose. The strength of protein/peptide interaction with immobilized metal ions is dependent on the type, number and spatial distribution of the amino acid side chains, and on the nature of the metal ion used. The chromatographic operating conditions (pH, type and concentration of salt, additives, etc.) also contribute to the observed interaction.

The presence of several adjacent histidines, such as a $(histidine)_6$-tag, increases the affinity for immobilized metal ions; in general this makes the histidine-tagged protein bind the strongest of all the proteins in an *E. coli* extract, or other samples.

In recent years, IMAC has been increasingly used for the purification of histidine-tagged, recombinant proteins.

2 Product description

IMAC Sepharose™ 6 Fast Flow consists of 90-µm beads of highly cross-linked agarose to which a chelating group has been covalently coupled.

The medium is available in 25 m, 100 ml, 1 l and 5 l packs, as well as prepacked in 1 ml and 5 ml HiTrap™ columns and 20 ml HiPrep™ 16/10 columns.

IMAC Sepharose 6 Fast Flow is highly stable and compatible with a wide range of common additives. This helps maintain biological activity and increase product yield, while at the same time greatly expanding the range of suitable operating conditions.

In addition, the medium is easy to pack and use, and its high flow properties make it excellent for scaling-up. The key characteristics of the medium are listed in *Table 1, on page 5*. A variety of compounds that are compatible with Ni^{2+} charged IMAC Sepharose 6 Fast Flow are listed in *Table 2, on page 7*.

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. BioProcess media cover all purification steps from capture to polishing.

Table 1. Medium characteristics.

Matrix	Highly cross-linked 6% spherical agarose
Dynamic binding capacity[1]	Approx. 40 mg $(histidine)_6$-tagged protein/ml medium (Ni^{2+} charged). Untagged protein: Approx. 25 mg/ml medium (Cu^{2+} charged), or approx. 15 mg/ml medium (Zn^{2+} or Ni^{2+} charged).
Metal ion capacity	Approx. 15 µmol Ni^{2+}/ml medium
Average particle size	90 µm
Max. linear flow rate[2]	600 cm/h (20 ml/min) using XK 16/20 columns with 5 cm bed height
Recommended flow rate[2]	<150 cm/h
Max. operating pressure[2]	0.1 MPa, 1 bar (when packed in XK columns. May vary if used in other columns)
Chemical stability[3]	Stable in:
	0.01 M HCl, 0.1 M NaOH. Tested for one week at 40°C.
	1 M NaOH, 70% acetic acid. Tested for 12 h.
	2% SDS. Tested for 1 h.
	30% 2-propanol. Tested for 30 min.
pH stability[3]	Short term (at least 2 h) 2–14
	Long term (≤one week) 3–12
Storage	4°C to 30°C in 20% ethanol

[1] Conditions for determining dynamic binding capacity:

Samples:	$(Histidine)_6$-tagged proteins: Capacity data were obtained for a protein (M_r 28 000) bound from an *E. coli* extract, and a pure protein (M_r 43 000; applied at 1 mg/ml in binding buffer; capacity at 10% breakthrough). Untagged protein: Capacities determined at 10% breakthrough for human apo-transferrin applied at 1 mg/ml in binding buffer.
Column volume:	0.25 ml or 1 ml.
Flow rate:	0.25 ml/min or 1 ml/min, respectively.
Binding buffer:	20 mM sodium phosphate, 0.5 M NaCl, 5 mM imidazole (1 mM for untagged protein), pH 7.4.
Elution buffer:	20 mM sodium phosphate, 0.5 M NaCl, 0.5 M imidazole (50 mM for untagged protein), pH 7.4.

Note:
Dynamic binding capacity is metal-ion- and protein-dependent.

[2] H_2O at room temperature.

[3] Metal ion-stripped medium.

Table 2. IMAC Sepharose 6 Fast Flow charged with Ni^{2+} is compatible with the following compounds, at least at the concentrations given.

Reducing agents[1]	5 mM DTE
	5 mM DTT
	20 mM ß-mercaptoethanol
	5 mM TCEP (Tris[2-carboxyethyl] phosphine)
	10 mM reduced glutathione
Denaturing agents	8 M Urea[2]
	6 M Gua-HCl[2]
Detergents	2% Triton™ X-100 (nonionic)
	2% Tween™ 20 (nonionic)
	2% NP-40 (nonionic)
	2% cholate (anionic)
	1% CHAPS (zwitterionic)
Other additives	20% ethanol
	50% glycerol
	100 mM Na_2SO_4
	1.5 M NaCl
	1 mM EDTA[3]
	60 mM citrate[3]
Buffer substances	50 mM Sodium phosphate, pH 7.4
	100 mM Tris-HCl, pH 7.4
	100 mM Tris-acetate, pH 7.4
	100 mM HEPES, pH 7.4
	100 mM MOPS, pH 7.4
	100 mM Sodium acetate, pH 4[2]

1 See Chapter 3.

2 Tested for one week at 40°C.

[3] The strong chelator EDTA has been used successfully in some cases at 1 mM (histidine-tagged proteins). Generally, chelating agents should be used with caution (and only in the sample, not in buffers). Any metal-ion stripping may be counteracted by adding a small excess of $MgCl_2$ before centrifugation/filtration of the sample. Note that metal ion stripping effects may vary with the applied sample volume.

3 General considerations

IMAC Sepharose 6 Fast Flow is supplied free of metal ions and thus needs to be charged with a suitable ion before use. The choice of metal ion is dependent on the type of application and the specific protein to be purified. The metal ions predominantly used in IMAC are Cu^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}. For choice of metal ion, see Chapter 7.

For histidine-tagged protein applications, imidazole at low concentrations is commonly used in the sample as well as in the binding/wash buffer to minimize binding of unwanted host cell proteins. At somewhat higher concentrations, imidazole may decrease the binding of histidine-tagged proteins. The imidazole concentration must therefore be optimized to ensure the best balance of high purity (low binding of unwanted proteins) and high yield (binding of all the histidine-tagged protein). In general, the concentration of imidazole that will give optimal purification results is protein-dependent, see Chapter 7.

The most frequently used elution procedure for histidine-tagged proteins is based on a competitive displacement by imidazole. Target proteins can also be eluted from the medium by several other methods or combinations of methods – for example, low-pH elution in the range of pH 7.5 to pH 4. Below pH 4, metal ions may be stripped off the medium. Elution can be done stepwise or with linear gradients.

Chelating agents such as EGTA or EDTA can be used to elute proteins by stripping the metal ions from the medium. The target protein pool will then contain the metal ions, which can be removed by desalting.

Elution of bound proteins with ammonium chloride or histidine has also been reported.

Leakage of Ni^{2+} and Co^{2+} from IMAC Sepharose 6 Fast Flow is generally low under normal conditions. For applications where very low leakage during purification is critical, it can be diminished even further by performing a blank run after charging the medium with metal ions, see *Charging the column with metal ions, on page 13*.

IMAC Sepharose 6 Fast Flow charged with Ni^{2+} has been shown to be compatible with reducing agents, see *Table 2, on page 7*. However, we recommend first removing any weakly bound metal ions by performing a blank run without reducing agents, see *Charging the column with metal ions, on page 13*. Do not leave IMAC Sepharose 6 Fast Flow in buffers containing reducing agents when not in use.

4 Column packing

IMAC Sepharose 6 Fast Flow is supplied preswollen 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 3. Recommended lab-scale columns for IMAC Sepharose 6 Fast Flow.

Empty column[1]	Packing flow rate (ml/min)		Max. recommended flow rate for chromatography (ml/min)
	first step	second step	
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

[1] For inner diameter and maximum bed volumes and bed heights, see Chapter 13.

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 medium and pour the slurry into the column in a single continuous operation. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.

Step	Action
4	When using a packing reservoir, immediately fill the remainder of the column and reservoir with water. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
5	Open the bottom outlet of the column and set the pump to run at the desired flow rate. Ideally, Sepharose 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. If the packing equipment does not include a pressure gauge, use a packing flow rate of 2.5 ml/min (XK 16/20 column) or 0.9 ml/min (Tricorn 10/100 column) in the first step, and 8.7 ml/min (XK 16/20 column) or 4.7 ml/min (Tricorn 10/100 column) in the second. See *Table 3, on page 10* for packing flow rates for other columns. If the recommended pressure or flow rate cannot be obtained, use the maximum flow rate your pump can deliver. This should also give a well-packed bed. ***Note:*** *For subsequent chromatography procedures, do not exceed 75% of the packing flow rate. See Table 3, on page 10 for chromatography flow rates.*
6	Maintain the packing flow rate for at least 3 bed volumes after a constant bed height is reached. Mark the bed height on the column.

Step	Action
7	Stop the pump and close the column outlet.
8	If a packing reservoir is used, disconnect the reservoir and fit the adapter to the column.
9	With the adapter inlet disconnected, push the adapter down into the column until it reaches the mark. Allow the packing solution to flush the adapter inlet. Lock the adapter in position.
10	Connect the column to a pump or a chromatography system and start equilibration. Re-adjust the adapter if necessary.

Packing large-scale columns

For general process-scale column packing instructions, please visit the support section at *cytiva.com/protein-purification*

5 Preparation before purification

Charging the column 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 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 at least 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.

Step	Action
5	**Optional: For critical applications,** where metal ion leakage during purification must be minimized, we recommend performing a blank run before use. Such treatment is intended to remove any weakly bound metal ions that might otherwise be desorbed later, during elution of the bound protein. Performing a blank run: Use buffers **without** reducing agents. **a.** Wash the column with 5 CV of distilled water. **b.** Wash with 5 CV of the buffer that has been chosen for the protein elution, for example imidazole elution buffer for competitive elution, or low-pH elution buffer. Do not use EDTA/EGTA elution buffer for a blank run. **c.** Equilibrate with 5–10 CV of binding buffer. Equilibration down to a very low concentration of imidazole, often used for binding of untagged proteins, may be slow. The equilibration can be monitored with the absorbance of imidazole, for example at 220 nm.

The column is now ready for use.

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 longer period of time in neutral solutions. We also advise stripping the immobilized Fe^{3+} ions after each run and re-charging the column as required. Strongly bound Fe^{3+} ions and ferric compounds can be removed by leaving the medium in 50 mM EDTA overnight.*

Binding and elution buffers

We recommend binding at neutral to slightly alkaline pH (pH 7–8) in the presence of 0.5–1.0 M NaCl (or similar neutral salt). Sodium phosphate buffers are often used. Tris-HCl can also be used, but should be avoided in cases where the metal-protein affinity is very low, since it may reduce the binding strength.

Avoid chelating agents such as EDTA or citrate in buffers, see *Table 2, on page 7*.

Addition of salt, for example 0.5–1.0 M NaCl in the buffers and samples eliminates ionic interactions but can also have a marginal effect on the retention of proteins.

If the recombinant histidine-tagged proteins are expressed as inclusion bodies, include up to 6 M Gua-HCl or 8 M urea in all buffers.

When using high concentrations of urea or Gua-HCl, proteins generally unfold. Refolding on-column (or after elution) is protein-dependent.

Tip: *Samples containing Urea can be analyzed directly by SDS-PAGE whereas samples containing Gua-HCl must be buffer-exchanged to a buffer with Urea before SDS-PAGE.*

The most frequently used elution procedure for histidine-tagged proteins is based on a competitive displacement by imidazole. In other instances, not least for untagged, naturally occurring proteins, elution by reducing the pH (linear or stepwise decrease in pH) is a frequently used method. Weakly bound proteins can be eluted at pH 6.0, while strongly bound proteins can be eluted successively when pH is lowered from 6.0 to 4.0. If the target proteins is strongly bound, it is advisable to check its stability in an acidic environment.

Tip: *If the proteins are sensitive to low pH, we recommend collecting the fractions in tubes containing 1 M Tris-HCl, pH 9.0 (60–200 µL/mL fraction) to quickly restore the pH to neutral. (Do not use NaOH).*

Imidazole concentration in binding buffer

The purity of recombinant histidine-tagged proteins can often be increased by washing with binding buffer containing as high a concentration of imidazole as possible. However, care must be taken not to use a wash concentration that causes elution of the histidine-tagged protein.

To obtain highest purity, first determine the optimal concentration of imidazole for sample loading, washing and elution, see Chapter 7.

When maximum binding and yield of the histidine-tagged protein (rather than purity) is the main objective, choose a low imidazole concentration for binding and wash, even if that concentration in some cases may lead to suboptimal purity.

Buffer preparation:

Water and chemicals used for buffer preparation should be of high purity. High purity imidazole gives very low or no absorbance at 280 nm. Filter buffers through a 0.45 µm filter before use.

Recommended buffers

Binding buffer:	20 mM sodium phosphate, 0.5-1.0 M NaCl, pH 7.4.
	For histidine-tagged proteins we recommend including imidazole in the binding buffer. The optimal imidazole concentration is protein-dependent; 20–40 mM is suitable for many histidine-tagged proteins when using Ni^{2+} or Co^{2+} ions.
Elution buffer:	
Imidazole elution:	20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4. The imidazole concentration required for elution is protein-dependent.
Elution at low pH:	Example: First stepwise with 50 mM sodium acetate, 0.5 M NaCl, pH 6, followed by a linear gradient to 50 mM sodium acetate, 0.5 M NaCl, pH 4.

Note: *Compared to histidine-tagged proteins, untagged proteins bind immobilized metal ions with lower affinity, so the imidazole concentrations that should be used with untagged proteins are generally much lower than the above recommendations, both for binding (sometimes no imidazole included) and elution.*

Sample preparation

For optimal growth, induction and cell lysis conditions, please refer to established protocols.

To avoid column clogging, centrifuge the sample and filter it through a 0.45-µm filter to remove cell debris and other particulate material. If the sample is dissolved in a buffer other than 20 mM phosphate, 0.5 M NaCl, pH 7.4, adjust the NaCl concentration to 0.5 M and the pH to 7–8. This can be done by adding concentrated stock solutions, diluting with binding buffer, or by buffer exchange. Do not use strong bases or acids to adjust pH (risk of precipitation).

Note: *To minimize the binding of unwanted host-cell proteins, add the same concentration of imidazole to the sample as to the binding buffer, see Chapter 7.*

6 Purification procedure

Please read the sections Chapter 3 and Chapter 5 before starting the purification

Step	Action
1	Charge the packed column with metal ions according to the procedure described earlier, see *Charging the column with metal ions, on page 13*.
2	If the column has been stored in 20% ethanol after metal ion charging, wash it with 2–5 column volumes (CV) of distilled water. Use a linear flow rate of 50–100 cm/h.
3	Equilibrate the column with at least 2–5 CV of binding buffer at a linear flow rate of 150 cm/h or higher.
4	Apply a correct prepared sample, see *Sample preparation, on page 17*.

Step	Action
5	Wash out unbound material with binding buffer until the absorbance is at or near the baseline.
6	Elute the bound protein with elution buffer using a stepwise or linear gradient. ***Note:*** *Use the elution buffer as blank when measuring absorbance manually.*

7 Optimization

Choice of metal ion

When choosing the most suitable metal ion to use, consider the structural requirements underlying of immobilized metal ions.

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

For purification of untagged proteins, Cu^{2+} ions have frequently been used. When the binding characteristics of an untagged target protein are unknown, it is advisable to test also other metal ions (e.g. Zn^{2+}, Ni^{2+}, Co^{2+}) to establish the most suitable metal ion to use. In some instances, a weak binding to a metal ion can be exploited to achieve selective elution (higher purity) of a target protein. In some special applications, Fe^{3+} and Ca^{2+} have also been used.

Concentration of imidazole in binding/wash buffer

Imidazole at low concentrations is commonly used in the binding/wash buffer to minimize co-adsorption of unwanted host cell proteins. It is important to also include imidazole in the sample (generally at the same concentration as in the binding/wash buffer). At somewhat higher concentrations, imidazole may decrease the binding of histidine-tagged proteins, leading to a lower yield. The concentration of imidazole must therefore be optimized to ensure the best balance of high purity (low binding of unwanted proteins), and high yield (binding of all of the histidine-tagged protein). The optimal concentration of imidazole is different for different histidine-tagged proteins/target proteins. Note that IMAC Sepharose 6 Fast Flow often requires a slightly higher concentration of imidazole in the binding/wash buffer than similar IMAC media on the market.

One optimization strategy is to elute with a linear or stepwise gradient of imidazole from 20 to 500 mM and test the fractions by SDS-PAGE and/or Western blotting for the presence of target protein and impurities. Finding the optimal imidazole concentration for a specific histidine-tagged

protein is a trial-and-error effort, but 20–40 mM in the sample as well as in the binding/wash buffer is a good starting point for many histidine-tagged proteins when using Ni^{2+} or Co^{2+}. Prepacked HiTrap IMAC FF columns (1 or 5 ml) are ideal for establishing the optimal chromatographic conditions to use.

For untagged target proteins, the concentrations of imidazole that should be used are generally much lower than for histidine-tagged proteins, for both binding (sometimes no imidazole is needed) and elution.

8 Regenerating the medium

When performing repeated purification cycles, the need for stripping and re-charging is highly dependent on the sample properties, sample volumes, metal ion, etc.

Before a new metal ion is immobilized, the medium must be stripped of the immobilized metal ions. 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, pH 7. Remove residual EDTA by washing with at least 2–3 column volumes of 0.5 M NaCl.

Re-charge the medium according to the method previously described, see *Charging the column with metal ions, on page 13*.

Strongly bound ferric ions and ferric compounds can be removed by leaving the medium in 50 mM EDTA overnight.

In some applications, substances such as denatured proteins or lipids are not removed during the regeneration procedures. These can be removed by Cleaning-In-Place.

9 Cleaning-In-Place (CIP)

Clean the column when backpressure increases, or to avoid cross-contamination between samples/target proteins. Before cleaning, strip off the metal ions using the recommended procedure, see Chapter 8. Use reversed flow direction. Stripping, without any additional CIP procedures, may sometimes give a satisfactory cleaning effect.

The stripped column can be cleaned by the following procedures:

- Remove ionically bound proteins by washing with at least 0.5 column volumes (CV) of 2 M NaCl. Then wash with at least 3 CV of distilled water.
- Remove precipitated proteins, hydrophobically bound proteins and lipoproteins by washing the column with 1 M NaOH, contact time usually 1–2 h (longer time may be required to inactivate endotoxins). Then wash with 3–10 CV of binding buffer, followed by at least 3 CV of distilled water.
- Remove hydrophobically bound proteins, lipoproteins and lipids by washing with 5–10 CV of 70% ethanol or 30% isopropanol for at least 15–20 min. Then wash with 3–10 CV of distilled water.

  Alternatively, wash with 2 CV of detergent in a basic or acidic solution. Use, for example, 0.1–0.5% nonionic detergent in 0.1 M acetic acid, contact time 1–2 h. After treatment, always remove residual detergent by washing with 5–10 CV of 70% ethanol. Then wash with 3–10 CV of distilled water.

10 Troubleshooting

The following tips may be of assistance. If you have any further questions about IMAC Sepharose 6 Fast Flow, please visit *cytiva.com/protein-purification* or contact our technical support team, or your local Cytiva representative.

Column has clogged:

- Cell debris in the sample may clog the column. Clean the column according to Cleaning-in-Place procedures.
- Centrifuge and/or filter the sample through a 0.22 µm or a 0.45 µm filter shortly before column application, see *Sample preparation, on page 17*.

Sample is too viscous:

- If the lysate is very viscous due to high concentrations of host nucleic acid, continue sonication until the viscosity is reduced, and/or add DNase I to 5 µg/ml, Mg^{2+} to 1 mM, and incubate on ice for 10–15 min. Alternatively, draw the lysate through a syringe needle several times.

Protein is difficult to dissolve or precipitates during purification:

- The following additives may help: 2% Triton X-100, 2% Tween 20, 2% NP-40, 2% cholate, 1% CHAPS, 1.5 M NaCl, 50% glycerol, 20 mM b-mercaptoethanol, 1–3 mM DTT or DTE (up to 5 mM is possible but depends on the sample and the sample volume), 5 mM TCEP, 10 mM reduced glutathione, 8 M Urea or 6 M Gua-HCl. Mix gently for 30

min to aid solubilization of the tagged protein (inclusion bodies may require much longer mixing). Note that Triton X-100 and NP-40 (but not Tween) have a high absorbance at 280 nm. Furthermore, detergents cannot be easily removed by buffer exchange.

Histidine-tagged protein found in the pellet:

SDS-PAGE analysis of samples collected during the preparation of the bacterial lysate may indicate that most of the histidine-tagged protein is located in the centrifugation pellet. Possible causes and solutions are:

- **Sonication may be insufficient:** Check cell disruption by microscopic examination or monitor by measuring the release of nucleic acids at 260 nm. Adding lysozyme (up to 0.1 volume of a 10 mg/ml lysozyme solution in 25 mM Tris-HCl, pH 8.0) prior to sonication may improve results. Avoid frothing and overheating as this may denature the target protein. Oversonication can also lead to co-purification of host proteins with the target protein.
- **The protein may be insoluble (inclusion bodies):** The protein can usually be solubilized (and unfolded) from inclusion bodies using common denaturants such as 4–6 M Gua-HCl, 4–8 M Urea or strong detergents.

  Prepare buffers containing 20 mM sodium phosphate, 0.5–1 M NaCl, 8 M Urea, or 6 M Gua-HCl, and suitable imidazole concentrations, pH 7.4–7.6. Use these buffers for sample preparation, as binding buffer, and as elution buffer. For sample preparation and binding buffer, use 10 mM imidazole or the concentration selected during optimization trials (including Urea or Gua-HCl). To minimize sample dilution, add solid Urea or Gua-HCl.

Histidine-tagged protein is found in the flowthrough and purified fractions:

- **Capacity of IMAC Sepharose 6 Fast Flow is exceeded:** Increase the volume of IMAC Sepharose 6 Fast Flow or decrease the sample volume used for your purification.
- **Buffer/sample composition is incorrect:** Check the pH and composition of sample and binding buffers. Ensure that the concentration of chelating or strong reducing agents, as well as of imidazole, is not too high.

No histidine-tagged protein in the purified fractions:

- **Elution conditions are too mild (histidine-tagged protein still bound):** Elute with an increasing imidazole gradient or decrease pH to determine optimal elution conditions.
- **The protein has precipitated in the column:** Try detergents, change NaCl concentration or elute under denaturing (unfolding) conditions (use 4–8 M urea or 4–6 M Gua-HCl) to remove precipitated proteins. For the next experiment, decrease the amount of sample, or decrease protein concentration by eluting with a linear imidazole gradient instead of stepwise elution.
- **Nonspecific hydrophobic or other interactions:** Add a nonionic detergent to the elution buffer (e.g. 0.2% Triton X-100) or change the NaCl concentration.
- **Concentration of imidazole in the sample and/or binding buffer is too high:** The protein is found in the flowthrough. Decrease the imidazole concentration.

- **Target protein may not be histidine-tagged as expected:** Verify DNA sequence of the gene. Analyze samples taken before and after induction of expression with, for example anti-histidine tag antibodies in Western blotting.
- **Histidine-tag may be insufficiently exposed:** The protein is found in the flowthrough. Perform purification of unfolded protein in urea or Gua-HCl as for inclusion bodies.

  To minimize dilution, add solid urea or Gua-HCl to the sample.
- **Buffer/sample composition is incorrect:** The protein is found in the flowthrough. Check pH and composition of sample and binding buffer. Ensure that the concentration of chelating or strong reducing agents, as well as imidazole, is not too high.

The eluted protein is not pure (multiple bands on SDS polyacrylamide gel):

- **Partial degradation of tagged protein by proteases:** Add protease inhibitors (use EDTA with caution, see *Table 2, on page 7*).
- **Contaminants have high affinity for the metal ion used:** Optimize the concentration of imidazole in the sample and binding buffer, see Chapter 7. Wash before elution with binding buffer containing as high a concentration of imidazole as possible, without causing elution of the target protein. A shallow imidazole gradient (20 column volumes or more) may separate proteins with similar binding strengths.

If optimized conditions do not remove contaminants, further purification by ion exchange chromatography and/or gel filtration may be necessary.

- **Contaminants are associated with tagged proteins:** Add detergent and/or reducing agents before sonicating the cells or shortly afterwards if foaming is a problem. Increase the detergent levels (e.g. up to 2% Triton X-100 or 2% Tween 20), change the NaCl concentration or add glycerol (up to 50%) to the wash buffer to minimize non-specific interactions.
- **Change metal ion:** The metal ion used may not be the most suitable, see Chapter 7.

11 Storage

Store the medium in 20% ethanol at 4°C to 30°C.

12 Further information

Visit *cytiva.com/protein-purification* for further information. Several handbooks also contain useful information, see Ordering information in the table below.

13 Ordering information

Product	Quantity	Product code
IMAC Sepharose 6 Fast Flow	25 ml	17092107
IMAC Sepharose 6 Fast Flow	100 ml	17092108
IMAC Sepharose 6 Fast Flow	1 l	17092109
IMAC Sepharose 6 Fast Flow	5 l	17092110

Larger quantities are available. Please contact Cytiva for more information.

Prepacked columns	Quantity	Product code
HiTrap IMAC FF	5 × 1 ml	17092102
HiTrap IMAC FF	5 × 5 ml	17092104
HiPrep IMAC FF 16/10	1 × 20 ml	17092106

Related products	Quantity	Product code
Ni IMAC Sepharose 6 Fast Flow	5 ml	17531806
Ni IMAC Sepharose 6 Fast Flow	25 ml	17531801
Ni IMAC Sepharose 6 Fast Flow	100 ml	17531802
Ni IMAC Sepharose 6 Fast Flow	500 ml	17531803
Ni IMAC Sepharose 6 Fast Flow	1 l	17531804
Ni IMAC Sepharose 6 Fast Flow	5 l	17531805
HisTrap™ FF	5 × 1 ml	17531901
HisTrap FF	5 × 5 ml	17525501
HisPrep™ FF 16/10	1 × 20 ml	17525601

Empty lab-scale columns	Quantity	Product code
Tricorn 10/20 column, 10 mm i.d.	1	28406413
Tricorn 10/50 column, 10 mm i.d.	1	28406414
Tricorn 10/100 column, 10 mm i.d.	1	28406415
XK 16/20 column, 16 mm i.d.	1	18877301
XK 26/20 column, 26 mm i.d.	1	18100072
XK 50/20 column, 50 mm i.d.	1	18100071
XK 50/30 column, 50 mm i.d.	1	18875101

Literature	Quantity	Product code
Recombinant Protein Purification Handbook, Principles and Methods	1	18114275
Affinity Chromatography Handbook, Principles and Methods	1	18102229

Literature	Quantity	Product code
Affinity Chromatography Columns and, Media Selection Guide	1	18112186
Ni Sepharose and IMAC Sepharose Selection Guide	1	28407092

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