Instructions 28-4046-25 AE

HiTrap™ IMAC FF, 1 ml and 5 ml

HiTrap IMAC FF is a ready to use column, prepacked with IMAC Sepharose™ 6 Fast Flow. The column is ideal for purification of histidine-tagged recombinant proteins and other proteins/peptides by immobilized metal ion affinity chromatography (IMAC).

HiTrap IMAC FF columns provide fast, simple and easy separations in a convenient format, and are a perfect start for scaling up.

HiTrap IMAC FF columns can be operated with a syringe, peristaltic pump, or liquid chromatography systems such as ÄKTA™.

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Please read these instructions carefully before using HiTrap columns.

Intended use

HiTrap columns are intended for research use only, and shall not be used in any clinical or *in vitro* procedures for diagnostic purposes.

Safety

For use and handling of the product in a safe way, please refer to the Safety Data Sheet.

1 Introduction

Immobilized metal ion affinity chromatography (IMAC) is a widely used separation method for purifying a broad range of proteins and peptides, either in capture steps or in subsequent intermediate or polishing purification steps. IMAC is based on the specific interaction between immobilized metal ions and certain amino acid side chains exposed on the surface of proteins (mainly His and to a lesser extent Cys and Trp). The strength of 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 interaction observed.

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

2 Product description

HiTrap column characteristics

The columns are made of biocompatible polypropylene that does not interact with biomolecules.

The columns are delivered with a stopper at the inlet and a snap-off end at the outlet. Table 1 lists the characteristics of HiTrap columns.

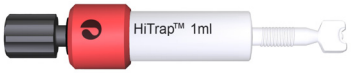


Fig 1. HiTrap, 1 ml column.

Fig 2. HiTrap, 5 ml column.

Note: *HiTrap columns cannot be opened or refilled.*

Note: *Make sure that the connector is tight to prevent leakage.*

Table 1. Characteristics of HiTrap columns.

Column volume (CV)	1 ml	5 ml
Column dimensions	0.7 × 2.5 cm	1.6 × 2.5 cm
Column hardware pressure limit	5 bar (0.5 MPa)	5 bar (0.5 MPa)

Note: *The pressure over the packed bed varies depending on a range of parameters such as the characteristics of the chromatography medium, sample/liquid viscosity and the column tubing used.*

Supplied Connector kit with HiTrap column

Connectors supplied	Usage	No. supplied
Union 1/16" male/ luer female	For connection of syringe to HiTrap column	1
Stop plug female, 1/16"	For sealing bottom of HiTrap column	2, 5 or 7

Medium properties

HiTrap IMAC FF 1 ml and 5 ml columns are prepacked with IMAC Sepharose 6 Fast Flow, which consists of 90 µm, highly cross-linked agarose beads with a covalently immobilized chelating group. The medium can easily be charged with Ni^{2+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+} or other metal ions.

The high flow-rate properties of IMAC Sepharose 6 Fast Flow make HiTrap IMAC FF columns ideal for establishing optimal chromatographic conditions for scaling up.

Table 2. HITrap IMAC HP characteristics.

Matrix	Highly cross-linked spherical agarose, 6%
Average particle size	90 µm
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
Recommended flow rates	1 ml/min and 5 ml/min for 1 ml and 5 ml column, respectively
Max. flow rates	4 ml/min and 20 ml/min for 1 ml and 5 ml column, respectively
Compatibility during use	*See* Table 3.
Chemical stability[3]	0.01 M HCl, 0.1 M NaOH. Tested for 1 week at 40°C. 1 M NaOH, 70% acetic acid. Tested for 12 hours. 2% SDS. Tested for 1 hour. 30% 2-propanol. Tested for 30 minutes.
Avoid in buffers	Chelating agents e.g. EDTA, EGTA, citrate (*see* Table 3)
pH stability[3]	
Short term (at least 2 hours)	2–14
Long term (≤ 1 week)	3–12
Storage	20% ethanol at 4°C to 30°C

[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, respectively
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 mM 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] Medium without metal ion.

Table 3. 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 β-mercaptoethanol
	5 mM TCEP (Tris[2-carboxyethyl]phosphine)
	10 mM reduced glutathione
Denaturing agents	8 M urea[2]
	6 M guanidine hydrochloride[2]
Detergents	2% Triton™ X-100 (nonionic)
	2% Tween™ 20 (nonionic)
	2% NP-40 (nonionic)
	2% cholate (anionic)
	1% CHAPS (zwitterionic)
Other additives	500 mM imidazole
	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* notes and blank run, under "Column preparation".
[2] Tested for 1 week at 40°C.
[3] The strong chelator EDTA has been used successfully in some cases, at 1 mM. Generally, chelating agents should be used with caution (and only in the sample, not the buffers). Any metal ion stripping may be counteracted by addition of a small excess of $MgCl_2$ before centrifugation/filtration of the sample. Note that stripping effects may vary with applied sample volume.

3 General considerations

HiTrap IMAC FF is supplied without bound 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* "Optimization".

We recommend protein binding at neutral to slightly alkaline pH (pH 7–8) in the presence of 0.5–1.0 M NaCl. Sodium phosphate buffers are often used. Tris-HCl can also be used, but should be avoided in cases where the metal ion-protein affinity is very low, since it may reduce binding strength. Avoid chelating agents such as EDTA or citrate in buffers, *see* Table 3.

Including salt e.g. 0.5–1.0 M NaCl in the buffers and samples eliminates ion-exchange effects but can also have a marginal effect on the retention of proteins.

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 host cell proteins) and high yield (binding all of the histidine-tagged protein). *See* "Optimization" for further info.

The most frequently used elution procedure for histidine-tagged proteins is based on a competitive displacement by imidazole. As alternatives to imidazole elution, target proteins can 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.

Note: *If the proteins are sensitive to low pH, we recommend collecting the eluted fractions in tubes containing 1 M Tris-HCl, pH 9.0 (60–200 µl/ml fraction) to quickly restore the pH to neutral.*

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 on a HiTrap Desalting, a PD-10 Desalting Column, or HiPrep™ 26/10 Desalting (*see* Table 4).

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 very low under all normal conditions. For applications where extremely low leakage during purification is critical, it can be even further diminished by performing a blank run, *see* "Column preparation".

Likewise, a blank run should also be performed before applying buffers/samples containing reducing agents, *see* "Column preparation".

Whatever conditions are chosen, HiTrap IMAC FF columns can be operated with a syringe, peristaltic pump, or chromatography system.

Table 4. Prepacked columns for desalting and buffer exchange

Column	Code No.	Loading volume	Elution volume	Comments	Application
HiPrep 26/10 Desalting	17-5087-01	2.5 - 15 ml	7.5 - 20 ml	Prepacked with Sephadex™ G-25 Fine. Requires a laboratory pump or a chromatography system to run.	For desalting and buffer exchange of protein extracts (M_r > 5000).
HiTrap Desalting	17-1408-01	0.25 - 1.5 ml	1.0 - 2.0 ml	Prepacked with Sephadex G-25 Superfine. Requires a syringe or pump to run.	
PD-10 Desalting	17-0851-01	1.0 - 2.5 ml[1] 1.75 - 2.5 ml[2]	3.5 ml[1] Up to 2.5 ml[2]	Prepacked with Sephadex G-25 Medium. Runs by gravity flow or centrifugation	For desalting, buffer exchange, and cleanup of proteins and other large biomolecules (M_r > 5000).
PD MiniTrap™ G-25	28-9180-07	0.1 - 0.5 ml[1] 0.2 - 0.5 ml[2]	1.0 ml[1] Up to 0.5 ml[2]		
PD MidiTrap™ G-25	28-9180-08	0.5 - 1.0 ml[1] 0.75 - 1.0 ml[2]	1.5 ml[1] Up to 1.0 ml[2]		

[1] Volumes with gravity elution
[2] Volumes with centrifugation

4 Operation

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 0.22 µm or a 0.45 µm filter before use.

If the recombinant histidine-tagged protein is expressed as inclusion bodies, include 6 M Gua-HCl or 8 M urea in all buffers and sample. Refolding of the denatured protein, on-column or after elution, is protein-dependent.

Recommended buffers

Binding buffer:	20 mM sodium phosphate, 0.5 M NaCl, 20–40 mM imidazole, pH 7.4. The optimal imidazole concentration is proteindependent, 20–40 mM is suitable for many histidine-tagged proteins when using Ni^{2+} or Co^{2+}.
Elution buffer:	20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4 The imidazole concentration required for elution is protein-dependent.

Note: *Compared to histidine-tagged proteins, untagged, naturally occurring proteins bind immobilized metal ions with lower affinity. Thus, the concentrations of imidazole that should be used with untagged proteins are lower than the above recommended, both for binding (sometimes no imidazole needs to be added) and elution.*

An alternative, especially for untagged target proteins, is elution at low pH, e.g., a linear gradient from pH 7.4 to pH 4. Like imidazole elution buffer, also low-pH elution buffers should contain 0.5 M NaCl. Example: First stepwise elution 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.

Column preparation

1 Prepare a 0.1 M solution of the chosen metal ion in distilled water. Salts of chlorides, sulfates, etc. can be used; e.g. 0.1 M $CuSO_4$ or 0.1 M $NiSO_4$ are commonly used.

Note: *Take extra precautions when working with Fe^{3+}. In neutral solutions, Fe^{3+} is easily reduced and forms compounds that can be hard to dissolve. Immobilize Fe^{3+} at low pH, approx. pH 3, to avoid precipitation of insoluble compounds.*

2 Fill the syringe or pump tubing with distilled water. Remove the stopper from the column and connect it to the syringe (with the connector provided), or to the pump tubing 'drop-to-drop' to avoid introducing air into the system.

3 Remove the snap-off end at the column outlet.

4 Wash the column with 5 ml or 15 ml distilled water for HiTrap IMAC FF 1 ml or 5 ml column, respectively. At this stage, do not use buffer to wash out the 20% ethanol as the metal ion can precipitate during step 5, depending on the buffer used.

5 Charge the water-washed column by loading at least 0.5 ml or 2.5 ml of the 0.1 M metal salt solution on the HiTrap IMAC FF 1 ml and 5 ml column, respectively.

6 Wash with distilled water, 5 ml or 15 ml for the 1 ml and 5 ml column, respectively.

7 The column is now ready for equilibration and sample application

8 **Optional:** In some cases, a blank run is recommended before using the column – *see* below.

Optional: Blank run

Note: *IMAC Sepharose 6 Fast Flow charged with Ni^{2+} is compatible with reducing agents,* see *Table 3. However, before applying buffer/sample including reducing agents we recommend removing any weakly bound metal ions by performing a blank run without reducing agents, see below. Do not leave HiTrap IMAC FF columns in buffers containing reducing agents when not in use.*

Note: *Leakage of Ni^{2+} and Co^{2+} ions from IMAC Sepharose 6 Fast Flow is low under all normal conditions. For very critical applications, performing a blank run (as described below) before loading sample can further decrease leakage during purification. Such a treatment is intended to remove any weakly bound metal ions that otherwise might be desorbed later, during the elution of bound protein.*

Performing a blank run:

Use binding buffer and elution buffer **without** reducing agents.

1 If the column has been stored in 20% ethanol after metal ion charging, wash it with 5 column volumes (CV) of distilled water.

2 Wash with 5 CV of the buffer that has been chosen for the protein elution, e.g. imidazole elution buffer for competitive elution, or low-pH elution buffer. Do not use EDTA/EGTA elution buffer for a blank run.

3 Equilibrate thoroughly with 5–10 CV of binding buffer.

Note: *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, e.g. at 220 nm.*

4 The column is now ready for sample application.

Sample preparation

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

Adjust the sample to the composition and pH of the binding buffer by adding buffer, NaCl, imidazole, and additives (as required) from concentrated stock solutions, by diluting the sample with binding buffer, or by buffer exchange. Do not use strong bases or acids for pH-adjustment (precipitation risk). Shortly before applying the sample to the column, centrifuge it and/or filter it through 0.45 or 0.22 µm filters.

Note: *To minimize the co-adsorption of unwanted host cell proteins, it is essential to include imidazole at a low concentration in the sample and binding buffer, see "Optimization".*

Purification

Please read the section "General considerations" before starting the purification.

1 After the column preparation, equilibrate with at least 5 column volumes (CV) of binding buffer. Recommended flow rates are 1 ml/min or 5 ml/min for the 1 ml and 5 ml columns, respectively.

Note: *In some cases, we recommend a blank run before final equilibration/sample application* - see *"Column preparation"*.

2 Apply the pretreated sample using a syringe or pump.

3 Wash with binding buffer until the absorbance reaches a steady baseline (generally, at least 10–15 CV).

4 Elute the bound proteins with elution buffer, stepwise or with a linear gradient. Five CV are usually sufficient if the protein of interest is eluted with one step. A shallow gradient, e.g. a linear gradient over 20 CV or more, may separate proteins with similar binding strengths.

Note: *If imidazole needs to be removed from the eluted protein, use HiTrap Desalting, a PD-10 Desalting Column, or HiPrep 26/10 Desalting depending on the sample volume,* see *Table 4*.

5 Optimization

Concentration of imidazole in binding/wash buffer

Imidazole at low concentrations is commonly used in binding/ wash buffers to minimize the binding of unwanted host cell proteins. For the same reason, 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 host cell proteins), and high yield (binding all of the histidine-tagged protein). This optimal concentration is different for different histidine-tagged proteins/target proteins, and is usually slightly higher for IMAC Sepharose 6 Fast Flow than for similar IMAC media on the market.

Finding the optimal imidazole concentration for a specific target 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+}.

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

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.

Use a high purity imidazole, which has essentially no absorbance at 280 nm.

Choice of metal ion

Ni^{2+} is usually the first choice for purifying most histidine-tagged recombinant proteins, and is also the metal ion most generally used. Nevertheless, it is not always possible to predict which metal ion will be the most suitable for a given protein. The strength of binding between a protein and an immobilized metal ion is affected by several factors, including the length, position, and exposure of the affinity tag on the protein, the type of metal ion used, and the pH, etc. of buffers. Some proteins may therefore be easier to purify with metal ions other than Ni^{2+}, e.g. Zn^{2+}, Co^{2+}, or Cu^{2+}.

For purification of untagged proteins, Cu^{2+} ions have frequently been used. When the binding characteristics of an untagged target protein are not known, it is advisable to test also other metal ions (e.g. Zn^{2+}, Ni^{2+}, Co^{2+}) in order 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.

6 Stripping and re-charging

Note: *The column does not have to be stripped and recharged between each purification cycle if the same protein is to be purified; it may be sufficient to strip and recharge it after 5–7 purifications, depending on the metal ion, sample properties, sample volume, target protein, etc.*

Recommended stripping buffer: 20 mM sodium phosphate, 0.5 M NaCl, 50 mM EDTA, pH 7.4

Strip the column by washing with at least 5–10 column volumes (CV) of stripping buffer. Wash with at least 5–10 CV of binding buffer and 5–10 CV of distilled water before re-charging the column.

Re-charge the water-washed column according to the method previously described, *see* "Column preparation".

7 Cleaning-in-place (CIP)

Clean the column when an increase in back-pressure is seen or when cross contamination between samples is to be prevented. Before cleaning, strip off the immobilized metal ions using the recommended procedure described above. Stripping, without any additional CIP procedures, may sometimes give a satisfactory cleaning effect.

After cleaning, store in 20% ethanol (wash with 5 CV) or re-charge with the preferred metal ion.

The stripped column can be cleaned by the following procedures. For difficult cases, use reversed flow direction:

- Remove ionically bound proteins by washing with several CV of 1.5 M NaCl. Then wash with at least 3 CV of distilled water.
- Remove precipitated proteins, hydrophobically-bound proteins and lipoproteins by washing 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 5–10 CV of distilled water.
- Remove hydrophobically bound proteins, lipoproteins, and lipids by washing the column with 5–10 CV 30% isopropanol for at least 15–20 min. Then wash with approx. 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 at least 5 CV of 70% ethanol. Then wash with 3-10 CV of distilled water.

8 Scaling up

Two or three HiTrap IMAC FF 1 ml or 5 ml columns can be connected in series for quick scale-up (note that backpressure will increase). Use HiPrep IMAC FF 16/10 (20 ml) prepacked column if further scale-up is necessary.

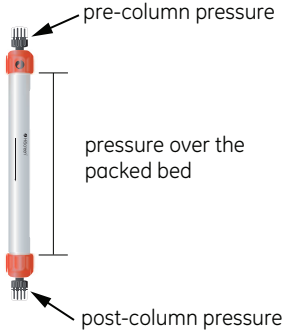
IMAC Sepharose 6 Fast Flow, the medium prepacked in HiTrap IMAC FF and HiPrep IMAC FF 16/10 columns, is supplied pre-swollen in 25 ml and 100 ml lab packs, *see* Ordering information). An alternative scale-up strategy is thus to pack the medium in empty columns – Tricorn™ and XK columns are suitable for this purpose.

9 Adjusting pressure limits in chromatography system software

Pressure generated by the flow through a column affects the packed bed and the column hardware, see Fig . Increased pressure is generated when running/using one or a combination of the following conditions:

- High flow rates
- Buffers or sample with high viscosity
- Low temperature
- A flow restrictor

Note: *Exceeding the flow limit (see Table 2) may damage the column.*



Pre-column and post-column measurements.

ÄKTA avant

The system will automatically monitor the pressures (pre-column pressure and pressure over the packed bed, Δp). The pre-column pressure limit is the column hardware pressure limit (see Table 1).

The maximum pressure the packed bed can withstand depends on media characteristics and sample/liquid viscosity. The measured value also depends on the tubing used to connect the column to the instrument.

ÄKTAexplorer, ÄKTApurifier, ÄKTAFPLC and other systems with pressure sensor in the pump

To obtain optimal functionality, the pressure limit in the software may be adjusted according to the following procedure:

1. Replace the column with a piece of tubing. Run the pump at the maximum intended flow rate. Note the pressure as *total system pressure*, P1.
2. Disconnect the tubing and run the pump at the same flow rate used in step 1. Note that there will be a drip from the column valve. Note this pressure as P2.
3. Calculate the new pressure limit as a sum of P2 and the column hardware pressure limit (see Table 1). Replace the pressure limit in the software with the calculated value.

   The actual pressure over the packed bed (Δp) will during run be equal to actual measured pressure - *total system pressure* (P1).

Note: *Repeat the procedure each time the parameters are changed.*

10 Storage

Store at 4°C to 30°C in 20% ethanol.

11 Troubleshooting

The following tips may be of assistance. If you have any further questions about your HiTrap IMAC FF column, please visit *www.gelifesciences.com/hitrap,* contact our technical support team, or your local representative.

Column has clogged:

- Cell debris in the sample may clog the column. Clean the column according to the section "Cleaning-in-place".
- Centrifuge and/or filter the sample through a 0.22 µm or a 0.45 µm filter, *see* "Sample preparation".

Sample is too viscous:

- If the lysate is very viscous due to a high concentration 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 be used:** 2% Triton X-100, 2% Tween 20, 2% NP-40, 2% cholate, 1% CHAPS, 1.5 M NaCl, 50% glycerol, 20 mM β-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 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.

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 the optimal elution conditions.
- **Protein has precipitated on the column:** For the next experiment, decrease the amount of sample or decrease protein concentration by eluting with a linear imidazole gradient instead of steps. Try detergents or changed NaCl concentration, or elute under denaturing (unfolding) conditions (use 4–8 M urea or 4–6 M Gua-HCl).
- **Nonspecific hydrophobic or other interaction:** Add a nonionic detergent to the elution buffer (e.g. 0.2% Triton X-100) or increase the NaCl concentration.
- **Concentration of imidazole in the sample and/or binding buffer is too high:** The protein is found in the flow-through. Decrease the imidazole concentration.
- **Histidine-tag may be insufficiently exposed:** The protein is found in the flow-through material; perform purification of unfolded protein in urea or Gua-HCl as for inclusion bodies. To minimize dilution of the sample, add solid urea or Gua-HCl.
- **Buffer/sample composition is incorrect:** The protein is found in the flow-through. Check pH and composition of sample and binding buffer. Ensure that chelating or strong reducing agents are not present in the sample at too high concentration, and that the concentration of imidazole is not too high.

Histidine-tagged protein found in the pellet:

SDS-PAGE 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:

- **Insufficient sonication:** Check cell disruption by microscopic examination or monitor it by measuring the release of nucleic acids at A_{260}. Adding of 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. Over-

sonication can also lead to copurification of host proteins with the target protein.

- **Protein is 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, 8 M urea, or 6 M Gua-HCl and suitable imidazole concentrations, pH 7.4–7.6. Buffers with urea should also include 500 mM NaCl. Use these buffers for sample preparation, as binding buffer and as elution buffer. For sample preparation and binding buffer, use 10–20 mM imidazole or the concentration selected during optimization trials (including urea or Gua-HCl). To minimize dilution of the sample, add solid urea or Gua-HCl.

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 3).
- **Contaminants have high affinity for the metal ions used:** Optimize the concentration of imidazole in the sample and binding buffer. Elute with a stepwise or linear imidazole gradient to determine optimal imidazole concentrations to use for binding and for wash; add imidazole to the sample to the same concentration as in the binding buffer. Wash before elution with binding buffer containing as a high 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 (HiTrap Q HP or HiTrap SP HP) and/or gel filtration (Superdex™ Peptide, Superdex 75 or Superdex 200) may be necessary.

- **Contaminants are associated with tagged proteins:** Add detergent and/or reducing agents before sonicating cells. Increase detergent levels (e.g. up to 2% Triton X-100 or 2% Tween 20), or add glycerol (up to 50%) to the wash buffer to disrupt nonspecific interactions.
- **Try another metal ion:** The metal ion used for purification may not be the most suitable, *see* "Optimization".

Histidine-tagged protein is eluted during sample loading/wash:

- **Buffer/sample composition is incorrect:** Check pH and composition of sample and binding buffer. Ensure that chelating or strong reducing agents are not present in the sample at too high a concentration, and that the concentration of imidazole is not too high.
- **Histidine-tag is partially obstructed:** Purify under denaturing conditions (use 4–8 M urea or 4–6 M Gua-HCl).
- **Column capacity is exceeded:** Join two or three HiTrap IMAC FF 1 ml columns together or change to a HiTrap IMAC FF 5 ml column or a prepacked HiPrep IMAC FF 16/10 column (20 ml).

12 Ordering information

Product	No. supplied	Code no.
HiTrap IMAC FF	5 × 1 ml	17-0921-02
HiTrap IMAC FF	5 × 5 ml	17-0921-04

Related products	No. supplied	Code no.
HiPrep IMAC FF 16/10	1 × 20 ml	28-9365-52
IMAC Sepharose 6 Fast Flow	25 ml	17-0921-07
	100 ml[1]	17-0921-08
HisTrap FF	5 × 1 ml	17-5319-01
	100 × 1 ml[2]	17-5319-02
	5 × 5 ml	17-5255-01
	100 × 5 ml[2]	17-5255-02
HisTrap FF crude	5 × 1 ml	11-0004-58
	100 × 1 ml[2]	11-0004-59
	5 × 5 ml	17-5286-01
	100 × 5 ml[2]	17-5286-02
HiTrap Desalting	1 × 5 ml	29-0486-84
	5 × 5 ml	17-1408-01
	100 × 5 ml[2]	11-0003-29
PD-10 Desalting Column	30	17-0851-01
HiPrep 26/10 Desalting	1 × 53 ml	17-5087-01
	4 × 53 ml	17-5087-02

[1] Larger quantities are available. Please contact your local representative for further information.
[2] Pack size available by special order.

Accessories	Quantity	Code No.
1/16" male/luer female *(For connection of syringe to top of HiTrap column)*	2	18-1112-51
Tubing connector flangeless/M6 female (*For connection of tubing to bottom of HiTrap column)*	2	18-1003-68
Tubing connector flangeless/M6 male *(For connection of tubing to top of HiTrap column)*	2	18-1017-98
Union 1/16" female/M6 male *(For connection to original FPLC System through bottom of HiTrap column)*	6	18-1112-57
Union M6 female /1/16" male *(For connection to original FPLC System through top of HiTrap column)*	5	18-3858-01
Union luerlock female/M6 female	2	18-1027-12
HiTrap/HiPrep, 1/16" male connector for ÄKTA design	8	28-4010-81
Stop plug female, 1/16" *(For sealing bottom of HiTrap column)*	5	11-0004-64
Fingertight stop plug, 1/16"	5	11-0003-55

Related literature	Code No.
Recombinant Protein Purification Handbook, Principles and Methods	18-1142-75
Affinity Chromatography Handbook, Principles and Methods	18-1022-29
Affinity Chromatography Columns and Media, Selection Guide	18-1121-86
Ni IMAC Sepharose and IMAC Sepharose, Selection Guide	28-4070-92

For local office contact information, visit:
www.gelifesciences.com/contact

GE Healthcare Bio-Sciences AB
Björkgatan 30
751 84 Uppsala
Sweden

www.gelifesciences.com/hitrap
www.gelifesciences.com/protein-purification

GE Healthcare Europe GmbH
Munzinger Strasse 5,
D-79111 Freiburg,
Germany

GE Healthcare UK Ltd
Amersham Place
Little Chalfont
Buckinghamshire, HP7 9NA
UK

GE Healthcare Bio-Sciences Corp
800 Centennial Avenue
P.O. Box 1327
Piscataway, NJ 08855-1327
USA

GE Healthcare Bio-Sciences KK
Sanken Bldg.
3-25-1, Hyakunincho
Shinjuku-ku, Tokyo 169-0073
Japan

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