instructions

HisTrap Kit

for purification of histidine-tagged proteins

HisTrap™ Kit is an affinity chromatography kit for fast and effective purification of histidine-tagged recombinant proteins.
HisTrap Kit contains everything necessary to perform a complete purification without using complicated equipment. It also eliminates time consuming buffer preparation and column packing.

71-5020-43 Edition AA

GE imagination at work

Table of contents

Code No.	Designation	No. supplied
17-1880-01	HisTrap Kit	
	containing:	
	HiTrap Chelating HP columns	3 x 1 ml
	Phosphate buffer, 8x stock solution, pH 7.4	2 x 50 ml
	2 M Imidazole, pH 7.4	50 ml
	0.1 M Nickel sulphate	10 ml
	Syringe, 5 ml	1
	Connectors	
	Luerlock female/M6 male	1
	Luerlock female/M6 female	1
	Tubing connector flangeless/M6 male	1
	Tubing connector flangeless/M6 female	1
	Domed nut	5
	Luer adaptor female/M6 male	1
	Instruction manual	1

I. Introduction

HisTrap Kit is designed for rapid, mild affinity purification of histidine-tagged fusion proteins. Using HisTrap Kit, histidine-tagged fusion proteins can be prepared to high purity in one step. Fusion proteins can be purified directly from bacterial lysates and are recovered from the matrix under mild elution conditions which preserve antigenicity and functionality of the protein.

The kit contains three ready-to-use, disposable HiTrap™ Chelating HP columns, accessories and buffer concentrates sufficient to perform 10-12 purifications when operated with a syringe. The special design of the column, together with a modern matrix, provides fast, easy and reproducible separations in a convenient format.

II. General Considerations

HiTrap Chelating HP, when charged with Ni^{2+} ions, will selectively retain proteins if complex-forming amino acid residues, in particular histidine, are exposed on the surface of the protein.

Histidine-tagged proteins can be desorbed from HiTrap Chelating HP with buffers containing imidazole (ref. 2).

There is, however, a balance between the amount of imidazole needed to prevent non-specific binding of contaminants and the amount of imidazole needed to elute the histidine-tagged protein. When optimum purity is needed this balance is easily determined by stepwise elution of the protein using buffers containing a constant concentration of phosphate buffer and increasing concentrations of imidazole. Stepwise elution - according to the Optimization Protocol - is technically simple and fast, and it is suitable for syringe operation. The results from the stepwise elution will provide information on which two buffers are needed for future purifications of identical protein samples. When high yield is more important than optimum purity, the Basic Purification Protocol may be used directly, without prior optimisation.

If the histidine-tagged proteins are expressed as inclusion bodies, see page 14 and ref. 1 and 3 for refolding and purification information.

III. Sample Preparation

For optimal conditions for growth, induction and cell lysis conditions of your recombinant histidine-tagged clones, please refer to recommended protocols. The following is a general protocol for sample preparation:

1. Harvest the cells from the culture by centrifugation (e.g. by centrifugation at 7 000 - 8 000 x g for 10 minutes or 1 000 - 1 500 x g for 30 minutes at +4 °C).
2. Discard the supernatant. Place the bacterial pellet on ice.
3. Using a pipette, completely suspend the cell pellet by adding 50 µl of ice-cold buffer pH 7-8.5 per ml of cell culture, e.g. 1x phosphate buffer, 10 mM imidazole, pH 7.4 (Binding buffer), prepared according to Appendix B.
4. Disrupt suspended cells.

   For example sonicate on ice in short bursts. Save an aliquot of the sonicate for analysis by SDS-PAGE. Sonicate for the minimum time necessary to disrupt the cells. Prolonged sonication may destroy protein functionality. Avoid frothing as this may denature the fusion protein and can also lead to co-purification of host proteins with the fusion protein.
5. Sediment the cell debris by centrifugation (e.g. at 12 000 x g for 10 minutes at +4 °C). Carefully transfer the supernatant, without disturbing the pellet, to a fresh container.

   Save aliquots of the supernatant and the cell debris pellet for analysis by SDS-PAGE. Samples containing 8 M urea can

be electrophoresed directly, but samples containing 6 M guanidine hydrochloride must be buffer exchanged (with e.g. PD-10 column or HiTrap Desalting) against buffer containing 8 M urea before loading on a gel. These samples can be used to identify the fraction in which the fusion protein is located.

6. The sample should be fully dissolved prior to loading the column. In order to avoid clogging in the column we recommend 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 1x phosphate buffer, 10 mM imidazole, pH 7.4, the sample should be adjusted to pH 7 - 8. This can be achieved by buffer exchange on a PD-10 or HiTrap Desalting column.

7. Store the sample at -20 °C, if necessary, before use.

IV. Histidine-tagged Protein Purification Protocols

Column preparation

1. Fill the syringe with distilled water. Remove the stopper and connect the column to the syringe with the provided adaptor "drop to drop" to avoid introducing air into the column.
2. Remove the twist-off end. Wash the column with 5 ml distilled water.

   Do not use buffer to wash away the 20% ethanol solution as nickel salt precipitation can occur in the next step. If air is trapped in the column, wash the column with distilled water until the air disappears.
3. Disconnect the syringe (with luer adaptor) from the column, fill the syringe with 0.5 ml of the 0.1 M nickel salt solution ($NiSO_4$) supplied and load it on the column.
4. Wash the column with 5 ml distilled water, using the syringe.

Basic Purification Protocol

When high yield is more important than optimum purity use the following protocol. When optimum purity is required proceed to the Optimization protocol.

Use the syringe supplied in all steps.

1. Prepare 24 ml binding buffer. Mix 3 ml Phosphate buffer 8x stock solution with 0.12 ml 2 M imidazole and add water up to 24 ml. Check pH and adjust to pH 7.4-7.6 if necessary. This buffer will contain 20 mM phosphate, 0.5 M NaCl and 10 mM imidazole.

2. Prepare 8 ml elution buffer. Mix 1 ml Phosphate buffer 8x stock solution with 2 ml 2 M imidazole and add distilled water up to 8 ml. Check pH and adjust to pH 7.4-7.6 if necessary. This buffer will contain 20 mM phosphate (1x), 0.5 M NaCl and 500 mM imidazole.
3. Using the syringe, equilibrate the column with 10 ml binding buffer.
4. Apply the sample with the syringe. Collect the flow-through fraction. A pump (e.g. a peristaltic P-1 pump) is convenient for large volumes (more than 15 ml). Flow rate 1-3 ml/min.
5. Wash with 10 ml binding buffer. Collect the wash fraction.
6. Elute with 5 ml elution buffer. Avoid dilution of the eluate by collecting the eluate in 1 ml fractions.
7. Check the purification by running an aliquot of the collected samples on SDS-PAGE. The purified protein is most likely to be found in the 2nd + 3rd ml of the elution step.

Note: 500 mM imidazole has A_{280} ~0.5 (5 mm cell). Use the elution buffer as blank. If imidazole needs to be removed use HiTrap Desalting or PD-10 columns.

8. After the protein has eluted, regenerate the column by washing it with Start buffer (according to step 3).

   The column is now ready for a new purification and there is no need to reload with metal. The reuse of HiTrap Chelating HP depends on the nature of the sample and should only be performed with identical recombinant proteins, to prevent cross-contamination.

Optimization Protocol

When optimum purity is needed the following protocol for stepwise gradient elution should be used. The next time the same protein is to be purified the number of steps will be reduced to those described under Basic Protocol. Use the syringe supplied in all steps.

1. Prepare buffers according to Table 1.

Table 1. General mixing table for one purification

Imidazole concentration in buffer	Phosphate buffer 8x stock solution pH 7.4	2 M Imidazole pH 7.4	Distilled water
10 mM	3.0 ml	0.12 ml	to 24 ml
20 mM	1.0 ml	0.08 ml	to 8 ml
40 mM	1.0 ml	0.16 ml	to 8 ml
60 mM	1.0 ml	0.24 ml	to 8 ml
100 mM	1.0 ml	0.40 ml	to 8 ml
300 mM	1.0 ml	1.20 ml	to 8 ml
500 mM	1.0 ml	2.00 ml	to 8 ml

Use 1x Phosphate buffer including 10 mM imidazole as binding buffer and 6 steps ranging up to 500 mM imidazole as elution buffers. Check pH after mixing and adjust to pH 7.4-7.6 if necessary. For larger volumes and other concentrations please refer to Appendix B.

Note: The high salt concentration in the buffer stock solution may cause salt crystals to form at low temperature. The crystals will dissolve at room temperature. We therefore recommend that the buffer stock solutions to be allowed to reach room temperature before use. The formation of

salt crystals which dissolves at room temperature does not affect the performance of the product.

2. Load the column with nickel ions according to ”Column preparation”.
3. Equilibrate the column with 10 ml binding buffer (1x phosphate buffer, 10 mM imidazole, pH 7.4), using the syringe.
4. Apply the sample. Collect the flow-through fraction.
5. Wash with 10 ml binding buffer. Collect the wash fraction.
6. Start elution with 5 ml 1x Phosphate buffer containing 20 mM imidazole. Avoid dilution by collecting the eluate in 1 ml fractions.
7. Proceed with the next imidazole concentration, i.e. elute with 5 ml 1x Phosphate buffer containing 40 mM imidazole. Collect the eluate in 1 ml fractions as above.
8. Proceed with the buffers of increasing imidazole concentration, as described in steps 6 and 7. The purified protein is most likely to be found in the 2nd + 3rd ml of one of the elution steps.
9. Check the different fractions, e.g. by SDS-PAGE and/or western blotting.

Note: 500 mM imidazole has A_{280} ~0.5 (5 mm cell). Use the elution buffers as blanks. If imidazole needs to be removed use HiTrap Desalting or PD-10 columns.

The results of these experiments enable the optimum binding and elution buffers to be found. The optimum elution buffer

is the one which eluted the histidine-tagged protein. The optimum binding buffer is the one from the step before, with a lower concentration of imidazole. Using the highest possible concentration of imidazole in the binding buffer will give the highest purity of the purified protein. Use these buffers for the next purification of an identical protein.

10. After the protein has eluted regenerate the column by washing it with binding buffer (according to step 3).

    The column is now ready for a new purification and there is no need to reload with metal. The reuse of HiTrap Chelating HP depends on the nature of the sample and should only be performed with identical recombinant proteins to prevent cross-contamination.

Scaling up

Two or three HiTrap columns can be connected in series for quick scale-up of purifications. HiTrap Chelating HP is also available as 5 ml columns. See Ordering Information.

Pump operation

The purification process can also be performed using ÄKTA™, FPLC™ or a low pressure laboratory pump (e.g. a Peristaltic Pump P-1). This would be an alternative in some situations, e.g. when large sample volumes have to be passed through the column. The connectors necessary are described below. Connect the column to the pump or chromatography system using the connectors supplied. The column can be connected directly to the monitor inlet of an GE Healthcare UV monitor to minimise the dead volume. Flow rate 1 - 4 ml per minute.

Note: If a P1-pump is used a max flow rate of 1-3 ml/min can be run on a HiTrap 1 ml column packed with Sepharose HP media.

System/ equipment	Connector
Syringe	Luer connector female/M6 male
FPLC systems	Flangeless/M6 male
ÄKTA systems	M6 female/1/16" male
Low pressure system with capillary tubing	Flangeless/M6 female
Systems with luer connections	Luer connector female/M6 male Luer connector female/M6 female

V. Column Cleaning and Storage

Cleaning

To remove the nickel ions prior to recharging or storage, wash the column with 10 ml 20 mM sodium phosphate, 0.5 M NaCl, 0.05 M EDTA, pH 7.4. To remove precipitated proteins, fill the column with 1 M NaOH and incubate for 2 hours. Wash out the dissolved proteins with 10 ml water and a buffer with pH ~7 until the pH of the flow-through reaches pH ~7.

Storage

Seal the column with the stopper and the domed nut to avoid dehydration The recommended storage temperature for the kit is +4 to +8°C. For longer storage the nickel ions should be removed from the column as descibed above, and the column filled with 20% ethanol.

VI. HisTrap Kit Trouble Shooting Guide

The following tips may be of assistance. If you have any further questions about the HiTrap Chelating HP column or the HisTrap Kit, please contact your local GE Healthcare representative.

Problem:
The sample is too viscous:

- If the lysate is very viscous due to the presence of a high concentration of host nucleic acid, continue sonication until viscosity is reduced, add RNase A to a final concentration of 10 µg/ml and DNase I to 5 µg/ml, and incubate on ice for 10-15 min. Alternatively, draw the lysate through a syringe needle several times.

Problem:
The protein is difficult to dissolve or precipitates during purification:

- The following additives may be used: 2% Triton™ X-100, 2% Tween™ 20, 50 mM CHAPS, 2 M NaCl, 50% glycerol, 20 mM β-mercaptoethanol, 8 M urea or 6 M guanidine hydrochloride. Mix gently for 30 minutes to aid in solubilization of the fusion protein. Note that Triton X-100 has a high A_{280} value - furthermore Triton X-100 cannot be removed by buffer exchange.

Problem:
The column has clogged:

- Cell debris in the sample may clog the column. Clean the column according to section V.

- It is important to filter the sample through a 0.45 µm filter, according to Sample preparation.

Problem:
No histidine-tagged protein in the purified fractions

SDS-PAGE analysis of samples collected during the preparation of the bacterial sonicate may indicate that the majority of the fusion protein is located in the post-sonicate pellet. Possible causes and solutions are:

- Sonication may be insufficient: Cell disruption may be checked by microscopic examination or monitored by measuring the release of nucleic acids at A_{260}. Addition of lysozyme (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 as this may denature the fusion protein. Over-sonication can also lead to co-purification of host proteins with the fusion protein.
- The protein may be insoluble (inclusion bodies): The protein can usually be solubilised from inclusion bodies using common denaturants such as 4-6 M guanidine hydrochloride, 4-8 M urea or detergents.
  Prepare buffers containing 20 mM sodium phosphate, 8 M urea or 6 M guanidine hydrochlorine, and different imidazole concentrations ranging from 10 mM to 500 mM pH 7.4-7.6. Use these buffers for Sample preparation, as binding buffer and as elution buffer. For sample preparation use the 10 mM imidazole concentration or the concentration selected in the Optimization Protocol. In this case step 4 (Disrupt suspended

cells) in Sample Preparation can be replaced by mixing for 1 hour, room temperature.
For purification follow the Basic Purification Protocol or the Optimization Protocol, using these buffers.

- The elution conditions are too mild: Elute with an increasing imidazole gradient or decreasing pH to determine the optimal elution conditions.
- The concentration of imidazole is too high in the binding buffer: The protein is found in the flow-through material. Decrease the imidazole concentration in the binding buffer.
- The protein has precipitated in the column: Elute under denaturing conditions (add 4-8 M urea or 4-6 M guanidine hydrochloride).
- Nonspecific hydrophobic interaction: Add a non-ionic detergent to the elution buffer (e.g. 0.2% Triton X-100) or increase the NaCl concentration.

Problem:
The protein is collected but is not pure (multiple bands on SDS-PAGE)

- Partial degradation of fusion proteins by proteases: Add a protease inhibitor.
- Not effective enough washing: Elute with a stepwise imidazole gradient to determine optimal conditions.
- Contaminants are associated with tagged proteins: 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 non-specific interactions. Add β-mercaptoethanol to a maximum of 20 mM to reduce disulphide links.

- Contaminants have high affinity for nickel ions: Add imidazole to the sample in the same concentration as the start buffer. If optimized washing conditions do not remove contaminants, further purification by ion exchange (HiTrap Q HP or HiTrap SP HP) or gel filtration (Superdex™ Peptide, Superdex 75 or Superdex 200) may be useful.

Problem:
The histidine-tagged protein elutes during the wash

- The buffer composition is incorrect: Check pH and composition of binding buffer according to the buffer table. Ensure that there are no chelating or reducing agents present in the sample, and that the concentration of imidazole is not too high.
- The histidine tag is partially hidden: Purify under denaturing conditions (add 4-8 M urea or 4-6 M guanidine hydrochloride).
- The column capacity is exceeded: Join 2-3 HiTrap Chelating HP 1 ml columns together or change to a HiTrap Chelating HP 5 ml column.
- The column is incorrectly prepared: Prepare the column according to Column preparation.

VII. Ordering Information

Designation	No. Supplied	Code No.
HisTrap Kit	1 kit	17-1880-01
HiTrap Chelating HP, 1 ml	5 x 1 ml	17-0408-01
HiTrap Chelating HP, 5 ml	1 x 5 ml	17-0409-01
HiTrap Desalting, 5 ml	5 x 5 ml	17-1408-01
HiTrap SP HP, 1 ml	5 x 1 ml	17-1151-01
HiTrap SP HP, 5 ml	5 x 5 ml	17-1152-01
HiTrap Q HP, 1 ml	5 x 1 ml	17-1153-01
HiTrap Q HP, 5 ml	5 x 5 ml	17-1154-01
PD-10 Disposible Column	30	17-0851-01

Accessories

Designation	No. Supplied	Code No.
Domed nut*	4	18-2450-01
Union Luerlock		
female/M6 female*	2	18-1027-12
female/M6 male*	2	18-1027-62
Tubing connector		
flangeless/M6 female*	2	18-1003-68
flangeless/M6 male*	2	18-1017-98
To connect columns with M6 connections to ÄKTAdesign:		
Union M6 female /1/16" male	5	18-3858-01
Union 1/16” female/M6 male	6	18-1112-57
Recombinant Protein Handbook,		
Protein Amplification and Simple Purification	1	18-1142-75
Affinity Chromatography Handbook,		
Principles & Methods	1	18-1022-29
Convenient Protein Purification,		
HiTrap Column Guide	1	18-1129-81
Affinity Chromatography Columns and Media		
Product Profile	1	18-1121-86

* included in HisTrap Kit

VIII. References

1. Colangeli R., *et al.* Three-step purification of lipopolysaccaride-free poly-histidine-tagged recombinant antigens of Myobacterium tuberculosis, *J. of Chromatography B* 714 (1998), 223-235.
2. Purification of Poly (His)-tagged Recombinant Proteins using HisTrap Kit, 18-1116-26, GE Healthcare.
3. Rapid and efficient purification and refolding of a $(His)_6$-tagged recombinant protein produced in *E.coli* as inclusion bodies, 18-1134-37, GE Healthcare.

Appendix A

Description & Characteristics

Column

HiTrap Chelating HP is packed with Chelating Sepharose™ High Performance. The column is made of medical grade polypropylene, which does not interact with biomolecules. Each column is delivered with a stopper on the inlet and a twist-off end on the outlet. Both ends have M6 connections (6 mm metric threads). The columns have porous top and bottom frits which allow high flow rates. Separations can be easily performed using the syringe supplied or alternatively, a low pressure laboratory pump, e.g. Peristaltic pump P-1.

Note: To prevent leakage it is essential to ensure that the adaptor is tight.

Matrix

Chelating Sepharose High Performance consists of highly cross-linked agarose beads to which imino-diacetic acid has been coupled by stable ether groups via a seven-atom spacer arm. This coupling technique gives both high capacity and high performance. The matrix is stable over the pH range 3-13, and tolerates all commonly used aqueous buffers and denaturants, such as 6 M guanidine hydrochloride, 8 M urea, and chaotropic salts.

Table 1. Characteristics of HiTrap Chelating HP 1 ml

Column volume	1 ml
Bed dimensions	0.7 x 2.5 cm
Chelating group	Imino-diacetic acid
Dynamic binding capacity	Approx. 12 mg pure (His)6-tagged protein (mw ~27,600) per column***
Average bead size	34 µm
Bead structure	Highly cross-linked spherical agarose
Maximum flow rate	4 ml/min
Recommended flow rate:	1-4 ml/min
Maximum pressure	0.3 MPa, 3 bar
Chemical stability	Stable in all commonly used aqueous
buffers and denaturants such as 6 M chaotropic salts.	guanidine hydrochloride, 8 M urea, and
pH stability :	
Short term*	2-14
Long term**	3-13
Storage	20% ethanol (antimicrobial agent)
Avoid (during purification)	Chelating agents (e.g., EDTA, EGTA) Reducing agents (e.g. DTT, DTE)

* Refers to the pH interval for cleaning (not loaded with metal).

** Refers to the pH interval where the gel is stable over a long period of time without adverse effects on its subsequent chromatographic performance (not loaded with metal).

*** Running conditions: Start buffer: 20 mM sodium phosphate, 0.5 M NaCl, pH 7.4; Elution buffer: 20 mM sodium phosphate, 0.5 M NaCl, 500 mM Imidazole, pH 7.4. Flow rate: 4 ml/min

Buffers

The buffers and the metal solution have been prepared using the highest quality salts and water and have been filtered through a 0.45 µm filter.

Note: The high salt concentration in the buffer stock solution may cause salt crystals to form at low temperature. The crystals will dissolve at room temperature. We therefore recommend that the buffer stock solutions to be allowed to reach room temperature before use. The formation of salt crystals which dissolves at room temperature does not affect the performance of the product.

Appendix B

Buffers, General Mixing Table for 50 ml Buffer

Imidazole concentration in buffer mM	Phosphate buffer 8x stock solution pH 7.4 ml	2 M Imidazole pH 7.4 ml	Distilled water ml
0	6.25	0	to 50 ml
10	6.25	0.25	to 50 ml
20	6.25	0.50	to 50 ml
30	6.25	0.75	to 50 ml
40	6.25	1.00	to 50 ml
50	6.25	1.25	to 50 ml
60	6.25	1.50	to 50 ml
70	6.25	1.75	to 50 ml
80	6.25	2.00	to 50 ml
90	6.25	2.25	to 50 ml
100	6.25	2.50	to 50 ml
150	6.25	3.75	to 50 ml
200	6.25	5.00	to 50 ml
250	6.25	6.25	to 50 ml
300	6.25	7.50	to 50 ml
400	6.25	10.00	to 50 ml
500	6.25	12.50	to 50 ml

To obtain the imidazole concentration indicated in the left-hand column, mix Phosphate buffer 8x stock solution, 2 M imidazole and distilled water according to the table. Check pH and adjust to pH 7.4 - 7.6 if necessary. These buffers will contain 20 mM phosphate, 0.5 M NaCl and the concentrations of imidazole indicated. For one purification prepare 24 ml of the binding buffer and 8 ml of each elution buffer.

Denaturing conditions

The buffers described above refer to native conditions. For denaturing conditions use 20 mM sodium phosphate, 8 M urea or 6 M guanidine hydrochloride and imidazole concentrations in the range 10 mM - 500 mM pH 7.4.

Buffers recipes

If larger volumes of solutions are needed than supplied in the kit they can be prepared as follows:

8x Phosphate buffer, pH 7.4

To 1.42 g $Na_2HPO_4 \cdot 2H_2O$ (177.99 g/mol), 1.11 g $NaH_2PO_4 \cdot H_2O$ (137.99 g/mol) and 23.38 g NaCl (58.44 g/mol), add distilled water to 90 ml and dissolve completely. Adjust pH, to 7.4. Add distilled water to 100 ml and filter through a 0.45 µm filter.

2 M Imidazole, pH 7.4

To 13.62 g imidazole (68.08 g/mol) add distilled water to 90 ml and dissolve completely. Adjust pH to 7.4. Add distilled water to 100 ml and filter through a 0.45 µm filter.

0.1 M $NiSO_4$

To 2.63 g $NiSO_4 \cdot 6H_2O$ (262.86 g/mol) add distilled water to 100 ml and dissolve completely. Filter through a 0.45 µm filter.

Important Information

GE Healthcare Bio-Sciences AB
SE-751 84 Uppsala
Sweden

GE Healthcare UK Ltd
GE Healthcare Place, Little Chalfont
Bucks HP7 9NA
England

GE Healthcare Bio-Sciences Inc
P.O. Box 1327
Piscataway, NJ 08855
USA

GE Healthcare Europe GmbH
Postfach 5480
D-79021 Freiburg
Germany

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

Produced by Aviator, printed by Wikströms, Sweden 1010336, 02.2001
Printed matter. Licence 341 051