

 HiTrap**Caution!**

Contains nickel.  
May cause an  
allergic reaction.

# HisTrap FF crude, 1 ml and 5 ml

HisTrap™ FF crude is a ready-to-use column, prepacked with precharged Ni Sepharose™ 6 Fast Flow. This pre-packed column is intended for preparative purification of histidine-tagged recombinant proteins by immobilized metal affinity chromatography (IMAC). After thorough cell disruption, it is possible to load the unclarified lysate on the column without precentrifugation and filtration of the sample. Extending the duration of the mechanical treatment of the sample to ensure a more complete lysis is recommended.

Ni Sepharose 6 Fast Flow has low nickel ion ( $\text{Ni}^{2+}$ ) leakage and is compatible with a wide range of additives used in protein purification. The special design of the column in combination with the medium, provide fast, simple, and convenient purifications. Short purification time generally minimizes deleterious effects, such as degradation and oxidation of sensitive target proteins, and is therefore of great importance.

HisTrap FF crude columns can be operated with a syringe, peristaltic pump, or liquid chromatography system such as ÄKTAdesign™ chromatography systems or FPLC™ System.



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# 1. Description

## Medium properties

HisTrap FF crude 1-ml and 5-ml columns are prepacked with the affinity medium Ni Sepharose 6 Fast Flow, which consists of highly cross-linked agarose beads with an immobilized chelating group. The medium has been precharged with Ni<sup>2+</sup> ions.

Several amino acids, for example histidine, form complexes with many metal ions. Ni Sepharose 6 Fast Flow selectively binds proteins if suitable complex-forming amino acid residues are exposed on the protein surface. An added histidine tag increases the affinity for Ni<sup>2+</sup> and generally makes the histidine-tagged protein the strongest binder among other proteins in samples such as *E. coli* lysates.

**Table 1.** HisTrap FF crude characteristics

Matrix	Highly cross-linked spherical agarose, 6%
Average bead size	90 μm
Metal ion capacity	~15 μmol Ni <sup>2+</sup> /ml medium
Dynamic binding capacity*	Approx. 40 mg (His) <sub>6</sub> -tagged protein/ml medium
Column volumes	1 ml or 5 ml
Column dimensions	i.d. × H: 0.7 × 2.5 cm (1 ml) 1.6 × 2.5 cm (5 ml)
Recommended flow rate	1 and 5 ml/min for 1-ml and 5-ml column, respectively
Max. flow rates	Depends on sample pretreatment and sample properties.
Max. back pressure†	0.3 MPa, 3 bar
Compatibility during use	Stable in all commonly used buffers, reducing agents, denaturants, and detergents. See Table 2.

*Continues on page 4*

Chemical stability <sup>†</sup>	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
Avoid in buffers	Chelating agents, e.g. EDTA, EGTA, citrate (see Table 2)
pH stability <sup>†</sup>	short term (2 h): 2–14 long term (one week): 3–12
Storage	20% ethanol
Storage temperature	+4 to +30 °C

\* Dynamic binding capacity conditions:

Sample:	1 mg/ml (His) <sub>6</sub> -tagged pure protein (M <sub>r</sub> 43 000) in binding buffer (Q <sub>B</sub> 10% determination) or (His) <sub>6</sub> -tagged protein (M <sub>r</sub> 28 000) bound from <i>E. coli</i> extract
Column volume:	0.25 ml (not prepacked) or 1 ml
Flow rate:	0.25 ml/min or 1 ml/min, for 0.25-ml or 1-ml column, respectively
Binding buffer:	20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, pH 7.4
Elution buffer:	20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4

**Note:** Dynamic binding capacity is protein-dependent.

<sup>†</sup> H<sub>2</sub>O at room temperature.

**Note:** When setting the total pressure limit, the back pressure of the system (including the flow restrictor) should also be added to the maximum back pressure of the column. For example, when using an ÄKTAdesign system with a flow restrictor, the pressure limit should be set to 0.5 MPa (5 bar), where the column and the flow restrictor give a pressure of 0.3 MPa and 0.2 MPa, respectively. The contribution from the ÄKTAdesign system itself is usually very low (not measurable), unless extremely narrow tubing is used.

<sup>‡</sup> Ni<sup>2+</sup>-stripped medium.

The Ni<sup>2+</sup>-charged medium is compatible with all commonly used aqueous buffers, reducing agents, denaturants such as 6 M Gua-HCl and 8 M urea, and a range of other additives (see Table 2).

**Table 2.** Ni Sepharose 6 Fast Flow is compatible with the following compounds at the concentrations given

Reducing agents*	5 mM DTE
	5 mM DTT
	20 mM $\beta$ -mercaptoethanol
	5 mM TCEP
	10 mM reduced glutathione
Denaturing agents†	8 M urea
	6 M Gua-HCl
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 <sub>2</sub> SO <sub>4</sub>
	1.5 M NaCl
	1 mM EDTA‡
Buffer	60 mM citrate‡
	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 <sup>†</sup>

\* Ni Sepharose 6 Fast Flow is compatible with reducing agents. However, for optimal performance, removal of any weakly bound Ni<sup>2+</sup> ions by performing a blank run without reducing agents (as described in Performing a purification, section 4) before applying buffer/sample including reducing agents is recommended. Do not leave HisTrap FF crude columns with buffers including reducing agents when not in use.

† Tested for one week at +40 °C.

‡ 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 in the buffers). Any metal-ion stripping may be counteracted by addition of a small excess of MgCl<sub>2</sub> before centrifugation/filtration of the sample. Note that stripping effects may vary with applied sample volume.

## Column properties

HisTrap FF crude is intended for preparative purification of histidine-tagged recombinant proteins. After thorough cell disruption, it is possible to load the unclarified lysate on the specially designed column without precentrifugation and filtration. The filters in the top and bottom of the column make it possible to directly load sonicated lysates on the column. The filter pore size is optimized to allow loading of sonicated lysates directly on the column without causing backpressure problems or leakage of the Ni Sepharose 6 Fast Flow beads.

HisTrap FF crude columns are made of biocompatible polypropylene that does not interact with biomolecules. Columns are delivered with a stopper on the inlet and a snap-off end on the outlet. The columns have porous top and bottom frits that allow loading of unclarified cell lysates directly on the column.

Columns can be operated with either a syringe and the supplied Luer adapter, a peristaltic pump, or a chromatography system such as ÄKTAdesign or FPLC System.

**Note:** HisTrap FF crude columns cannot be opened or refilled.

**Note:** To prevent leakage, ensure that the adapter is tight.

## 2. General considerations

HisTrap FF crude columns are supplied precharged with  $\text{Ni}^{2+}$  ions. In general,  $\text{Ni}^{2+}$  is the preferred metal ion for purification of recombinant histidine-tagged proteins. Note, however, that in some cases it may be wise to test other metal ions, e.g. zinc ( $\text{Zn}^{2+}$ ) and cobalt ( $\text{Co}^{2+}$ ), as the strength of binding depends on the nature of the histidine-tagged protein as well as the metal ion (see Optimization of purification performance in section 5).

We recommend 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 generally be used, but should be avoided in cases where the metal-protein affinity is very weak, since it may reduce binding strength. Avoid chelating agents such as EDTA or citrate in buffers (see Table 2). In general, imidazole is used for elution of histidine-tagged proteins.

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 binding and wash buffers to minimize binding of host cell proteins. Imidazole is also included in the sample (generally at the same concentration as in the binding buffer) to further minimize binding of host cell proteins. At somewhat higher concentrations, imidazole may also 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 host cell proteins) and high yield (strong binding of histidine-tagged target protein). The concentration of imidazole that will give optimal purification results is protein-dependent, and is usually slightly higher for Ni Sepharose 6 Fast Flow than for similar IMAC media on the market (see Data File 11-0008-86). Use highly pure imidazole, which gives essentially no absorbance at 280 nm.

As alternatives to imidazole elution, histidine-tagged proteins can be eluted from the medium by several other methods or combinations of methods, for example a lowering of pH within the range of 2.5–7.5 can be used. Below pH 4, metal ions will be stripped off the medium.

**Note:** If the protein is sensitive to low pH, we recommend collection of the eluted fractions in tubes containing 1 M Tris-HCl, pH 9.0 (60–200  $\mu$ l/ml fraction) to restore the pH to neutral.

EGTA and EDTA will strip metal ions from the medium and thereby cause protein elution, but the target pool will then contain chelated Ni<sup>2+</sup> ions. In this case, the Ni<sup>2+</sup> ions can be removed by desalting on HiTrap™ Desalting, PD-10 Desalting, or HiPrep™ 26/10 Desalting columns (see Table 3).

**Table 3.** Prepacked columns for desalting and buffer exchange

<b>Code No.</b>	<b>Column</b>	<b>Load</b>	<b>Elute</b>	<b>Comments</b>	<b>Application</b>
17-0851-01	PD-10 Desalting	2.5 ml	3.5 ml	Prepacked with Sephadex™ G-25. Requires only gravity to run.	For desalting and buffer exchange of protein extracts ( $M_r > 5000$ ).
17-1408-01	HiTrap Desalting	0.1– 1.5 ml	1.3– 4.0 x applied volume	Prepacked with Sephadex G-25 Superfine. Requires a syringe or pump to run.	For desalting and buffer exchange of protein extracts ( $M_r > 5000$ ).
17-5087-01	HiPrep 26/10 Desalting	Up to 15 ml	15– 20 ml	Prepacked with Sephadex G-25 Fine. Requires a pump to run.	For desalting and buffer exchange of protein extracts ( $M_r > 5000$ ).
17-0855-01	NICK™	0.1 ml	0.4 ml	Prepacked with Sephadex G-25. Requires only gravity to run.	For separation of proteins ( $M_r > 5000$ ) and nick-translated DNA from radiolabelled nucleotides not shorter than 120 mers, and similar separations.
17-0853-01	NAP™-5	0.5 ml	1.0 ml	Prepacked with Sephadex G-25 DNA grade. Requires only gravity to run.	For purification of proteins ( $M_r > 5000$ ), DNA and oligonucleotides greater than 10 bases in length.
17-0854-01	NAP-10	1.0 ml	1.5 ml		
17-0852-01	NAP-25	2.5 ml	3.5 ml		

Leakage of Ni<sup>2+</sup> from Ni Sepharose 6 Fast Flow is very low under all normal conditions, lower than for other IMAC media tested (see Data File 11-0008-86). For applications where extremely low leakage during purification is critical, leakage can be even further reduced by performing a blank run (see page 15). Likewise, a blank run should also be performed before applying buffers/samples containing reducing agents (see page 15). Whatever conditions are chosen, HisTrap FF crude columns can be operated with a syringe, peristaltic pump, or chromatography system.

HisTrap FF crude columns are designed for purification of histidine-tagged proteins directly from unclarified cell lysates. Sample preparation is performed by enzymatic and mechanical lysis. No centrifugation and filtration are needed before loading the sample onto the column. Extending the duration of mechanical treatment of the sample to ensure a more complete lysis is recommended (keep the sample on ice to prevent overheating).

### **Recommended buffers**

**Binding buffer\*:** 20 mM sodium phosphate, 500 mM NaCl, 20–40 mM imidazole, pH 7.4  
(The optimal imidazole concentration is protein-dependent; 20–40 mM is suitable for many proteins).

**Elution buffer\*:** 20 mM sodium phosphate, 500 mM NaCl, 500 mM imidazole, pH 7.4  
(The imidazole concentration required for elution is protein-dependent).

\* See Appendix A for the buffer recipes.

If the recombinant histidine-tagged protein is expressed as inclusion bodies, include 6 M Guanidine-HCl (Gua-HCl) or 8 M urea in all buffers and sample to promote protein unfolding. On-column refolding of the denatured protein may be possible, but depends on the protein. Advice for overcoming problems associated with inclusion bodies is described in Troubleshooting, section 7.

**Tips:** 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.

### 3. Sample preparation

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

#### **Recommended four-step protocol for cell lysis**

The protocol below has been used successfully in our own laboratories, but other established procedures may also work.

**1. Dilution of cell paste:** Add 5–10 ml of binding buffer for each gram of cell paste. To prevent the binding of host cell proteins with exposed histidines, it is essential to include imidazole at a low concentration in the sample and binding buffer (see Optimization of purification performance in section 5).

**2. Enzymatic lysis:** 0.2 mg/ml lysozyme, 20 µg/ml DNase, 1 mM MgCl<sub>2</sub>, 1 mM Pefabloc™ SC or PMSF (final concentrations). Stir for 30 min at room temperature or +4 °C depending on the sensitivity of the target protein.

**3. Mechanical lysis\*:** Sonication on ice, approx. 10 min

*or*

Homogenization with a French press or other homogenizer

*or*

Freeze/thaw, repeated at least five times

\* Mechanical lysis time may have to be extended compared with standard protocols to secure an optimized lysate for sample loading (to prevent clogging of the column and back pressure problems). Different proteins have different sensitivity to cell lysis and caution has to be taken to avoid frothing and overheating of the sample.

**4. Adjust the pH of the lysate:** Do not use strong bases or acids for pH-adjustment (precipitation risk). Apply the unclarified lysate on the column directly after preparation.

**Note:** If the sonicated or homogenized unclarified cell lysate is frozen before use, precipitation and aggregation may increase. New sonication of the lysate can then prevent increased back pressure problems when loading on the column.

## 4. Performing a purification

1. Fill the pump tubing or syringe with distilled water. Remove the stopper and connect the column to the chromatography system tubing, syringe (use the adapter provided) or laboratory pump “drop-to-drop” to avoid introducing air into the system.
2. Remove the snap-off end at the column outlet.
3. Wash out the ethanol with 3–5 column volumes of distilled water.
4. Equilibrate the column with at least five column volumes of binding buffer. Recommended flow rates are 1 ml/min or 5 ml/min for the 1-ml and 5-ml columns, respectively. In some cases, a blank run is recommended before final equilibration/sample application.
5. Apply the unclarified lysate with a pump or a syringe.

Continuous stirring of the sample during sample loading is recommended to prevent sedimentation.

Typical loading volumes of unclarified lysate (highly dependent on specific sample, sample pretreatment, and temperature at sample loading):

**HisTrap FF crude 1 ml:** Up to 100 ml

**HisTrap FF crude 5 ml:** Up to 500 ml

**Note:** Sample loading at +4 °C may increase the viscosity of the

sample. An adverse effect of increased sample viscosity is that maximum back pressure for the column is reached at a lower sample volume loading on the column.

Do not exceed the binding capacity of the column.

Large volumes may increase back pressure, making the use of a syringe more difficult.

6. Wash with binding buffer until the absorbance reaches a steady baseline (generally at least 10–15 column volumes).

**Note:** Purification results are improved by using imidazole in sample and binding buffer (see Optimization of purification performance in section 5).

7. Elute with elution buffer using a one-step procedure or a linear gradient. For step elution, five column volumes of elution buffer is usually sufficient. A shallow gradient, e.g. a linear gradient over 20 column volumes or more, can separate proteins with similar binding strengths.

**Note:** Unclarified lysates may cause increased air bubble formation during purification. An attached flow restrictor in the chromatography system can prevent this if it causes problems. If a flow restrictor is attached, it is important to change the pressure limit to 0.5 MPa (5 bar) on the ÄKTA design system (where the column and the flow restrictor give a pressure of 0.3 MPa and 0.2 MPa, respectively).

**Note:** If imidazole needs to be removed from the protein, use HiTrap Desalting, PD-10 Desalting, or HiPrep 26/10 Desalting columns depending on the sample volume (see Table 3).

**Note:** Ni Sepharose 6 Fast Flow is compatible with reducing agents. However, for optimal performance removal of any weakly bound Ni<sup>2+</sup> ions by performing a blank run without reducing agents (as described below) before applying buffer/sample including reducing agents is recommended. Do not leave HisTrap FF crude columns with buffers including reducing agents when not in use.

**Note:** Leakage of Ni<sup>2+</sup> from Ni Sepharose 6 Fast Flow is low under all normal conditions. The leakage is lower than for other IMAC media tested. For very critical applications, leakage during purification can be even further reduced by performing a blank run (as described below) before loading the sample.

### **Performing a blank run**

Use binding and elution buffers without reducing agents.

1. Wash the column with five column volumes of distilled water (to remove the 20% ethanol).
2. Wash with five column volumes of binding buffer.
3. Wash with five column volumes of elution buffer.
4. Equilibrate with 10 column volumes of binding buffer.

**Note:** If the column has clogged it may be possible to perform cleaning-in-place, see section 6. If cleaning-in-place is unsuccessful, replace the column. Optimize sample pretreatment before the next sample loading.

## **5. Optimization of purification performance**

### **Concentration of imidazole**

Imidazole at low concentrations is commonly used in the binding and the wash buffers to minimize binding of host cell proteins. Imidazole is also included in the sample (generally at the same concentration as in the wash buffer) to further minimize binding of host cell proteins. At somewhat higher concentrations, imidazole may also 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 host cell proteins), and high yield (strong binding of histidine-tagged target protein). This optimal concentration is different for different histidine-tagged proteins, and is usually slightly higher for Ni Sepharose 6 Fast Flow than for similar IMAC media on the market (see Data File 11-0008-86). Finding the optimal imidazole concentration for a specific histidine-tagged protein is a trial-and-error effort, but 20–40 mM in the binding and wash buffers is a good starting point for many proteins. Use high purity imidazole, which gives essentially no absorbance at 280 nm.

## Choice of metal ion

$\text{Ni}^{2+}$  is usually the first choice metal ion for purifying most (His)<sub>6</sub>-tagged recombinant proteins from host cell proteins, and also the ion most generally used. The strength of binding between a protein and a metal ion is affected by several factors, including the length, position, and exposure of the affinity tag on the protein, the type of ion used, and the pH of buffers, so some proteins may be easier to purify with ions other than  $\text{Ni}^{2+}$ .

## Stripping and recharging

**Note:** The column does not have to be stripped and recharged between each purification if the same protein is to be purified. It may be sufficient to strip and recharge it after approximately two to five purifications, depending on the specific sample, sample pretreatment, sample volume, etc.

**Recommended stripping buffer:** 20 mM sodium phosphate, 500 mM NaCl, 50 mM EDTA, pH 7.4

Strip the column by washing with at least 5–10 column volumes of stripping buffer. Wash with at least 5–10 column volumes of binding buffer and 5–10 column volumes of distilled water before recharging the column.

Recharge the water-washed column by loading 0.5 ml or 2.5 ml of 0.1 M  $\text{NiSO}_4$  in distilled water onto the HisTrap FF crude 1-ml and 5-ml columns, respectively. Wash with five column volumes of distilled water, and five column volumes binding

buffer (to adjust pH) before storage in 20% ethanol. Salts of other metals, chlorides, or sulfates, may also be used (see Optimization of purification performance in this section).

### **Scaling up**

Scaling up from a HisTrap FF crude 1-ml column to a 5-ml column while keeping the same linear flow rate provides highly consistent results.

## **6. Column cleaning and storage**

### **Cleaning-in-place**

When an increase in back pressure is seen, the column should be cleaned. Before cleaning, strip off Ni<sup>2+</sup> ions using the recommended procedure described above.

The Ni<sup>2+</sup>-stripped column can be cleaned by the following methods:

- Remove ionically bound proteins by washing with several column volumes of 1.5 M NaCl; then wash with approx. 10 column volumes 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 (12 h or more for endotoxin removal). Then wash with approx. 10 column volumes of binding buffer, followed by 5–10 column volumes of distilled water.

- Remove hydrophobically bound proteins, lipoproteins, and lipids by washing with 5–10 column volumes of 30% isopropanol for about 15–20 min. Then wash with approx. 10 column volumes of distilled water.

Alternatively, wash with two column volumes 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 five column volumes of 70% ethanol. Then wash with approx. 10 column volumes of distilled water.

**Note:** Reversed flow may improve the efficiency of the cleaning-in-place procedure.

After cleaning, store in 20% ethanol (wash with five column volumes) or recharge with  $\text{Ni}^{2+}$  prior to storage in ethanol.

Clean the chromatography system according to the manufacturer's recommendations.

## **Storage**

Store HisTrap FF crude columns in 20% ethanol at +4 to +30 °C.

## **7. Troubleshooting**

The following tips may be of assistance. If you have any further questions about your HisTrap FF crude column, please visit [www.hitrap.com](http://www.hitrap.com), contact our technical support, or your local GE Healthcare, Protein Separations representative.

### **Increased back pressure:**

- Increase the efficiency of the mechanical cell disruption (for example increase sonication time). Keep the sample on ice to avoid frothing and overheating as this may denature the target protein. Over-sonication can also lead to co-purification of host proteins with the target protein.
- Increase dilution of the cell paste before sonication or dilute after the sonication to reduce viscosity.
- 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 an additional dose of DNase and  $Mg^{2+}$  (see Sample preparation, section 3). Alternatively, draw the lysate through a syringe needle several times.
- Freeze/thaw of the unclarified lysate may increase precipitation and aggregation. Sonication of the thawed lysate can prevent increased back pressure problems when loading on the column.
- If the purification has been performed at +4 °C, move to room temperature if possible (sample viscosity is reduced at room temperature).
- Decrease flow rate during sample loading.

### **Column has clogged:**

- If cleaning-in-place is unsuccessful, replace the column. Optimize sample pretreatment before the next sample loading.

## **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  $\beta$ -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.
- **The protein might 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, 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 well 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, solid urea or Gua-HCl can be added.

## 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 decreasing pH to determine the optimal elution conditions.
- **Protein has precipitated in the column:** Decrease amount of sample, or decrease protein concentration by eluting with linear imidazole gradient instead of imidazole steps. Try detergents or change 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.
- **Protein found in the flowthrough:** Concentration of imidazole in the sample and/or binding buffer is too high; decrease imidazole concentration.
- **Protein found in the flowthrough:** Histidine tag may be insufficiently exposed; perform purification of unfolded protein in urea or Gua-HCl as for inclusion bodies. To minimize dilution of the sample, solid urea or Gua-HCl can be added.
- **Protein found in the flowthrough:** Buffer/sample composition is not optimal; check pH and composition of sample and binding buffer. Ensure that the concentration of chelating or strong reducing agents in the sample as well as the concentration of 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).
- **Contaminants have high affinity for nickel ions:** 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 in the same concentration as the binding buffer. Wash thoroughly before elution with binding buffer containing the highest possible imidazole concentration (chosen imidazole concentration must not cause elution of the histidine-tagged 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.

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

- **Buffer/sample composition is not optimal:** Check pH and composition of sample and binding buffer. Ensure that the concentration of chelating or strong reducing agents in the sample, as well as 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:** If HisTrap FF crude 1-ml columns have been used, change to a larger column, HisTrap FF crude 5 ml.

## Unwanted air bubble formation

- Unclarified lysates may cause increased air bubble formation during purification. An attached flow restrictor in the chromatography system can prevent this. If a flow restrictor is attached, it is important to change the pressure limit to 0.5 MPa (5 bar) on the ÄKTAdesign system (where the column and the flow restrictor give a pressure of 0.3 MPa and 0.2 MPa, respectively).

## 8. Ordering information

Designation	No. Supplied	Code No.
HisTrap FF crude	5 × 1 ml	11-0004-58
HisTrap FF crude	100 × 1 ml*	11-0004-59
HisTrap FF crude	5 × 5 ml	17-5286-01
HisTrap FF crude	100 × 5 ml*	17-5286-02

\* Pack size available by special order.

<b>Related products</b>	<b>No. Supplied</b>	<b>Code No.</b>
HisTrap FF	5 × 1 ml	17-5319-01
HisTrap FF	100 × 1 ml*	17-5319-02
HisTrap FF	5 × 5 ml	17-5255-01
HisTrap FF	100 × 5 ml*	17-5255-02
HisPrep™ FF 16/10	1 (20 ml)	17-5256-01
Ni Sepharose 6 Fast Flow	25 ml	17-5318-01
Ni Sepharose 6 Fast Flow	100 ml	17-5318-02
Ni Sepharose 6 Fast Flow	500 ml	17-5318-03
HiTrap Desalting	5 × 5 ml	17-1408-01
HiTrap Desalting	100 × 5 ml*	11-0003-29
PD-10 Desalting Column	30	17-0851-01
HiPrep 26/10 Desalting	1 (53 ml)	17-5087-01
HiPrep 26/10 Desalting	4 (53 ml)	17-5087-02

\* Pack size available by special order.

<b>Accessories</b>	<b>No. Supplied</b>	<b>Code No.</b>
1/16" male/luer female	2	18-1112-51
Union luerlock female/M6 female	2	18-1027-12
Union 1/16" female/M6 male	6	18-1112-57
Tubing connector flangeless/M6 male	2	18-1017-98
Tubing connector flangeless/M6 female	2	18-1003-68
Union M6 female /1/16" male	5	18-3858-01
Stop plug female, 1/16"	2, 5, or 7*	11-0004-64
Fingertight stop plug, 1/16"	5†	11-0003-55

\* Two, five, or seven stop plug female, 1/16" are included in the HiTrap packages (depending on the product).

† One fingertight stop plug is connected to each HiTrap column supplied.

<b>Literature</b>	<b>Code no.</b>
Recombinant Protein Handbook, Protein Amplification and Simple Purification	18-1142-75
Affinity Chromatography Handbook, Principle and Methods	18-1022-29
Affinity Chromatography Columns and Media Product Profile	18-1121-86
Convenient Protein Purification, HiTrap Column Guide	18-1129-81
ÅKTA design brochure	11-0007-17

## Appendix A

### Phosphate buffer (containing imidazole for binding and elution buffers)

*(20 mM sodium phosphate, 500 mM NaCl, 20–500 mM imidazole in 1 l)*

To 1.78 g  $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$  (177.99 g/mol), 1.38 g  $\text{NaH}_2\text{PO}_4 \times \text{H}_2\text{O}$  (137.99 g/mol) and 29.22 g NaCl (58.44 g/mol) and X g imidazole (depending on the chosen imidazole binding and elution concentrations, see Table below) add distilled water to 900 ml and dissolve completely.

Adjust pH from basic to 7.4 with HCl. Add distilled water to 1000 ml and filter through a 0.45- $\mu\text{m}$  filter.

This gives a final concentration of 20 mM sodium phosphate, 500 mM NaCl, 10–500 mM imidazole, pH 7.4.

Use high purity imidazole as this will give very low or no absorbance at 280 nm (imidazole, 68.08 g/mol).

Imidazole concentration in buffer (mM)	Weight of imidazole in Phosphate buffer (g)
10	0.7
20	1.4
30	2.0
40	2.7
50	3.4
60	4.1
70	4.8
80	5.4
90	6.1
100	6.8
200	13.6
300	20.4
400	27.2
500	34.0



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

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