Ni Sepharose™ 6 Fast Flow

Immobilized metal ion affinity chromatography (IMAC) exploits the interaction between chelated transition metal ions and side-chains of certain amino acids (mainly histidine) on proteins. In general, Ni^{2+} is the preferred metal ion for purification of histidine-tagged proteins. Ni Sepharose 6 Fast Flow is a BioProcess™ IMAC medium that consists of 90 µm beads of highly cross-linked agarose, to which a chelating group has been coupled. This chelating group has then been charged with nickel (Ni^{2+}) ions.

Ni Sepharose 6 Fast Flow has low Ni^{2+} leakage, high proteinbinding capacity, and is compatible with a wide range of additives used in protein purification. Its high flow properties make it excellent for scale-up.

Ni Sepharose 6 Fast Flow is available in 5, 25, 100, 500 ml, 1L, and 5L bulk packs. Ni Sepharose 6 Fast Flow is also available prepacked in:

- HisTrap™ FF, 1 and 5 ml
- HisTrap FF crude, 1 and 5 ml
- His MultiTrap™ FF 96 well filter plate
- HiScreen™ Ni FF
- HisPrep™ FF 16/10 columns, 20 ml
- His GraviTrap™ gravity-flow columns, 1 ml

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Please read these instructions carefully before using the products.

Safety

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

1 Product description

Ni Sepharose 6 Fast Flow is highly stable and compatible with a wide range of common additives. This helps to 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*. A variety of compounds that are compatible with Ni Sepharose 6 Fast Flow are listed in *Table 2*.

Table 1. Medium characteristics

Matrix	Highly cross-linked spherical agarose, 6%
Dynamic binding capacity [1]	Approx. 40 mg (histidine)$_6$-tagged protein/ml medium
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 column 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 (for medium without metal ion)	Stable in: 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 min.
pH stability [3] (for medium without metal ion)	
Working range	3 to 12
Cleaning-in-place	2 to 14
Storage	20% ethanol
Storage temperature	4°C to 30°C

[1] Dynamic binding capacity conditions:

Sample:	1 mg/ml (histidine)$_6$-tagged pure protein (M_r 43 000) in binding buffer (capacity at 10% breakthrough) or (histidine)$_6$-tagged protein (Mr 28 000) bound from *E. coli* extract
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, pH 7.4
Elution buffer:	20 mM sodium phosphate, 0.5 M NaCl, 500 mM imidazole, pH 7.4

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

[2] H_2O at room temperature

[3] Working range: pH interval where the medium can be handled without significant change in function.

Cleaning-in-place: pH interval where the medium can be subjected to cleaning-in-place without significant change in function.

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

Reducing agents [1]	5 mM DTE 5 mM DTT 20 mM β-mercaptoethanol 5 mM TCEP 10 mM reduced glutathione
Denaturing agents [2]	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_2SO_4 1.5 M NaCl 1 mM EDTA [3] 60 mM citrate
Buffer	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] Ni Sepharose 6 Fast Flow is compatible with reducing agents. However, for optimal performance, removal of any weakly bound Ni^{2+} ions by performing a blank run without reducing agents (as described in Section *Blank run, on page 15*.) before applying buffer/sample including reducing agents is recommended. Do not leave Ni Sepharose 6 Fast Flow columns with buffers including reducing agents when not in use.

[2] Tested for one 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 in 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.

2 General considerations

Introduction

This section describes important information that should be considered when using Ni Sepharose 6 Fast Flow in order to achieve the best results. The actions for minimizing nickel leakage and discoloring are normally not needed but can be performed for sensitive applications.

Imidazole concentration

The recommended binding buffer is:

- 20 mM sodium phosphate, 500 mM NaCl, **20 to 40 mM imidazole**, pH 7.4

The imidazole concentration in sample and binding buffer can be further increased if there is a need for higher final purity. If, on the other hand, there is a need for higher yield the imidazole concentration can be lowered (this may result in lower final purity).

Minimize nickel-ion leakage

- Leakage of Ni-ions from Ni Sepharose 6 Fast Flow is very low under all normal conditions. For applications where extremely low leakage during purification is critical, leakage can be diminished by performing a blank run. See Section *Blank run, on page 15*.
- Use binding and elution buffers without reducing agents.

Reduce discoloring when reducing agents are used

Ni Sepharose 6 Fast Flow is compatible with reducing agents as listed in *Table 2*. Discoloring is always seen when using high concentrations of reducing agents. In most cases this does not affect the performance of the chromatography medium. To minimize the discoloring, perform a blank run using buffers without reducing agents before the purification. See Section *Blank run, on page 15*.

Table 3. Prepacked columns for desalting

Column	Code No.	Loading volume	Elution volume	Comments	Application
HiPrep™ 26/10 Desalting	17-5087-01	2.5 to 15 ml	7.5 to 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 to 1.5 ml	1.0 to 2.0 ml	Prepacked with Sephadex G-25 Superfine. Requires a syringe or pump to run.	
PD-10 Desalting	17-0851-01	1.0 to 2.5 ml[1] 1.75 to 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 to 0.5 ml[1] 0.2 to 0.5 ml[2]	1.0 ml[1] up to 0.5 ml[2]		
PD MidiTrap™ G-25	28-9180-08	0.5 to 1.0 ml[1] 0.75 to 1.0 ml[2]	1.5 ml[1] up to 1.0 ml[2]		

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

3 Recommended columns and packing parameters

Ni 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 4. Recommended lab-scale columns forNi 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	1.0	4.7	2
Tricorn 10/50	1.0	4.7	2
Tricorn 10/100	1.0	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 *Section 15 Ordering information, on page 26*.

Table 5. Recommended process-scale columns for Ni Sepharose 6 Fast Flow

Column	**Inner diam. (mm)**	**Bed volume (l)**	**Bed height max (cm)**
BPG™ 100/500	100	up to 2.0 l	26
BPG 140/500	140	up to 4.0 l	26
BPG 200/500	200	up to 8.2 l	26
BPG 300/500	300	up to 18.0 l	26
BPG 450/500	450	up to 36.0 l	23
Chromaflow™ 400/100–30	400	13–37 l	30
Chromaflow 600/100–300	600	28–85 l	30

4 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 motion. Pouring the slurry down a glass rod held against the column wall will minimize the introduction of air bubbles.
4	If using a packing reservoir, immediately fill the remainder of the column and reservoir with water. Mount the adapter or lid of the packing reservoir and connect the column to a pump. Avoid trapping air bubbles under the adapter or in the inlet tubing.
5	Open the bottom outlet of the column and set the pump to run at the desired flow rate. Ideally, Sepharose 6 Fast Flow 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 step. • 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 1.0 ml/min (Tricorn 10/100 column) in the first step, and 8.7 ml/min (XK 16/20 column) or 4.7 ml/ min (Tricorn 10/100 column) in the second step. See *Table 4* 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 wellpacked bed. ***Note:*** *For subsequent chromatography procedures, do not exceed 75% of the packing flow rate. See Table 4 for flow rates for chromatography.*

Step	Action
6	Maintain packing flow rate for at least 3 bed volumes after a constant bed height is reached. Mark the bed height on the column.
7	Stop the pump and close the column outlet.
8	If using a packing reservoir, 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.

5 Packing process-scale columns

For general process-scale column packing instructions, please visit support section at www.gelifesciences.com

6 Evaluation of column packing

Intervals

Test the column efficiency to check the quality of packing. Testing should be done after packing and at regular intervals during the working life of the column and also when separation performance is seen to deteriorate.

Column efficiency testing

The best method of expressing the efficiency of a packed column is in terms of the height equivalent to a theoretical plate (HETP) and the asymmetry factor (A_S). These values are easily determined by applying a sample such as 1% acetone solution to the column. Sodium chloride can also be used as a test substance. Use a concentration of 0.8 M NaCl in water as sample and 0.4 M NaCl in water as eluent.

For more information about column efficiency testing, consult the application note *Column efficiency testing* (28-9372-07).

Note: *The calculated plate number will vary according to the test conditions and it should only be used as a reference value. It is important that test conditions and equipment are kept constant so that results are comparable. Changes of solute, solvent, eluent, sample volume, flow velocity, liquid pathway, temperature, etc. will influence the results.*

Sample volume and flow velocity

For optimal column efficiency results, the sample volume should be approximately 1% of the column volume and the flow velocity 30 cm/h. If an acceptance limit is defined in relation to column performance, the column plate number can be used as one of the acceptance criteria for column use.

Method for measuring HETP and A_s

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

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

L = bed height (cm)
N = number of theoretical plates

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

V_R = volume eluted from the start of sample application to the peak maximum
W_h = peak width measured as the width of the recorded peak at half of the peak height
V_R and W_h are in the same units

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

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

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

d_{50v} = mean diameter of the beads (cm)

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

The peak should be symmetrical, and the asymmetry factor as close to 1 as possible (a typical acceptable range could be $0.8 < A_S < 1.8$).

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

Peak asymmetry factor calculation:

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

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

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

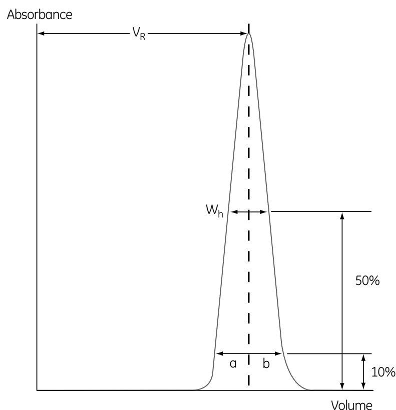

Fig 1. A typical test chromatogram showing the parameters used for HETP and A_s calculations.

7 Preparation before purification

General recommendations

We recommend binding at neutral to slightly alkaline pH (pH 7 to 8) in the presence of 0.5 to 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. Addition of salt, for example 0.5 to 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.

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

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, protein unfolding generally takes place. 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. See Table 3 for column selection.*

As an alternative to imidazole elution, histidine-tagged proteins can be eluted from the medium by several other methods or combinations of methods. Lowering pH within the range 2.5 to 7.5 can be used, for example. At pH values below 4, metal ions will be stripped off the medium.

EGTA and EDTA can be used for elution. Chelating agents such as EGTA and EDTA cause protein elution by stripping the metal ions from the medium. The target protein pool will then include Ni^{2+} ions.

Elution with ammonium chloride or histidine has also been reported.

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 of imidazole 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. This can be done by eluting with a linear or stepwise gradient of imidazole from 20 to 500 mM, and testing fractions for the presence of target protein and impurities. See *Section 9 Optimization, on page 18*.

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. Filter buffers through a 0.45 μm filter before use.

Use a high purity imidazole as this will give a very low or no absorbance at 280 nm.

Recommended buffers

Binding buffer: 20 mM sodium phosphate, 0.5 M NaCl, 20 to 40 mM imidazole, pH 7.4 (the optimal imidazole concentration is protein-dependent; 20 to 40 mM is suitable for many histidine-tagged proteins).

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).

Ni^{2+} leakage

Leakage of Ni^{2+} from Ni Sepharose 6 Fast Flow is low under all normal conditions. In order to reduce leakage even further it is recommended to perform a blank run or alternatively perform an acidic wash. Such a treatment is intended to remove any weakly bound metal ions that otherwise might be desorbed later, during the elution of the bound protein.

Blank run

Use buffers **without** reducing agents.

Step	Action
1	Wash the column with 5 column volumes (CV) of distilled water.
2	Wash with 5 CV elution buffer.
3	Equilibrate with 5 to 10 CV binding buffer.

Alternative to blank run

Acidic wash is especially recommended when binding buffer contain no or low concentration of imidazole and in applications when the proteins to be purified are not histidine-tagged.

Use buffers **without** reducing agents.

Step	Action
1	Wash the column with 5 column volumes (CV) of distilled water.
2	Wash the column with 5 column volumes of an acidic buffer (for example 0.02 M sodium acetate, 0.5 to 1.0 M NaCl, pH 4.0).
3	Equilibrate the column with 2 to 5 CV binding buffer, or until the desired pH is obtained.

Sample preparation

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

The sample should be fully dissolved. To avoid column clogging, we recommend centrifugation and filtration through a 0.45 μm filter to remove cell debris or other particulate material. If the sample is dissolved in a buffer other than 20 mM phosphate buffer with 0.5 M NaCl pH 7.4, adjust its NaCl concentration to 0.5 M and pH to 7–8. Do not use strong bases or acids for pH-adjustments (precipitation risk).

Note: *To prevent binding of unwanted host cell proteins with exposed histidine, add the same concentration of imidazole to the sample as to the binding buffer (see* *Section 9 Optimization, on page 18**).*

8 Purification

Please read *Section 2 General considerations, on page 6* and *Section 7 Preparation before purification, on page 13* before performing the purification.

Step	Action
1	If the column contains 20% ethanol, wash it with 2 to 5 column volumes (CV) of distilled water. Use a linear flow rate of 50 to 100 cm/h.
2	Equilibrate the column with at least 2 CV binding buffer. Recommended linear flow rate: 150 cm/h. In some cases, a blank run or an acidic wash is recommended before final equilibration/sample application (see Section *Blank run, on page 15* or Section *Alternative to blank run, on page 16*).
3	Apply the pre-treated sample (see Section *Sample preparation, on page 16*).
4	Wash with binding buffer until the absorbance reaches the baseline.
5	Elute with elution buffer using a stepwise or linear gradient. In most process applications, it is recommended to use a stepwise gradient.

9 Optimization

Concentration of imidazole in binding/wash buffer

Imidazole at low concentrations is commonly used in the binding/wash buffer to minimize binding of unwanted host cell proteins. It is important to include imidazole also in the sample (generally, at the same concentration as in the wash buffer). 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 of the histidine-tagged protein). This optimal concentration is different for different histidine-tagged proteins. Note that Ni Sepharose 6 Fast Flow often requires a slightly higher concentration of imidazole in the binding/wash buffer than similar IMAC media on the market.

Finding the optimal imidazole concentration for a specific histidine-tagged protein is a trial-and-error effort, but 20 to 40 mM in the binding/wash buffer is a good starting point. for many proteins. Prepacked HiScreen Ni FF columns (4.6 ml, bed height 10 cm) are ideal for optimization of running conditions before scaling up. HisTrap FF columns (1 and 5 ml) can also be used for optimization.

Choice of metal ion

Ni^{2+} is usually the first choice metal ion for purifying most histidine-tagged recombinant proteins from cellular contaminants.

The strength of binding between a protein and a metal ion is affected by several factors, including the length and position of the affinity tag on the protein, the type of ion used, and the pH of buffers. Some proteins may therefore be easier to purify with ions other than Ni^{2+}, for example Zn^{2+} or Co^{2+}.

Prepacked HiTrap™ IMAC FF columns or IMAC Sepharose 6 Fast Flow (not metal-ion charged) can be used to test this possibility. These products can be charged with different metal ions, e.g., Cu^{2+}, Co^{2+}, Zn^{2+}, Ca^{2+}, Ni^{2+} or Fe^{3+}.

A study to compare the purification of six histidine-tagged recombinant proteins, including three variants of histidine-tagged maltose binding protein, with different metal ions has indicated that Ni^{2+} generally gives best selectivity between histidine-tagged proteins and nontagged host cell proteins (see Application Note 18-1145-18).

10 Troubleshooting

The following tips may be of assistance. If you have any further questions about Ni Sepharose 6 Fast Flow, please visit www.gelifesciences.com/protein-purification or contact our technical support, or your local GE representative.

Column has clogged

- Cell debris in the sample may clog the column. Clean the column according to the *Section 12 Cleaning-in-Place (CIP), on page 24*.
- Centrifuge and/or filter the sample through a 0.22 μm or a 0.45 μm filter, see Section *Sample preparation, on page 16*.

Sample is too viscous

- If the lysate is very viscous due to 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 to15 minutes. Alternatively, draw the lysate through a syringe needle several times.

Protein is difficult to dissolve or precipitates during purification

- **See *Table 2* for reducing agents, detergents, glycerol and denaturing agents that may be used.**

  Mix gently for 30 min after addition of additive 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.

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 A_{260}. Addition 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. 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 to 6 M Gua-HCl, 4 to 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 to 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, solid urea or Gua-HCl can be added.

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

- **Capacity of Ni Sepharose 6 Fast Flow is exceeded:** Increase the volume of Ni Sepharose 6 Fast Flow used for your purification.

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.
- **The protein has precipitated in the column:** Try detergents or changed NaCl concentration or elute under denaturing (unfolding) conditions (use 4 to 8 M urea or 4 to 6 M Gua-HCl) to remove precipitated proteins. For the next experiment, decrease amount of sample, or decrease protein concentration by eluting with a linear imidazole gradient instead of imidazole steps.
- **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 flowthrough material. 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-His antibodies in Western blotting.
- **Histidine-tag may be insufficiently exposed:** The protein is found in the flowthrough material. Perform purification of unfolded protein in urea or Gua-HCl as for inclusion bodies.

  To minimize dilution, solid urea or Gua-HCl can be added to the sample.
- **Buffer/sample composition is incorrect:** The protein is found in the flowthrough material. Check pH and composition of sample and binding buffer. Ensure that the concentration of chelating or strong reducing agents, as well as imidazole, in the solution 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:** Optimize imidazole concentration for binding buffer, see *Section 9 Optimization, on page 18*. If optimized conditions do not remove contaminants, further purification by ion exchange chromatography (HiTrap Q HP or HiTrap SP HP) and/or size exclusion chromatography (Superdex™ Peptide, Superdex 75 or Superdex 200 Increase) may be necessary.
- **Contaminants are associated with tagged proteins:** Add detergent and/or reducing agents before sonicating the cells. Increase the 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.

11 Regenerating the medium

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

Stripping

To remove residual Ni^{2+}, wash with 5 column volumes 20 mM sodium phosphate, 0.5 M NaCl, 50 mM EDTA, pH 7.4. Remove residual EDTA by washing with at least 5 column volumes of binding buffer followed by 5 column volumes of distilled water before recharging the column.

Recharging

To recharge the water-washed column, load 0.5 column volumes of 0.1 M $NiSO_4$ in distilled water. Salts of other metals, chlorides or sulfates, may also be used (see *Section 9 Optimization, on page 18*).

Wash with 5 column volumes of distilled water followed by 5 column volumes of binding buffer (to adjust pH) before storage in 20% ethanol.

In some applications, substances such as denatured proteins or lipids cannot be eluted in the regeneration. These can be removed by Cleaning-in- Place.

12 Cleaning-in-Place (CIP)

When reduced performance or an increase in back-pressure are noted, the column should be cleaned. Before cleaning, strip off the Ni^{2+} ions using the recommended procedure (see *Section 11 Regenerating the medium, on page 23*). Use reversed flow direction for cleaning.

After cleaning, store in 20% ethanol or recharge with Ni^{2+} prior to storage in ethanol.

The Ni^{2+} stripped column can be cleaned by the following methods:

CIP protocols

Ionically bound proteins	Wash with several column volumes of 1.5 M NaCl. Then wash with several column volumes of distilled water.
Precipitated proteins, hydrophobically bound proteins, and lipoproteins	Wash the column with 1 M NaOH, contact time usually 1 to 2 hours (12 hours or more to remove endotoxins). Then wash with approximately 10 column volumes of binding buffer, followed by 10 column volumes of distilled water.
Hydrophobically bound proteins, lipoproteins and lipids	Wash with 5 to 10 column volumes of 30% isopropanol for about 15 to 20 minutes. Then wash with approximately 10 column volumes of distilled water. Alternatively, wash with 2 column volumes of detergent in a basic or acidic solution. Use, for example, 0.1 to 0.5% nonionic detergent in 0.1 M acetic acid, contact time 1 to 2 hours. After treatment, always remove residual detergent by washing with 5 to 10 column volumes of 70% ethanol [1]. Then wash with approximately 10 column volumes of distilled water.

1 Specific regulations may apply when using 70% ethanol since the use of explosion proof areas and equipment may be required.

13 Storage

Store the medium for longer periods of time in 20% ethanol at 4°C to 30°C.

14 Further information

Check www.gelifesciences.com for further information. Several handbooks also contain useful information, see *Section 15 Ordering information, on page 26*.

15 Ordering information

Product	Quantity	Code No.
Ni Sepharose 6 Fast Flow	5 ml	17-5318-06
	25 ml	17-5318-01
	100 ml	17-5318-02
	500 ml	17-5318-03
	1 l	17-5318-04
	5 l	17-5318-05

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

Prepacked columns	Quantity	Code No.
HisTrap FF	5 × 1 ml	17-5319-01
	100 × 1 ml [1]	17-5319-02
	5 × 5 ml	17-5255-01
	100 × 5 ml [1]	17-5255-02
	1 × 20 ml	17-5256-01
HisTrap FF crude	5 × 1 ml	11-0004-58
	100 × 1 ml [1]	11-0004-59
	5 × 5 ml	17-5286-01
	100 × 5 ml [1]	17-5286-02
HiScreen Ni FF	1 × 4.7 ml	28-9782-44
His MultiTrap FF	4 × 96-well filter plates	28-4009-90
HisPrep FF 16/10	1 × 20 ml	28-9365-51
His GraviTrap	10 × 1 ml	11-0033-99

[1] Special pack delivered on specific customer order.

Empty lab-scale columns	Quantity	Code No.
Tricorn 10/20 column, 10 mm i.d.	1	28-4064-13
Tricorn 10/50 column, 10 mm i.d.	1	28-4064-14
Tricorn 10/100 column, 10 mm i.d.	1	28-4064-15
XK 16/20 column, 16 mm i.d.	1	18-8773-01
XK 26/20 column, 26 mm i.d.	1	18-1000-72
XK 50/20 column, 50 mm i.d.	1	18-1000-71
XK 50/30 column, 50 mm i.d.	1	18-8751-01

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 Product Profile	18-1121-86
Ni Sepharose and IMAC Sepharose Selection Guide	28-4073-92
Datafile BPG columns	18-1115-23
Datafile BPG 450 columns	18-1160-59
Datafile Chromaflow columns	18-1138-92

For local office contact information, visit
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