

# Purification of Poly(His)-tagged Recombinant Proteins using HisTrap

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## Abstract

Recombinant proteins engineered to have a poly(His) tail at either the carboxyl or amino terminus can easily be purified using HisTrap™ purification kit. The basic method can easily be modified to give optimal results for proteins with slightly different contents of histidine.

HisTrap contains three prepacked HiTrap™ Chelating 1 ml columns and all necessary accessories, including buffers, to perform specific purifications of histidine tagged proteins easily and conveniently.

This work presents purifications of both cytoplasmatic and periplasmatic expressed proteins with (His)<sub>10</sub> or (His)<sub>6</sub> tails under native or denaturing conditions. The purified material was characterised by SDS-PAGE, ELISA and Western Blot. All purified histidine tagged proteins were immunological active and >90% pure. The purifications could be performed within 15 minutes without using complicated instrumentation. The column was operated using a simple syringe or a low pressure pump.

The binding capacity for pure proteins with a (His)<sub>6</sub> tail is ~12 mg/ml gel, at both 1 ml/min and 4 ml/min. Five repetitive purifications of Glutathione S-transferase-(His)<sub>6</sub> at 0.5 ml/min and 4 ml/min (without re-loading the column with Ni<sup>2+</sup>) showed very similar results. The column could be run at different flow rates, and re-used to give the same good results.

The binding capacity was not sensitive to low levels of expression. Sample concentrations between 0.03 - 3.1 mg/ml of the tagged protein, result in recoveries of 60 - 71%.

## Introduction

HiTrap Chelating, when charged with Ni<sup>2+</sup> ions, will selectively bind proteins if complex-forming amino acid residues, in particular histidine, are exposed on the surface of the protein, e.g. poly-histidine tagged recombinant proteins.

Using HisTrap, 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, using imidazole, which preserve antigenicity and functionality of the protein.

The purifications can be performed with the same good results under both native and denaturing conditions, e.g. purification of inclusion bodies in 8 M urea or 6 M guanidine hydrochloride.

The properties of the matrix give the opportunity to perform a complete purification in just 10-15 minutes maintaining high selectivity and binding capacity. No special equipment is needed, just a syringe is enough to perform a purification.



## Material & Methods

Column:	HiTrap Chelating, 1 ml
Samples:	Different <i>E.coli</i> extracts, pH 7.4 containing poly-histidine tagged recombinant proteins, filtered through a Millipore 0.45 µm filter, SBHA025SB
8 x Binding Buffer:	0.16 M phosphate buffer, 4 M NaCl pH 7.4
Elution Buffer:	2 M imidazole pH 7.4
Nickel solution:	0.1 M NiSO <sub>4</sub> x 6 H <sub>2</sub> O
Buffer pretreatment:	Dilution 1:8 of Binding Buffer, and addition of 2 M imidazole according to protocol

### Loading of Ni<sup>2+</sup> to the column using a syringe

1. Wash the column with 5 ml distilled water
2. Load 0.5 ml 0.1 M NiSO<sub>4</sub>
3. Wash the column with 5 ml distilled water
4. Wash with 10 ml Binding Buffer

## Characteristics of HisTrap

<b>Columns</b>	3 x HiTrap Chelating, 1 ml
Chelating group:	Imino-diacetic acid
Binding capacity:	~12 mg pure (His) <sub>6</sub> -tagged protein
Bead structure:	Highly cross-linked spherical agarose, 34 µm particle size
Recommended flow rate:	1-4 ml/min, 156-624 cm/h
Maximum back pressure:	3 bar, 0.3 MPa, 43 psi
pH stability, short term:	pH 2-14

### Buffers

Phosphate buffer, 8 x stock solution, pH 7.4	2 x 50 ml
2 M Imidazole, pH 7.4	50 ml
0.1 M Nickel sulphate	10 ml

Connectors, syringe, instructions

## Analysis

### Concentration determination

Absorbance measurement at 280 nm using Ultrospec Plus, Amersham Pharmacia Biotech

Maltose Binding Protein, MBP-(His)<sub>6</sub>, A<sub>280</sub><sup>1%</sup>: 14.8

Glutathione S-transferase, GST-(His)<sub>6</sub>, A<sub>280</sub><sup>1%</sup>: 14.8

### Purity check

#### SDS-PAGE electrophoresis

Gel:	PhastGel™ Gradient 10-15
Sample pretreatment:	Dilution 1:5 with 15% SDS, 60 mM Tris™, 6 mM EDTA, 0.06% Bromophenol Blue, pH 8.0 Heating, 3 min, 90°C
Sample volume:	1 µl
Molecular weight standard:	Low Molecular Weight Calibration Kit (LMW), Amersham Pharmacia Biotech
Staining:	Silver, according to the manufacturer's standard protocol
Instrumentation:	PhastSystem™, Amersham Pharmacia Biotech

### Activity and Identity check

#### Western Blotting for MBP-(His)<sub>6</sub> or ScFv-(His)<sub>6</sub>

Gel:	PhastGel Gradient 10-15 (SDS-PAGE, 60 Vh)
Nitrocellulose paper:	Schleicher & Schnell, BA85
Transfer buffer:	25 mM Tris, 192 mM glycine, 20% methanol pH 8.3
Blotting:	Electroblotting according to the manufacturer's instructions, 6 Vh, ~20 min
Washing buffer:	PBS, 0.05% Tween™ 20 pH 7.4
Blocking Buffer:	15 g non-fat dry milk in 500 ml PBS pH 7.4
Primary antibody:	MBP-(His) <sub>6</sub> : Rabbit-anti-MBP, dilution 1:400, New England BioLabs, 800-30S ScFv-(His) <sub>6</sub> : Mouse anti-9E10, dilution 1:1000, Amersham Pharmacia Biotech
Secondary antibody:	MBP-(His) <sub>6</sub> : Mouse anti-rabbit IgG alkaline phosphatase, dilution 1:1000, Sigma A-2556 ScFv-(His) <sub>6</sub> : Goat anti-mouse IgG alkaline phosphatase, dilution 1:1000, Sigma A-7434
Substrate:	BCIP/NBT, Sigma B-5655
Instrumentation:	PhastSystem with PhastTransfer™

#### ELISA for ScFv-(His)<sub>6</sub>

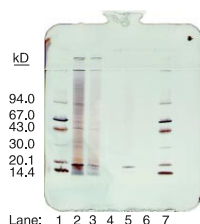
Microtiter plate:	Polyvinyl microtiter plate, Falcon 3912
Coating buffer:	0.05 M sodium carbonate pH 9.6
Washing buffer:	PBS, 0.05% Tween 20 pH 7.4
Blocking Buffer:	15 g non-fat dry milk in 500 ml PBS pH 7.4
Antigen:	10 µg/ml bovine FITC-albumin, Sigma L-9771
Primary antibody:	4 µg/ml Mouse anti-9E10, Amersham Pharmacia Biotech
Secondary antibody:	Goat anti-mouse IgG alkaline phosphatase, dilution 1:2000, Sigma A-7434
Substrate:	pNPP, Sigma N-2770

## Purification under denaturing conditions

Samples:	8 ml cell extract containing (His) <sub>10</sub> -tagged protein
Binding Buffers:	1 x Binding Buffer, 100 mM imidazole, 8 M urea <b>or</b> 6 M guanidine hydrochloride pH 7.4
Elution Buffers:	1 x Binding Buffer, 500 mM imidazole, 8 M urea <b>or</b> 6 M guanidine hydrochloride pH 7.4
Flow rate:	~4 ml/min, ~624 cm/h
Instrumentation:	Syringe

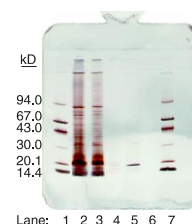
### SDS-PAGE, Purification using 8 M Urea

Lane 1:	LMW
Lane 2:	Starting material, cell extract, dil. 1:20
Lane 3:	Flow-through, dil. 1:10
Lane 4:	Wash
Lane 5:	Elution Ia (two first mls)
Lane 6:	Elution Ib (two last mls)
Lane 7:	LMW



### SDS-PAGE, Purification using 6 M Guanidine hydrochloride

Lane 1:	LMW
Lane 2:	Starting material, cell extract, dil. 1:10
Lane 3:	Flow-through
Lane 4:	Wash
Lane 5:	Elution Ia (two first mls)
Lane 6:	Elution Ib (two last mls)
Lane 7:	LMW



## Purification under native conditions

### Cytoplasmic cell extract

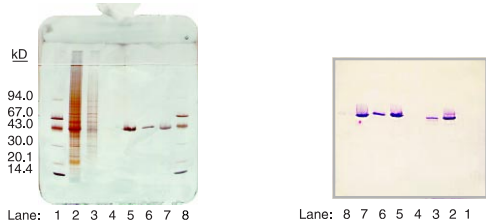
Sample: 1 ml cytoplasmic cell extract containing MBP-(His)<sub>6</sub>  
 Binding Buffer: 1 x Binding Buffer, 10 mM imidazole pH 7.4  
 Elution Buffer: 1 x Binding Buffer, 300 mM imidazole pH 7.4  
 Flow rate: ~4 ml/min, ~624 cm/h  
 Instrumentation: Syringe

### SDS-PAGE and Western Blotting

Lane 1: LMW  
 Lane 2: Starting material, cytoplasmic cell extract, dil. 1:10  
 Lane 3: Flow-through, dil. 1:10  
 Lane 4: Wash  
 Lane 5: Eluted MBP-(His)<sub>6</sub>  
 Lane 6: Eluted MBP-(His)<sub>6</sub>, dil. 1:5  
 Lane 7: MBP standard, 0.3 mg/ml  
 Lane 8: LMW

### Result:

Eluted MBP-(His)<sub>6</sub>, 4 ml, A<sub>280</sub>: 0.52  
 Total amount: 1.4 mg



### Periplasmic cell extract

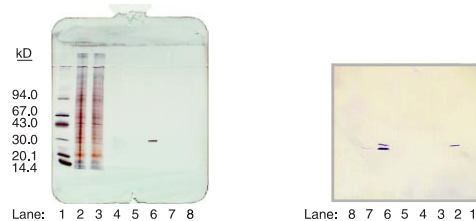
Sample: 30 ml periplasmic cell extract containing ScFv-(His)<sub>6</sub>  
 Binding Buffer: 1 x Binding Buffer, 10 mM imidazole pH 7.4  
 Elution Buffers: 1) 1 x Binding Buffer, 60 mM imidazole pH 7.4  
 2) 1 x Binding Buffer, 100 mM imidazole pH 7.4  
 3) 1 x Binding Buffer, 200 mM imidazole pH 7.4  
 4) 1 x Binding Buffer, 300 mM imidazole pH 7.4

Flow rate: ~4 ml/min, ~624 cm/h

Instrumentation: Syringe

### SDS-PAGE and Western Blotting

Lane 1: LMW  
 Lane 2: Starting material, periplasmic cell extract  
 Lane 3: Flow-through  
 Lane 4: Wash  
 Lane 5: Elution with 1 x Binding Buffer, 60 mM imidazole pH 7.4  
 Lane 6: " 1 x Binding Buffer, 100 mM imidazole pH 7.4  
 Lane 7: " 1 x Binding Buffer, 200 mM imidazole pH 7.4  
 Lane 8: " 1 x Binding Buffer, 300 mM imidazole pH 7.4

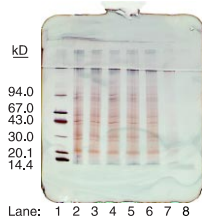


### Periplasmic cell extract

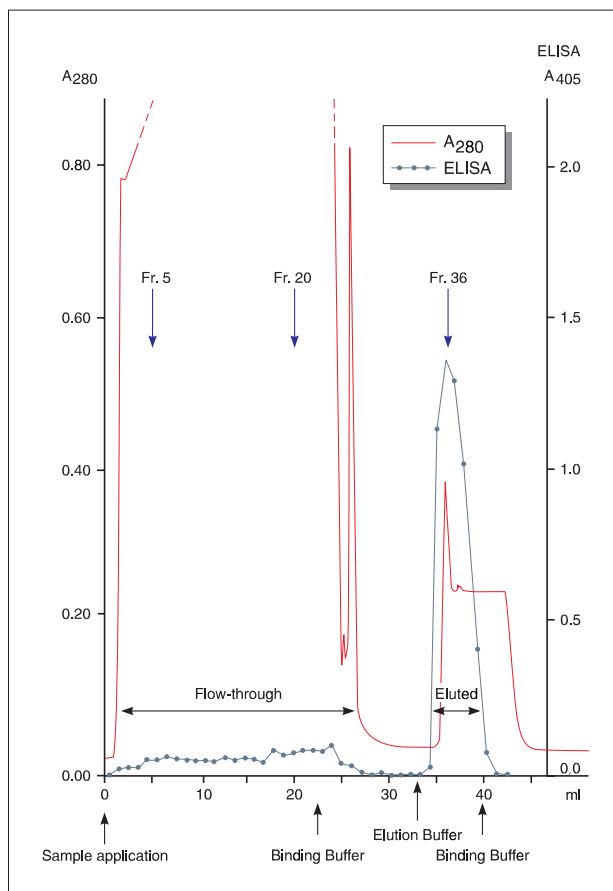
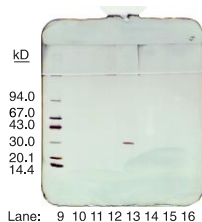
Sample: 23 ml periplasmic cell extract containing ScFv-(His)<sub>6</sub>  
 Binding Buffer: 1 x Binding Buffer, 10 mM imidazole pH 7.4  
 Elution Buffer: 1 x Binding Buffer, 200 mM imidazole pH 7.4  
 Flow rate: 1 ml/min, 156 cm/h  
 Instrumentation: FPLC™ System with FPLCdirector™

### SDS-PAGE

Lane 1: LMW  
 Lane 2: Starting material, cell extract  
 Lane 3: Fraction 5  
 Lane 4: " 10  
 Lane 5: " 15  
 Lane 6: " 20  
 Lane 7: " 25  
 Lane 8: " 26



Lane 9: LMW  
 Lane 10: Fraction 28  
 Lane 11: " 34  
 Lane 12: " 35  
 Lane 13: " 36  
 Lane 14: " 37  
 Lane 15: " 38  
 Lane 16: " 39



## Binding capacity at different flow rates

Samples: 1) 16.1 mg/run pure MBP-(His)<sub>6</sub>  
 2) 16.4 mg/run pure GST-(His)<sub>6</sub>

Binding Buffer: 1 x Binding Buffer pH 7.4

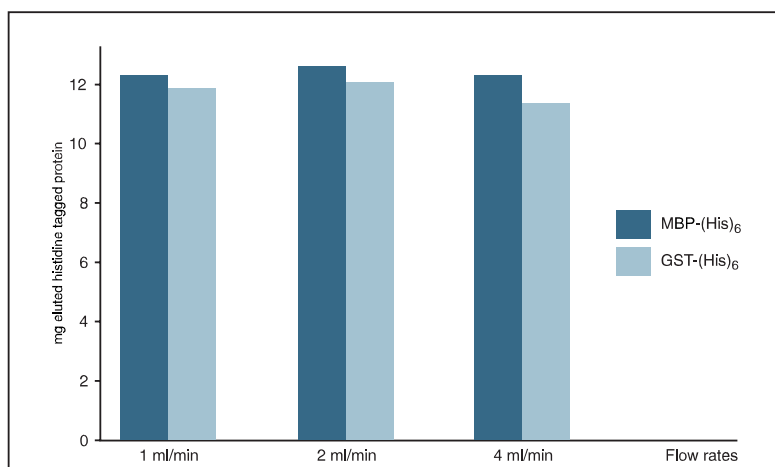
Elution Buffer: 1 x Binding Buffer, 300 mM imidazole pH 7.4

Flow rates: 1) 1 ml/min, 156 cm/h  
 2) 2 ml/min, 312 cm/h  
 3) 4 ml/min, 624 cm/h

### Result:

	1 ml/min	2 ml/min	4 ml/min
Eluted MBP-(His) <sub>6</sub>	12.3 mg	12.6 mg	12.3 mg
Eluted GST-(His) <sub>6</sub>	11.9 mg	12.1 mg	11.4 mg

Instrumentation: FPLCSystem with FPLCdirector



## Dependence of binding on sample concentration

Samples: 1 ml pure MBP-(His)<sub>6</sub>, diluted to different loading concentrations with Binding Buffer pH 7.4

1) 1 ml 3.1 mg/ml  
 2) 2 ml 1.6 mg/ml  
 3) 10 ml 0.31 mg/ml  
 4) 50 ml 0.06 mg/ml  
 5) 100 ml 0.03 mg/ml

Total amount MBP-(His)<sub>6</sub> loaded/run: 3.1 mg

### Result:

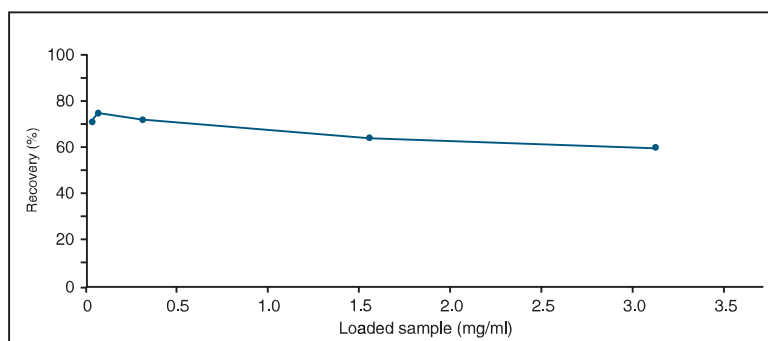
Loading sample volume, ml	Loading sample conc. mg/ml	Eluted ml	Eluted A <sub>280</sub>	Eluted MBP-(His) <sub>6</sub> total mg
1	3.1	5.8	0.482	1.9
2	1.6	5.8	0.514	2.0
10	0.31	5.8	0.578	2.3
50	0.06	5.8	0.599	2.4
100	0.03	5.8	0.566	2.2

Binding buffer: 1 x Binding Buffer pH 7.4

Elution buffer: 1 x Binding Buffer, 300 mM imidazole pH 7.4

Flow rate: 1 ml/min, 156 cm/h

Instrumentation: FPLCSystem with FPLCdirector



## Stability of the Ni<sup>2+</sup>-loaded HiTrap Chelating column

### 5 repetitive purifications of GST-(His)<sub>6</sub> at two different flow rates

Samples: 10 ml GST-(His)<sub>6</sub> cell extract  
 Binding buffer: 1 x Binding Buffer, 20 mM imidazole pH 7.4  
 Elution buffer: 1 x Binding Buffer, 500 mM imidazole pH 7.4  
 Flow rates: 1) 0.5 ml/min, 78 cm/h  
 2) 4 ml/min, 624 cm/h

Instrumentation: FPLCSystem with FPLCdirector

**Note: No Ni<sup>2+</sup> re-loading of the column between the runs**

#### Result:

##### 0.5 ml/min

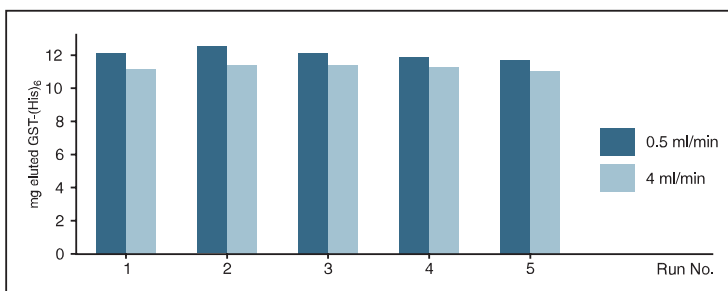
Run No.	Eluted ml	Eluted A <sub>280</sub>	Eluted mg/ml	Eluted GST-(His) <sub>6</sub> total mg
1	5.6	3.208	2.17	12.1
2	5.6	3.304	2.23	12.5
3	5.6	3.196	2.16	12.1
4	5.6	3.134	2.12	11.9
5	5.6	3.098	2.09	11.7

Average: 12.1 mg

##### 4.0 ml/min

Run No.	Eluted ml	Eluted A <sub>280</sub>	Eluted mg/ml	Eluted GST-(His) <sub>6</sub> total mg
1	5.6	2.958	2.00	11.2
2	5.6	3.022	2.04	11.4
3	5.6	3.020	2.04	11.4
4	5.6	2.988	2.02	11.3
5	5.6	2.912	1.98	11.0

Average: 11.3 mg



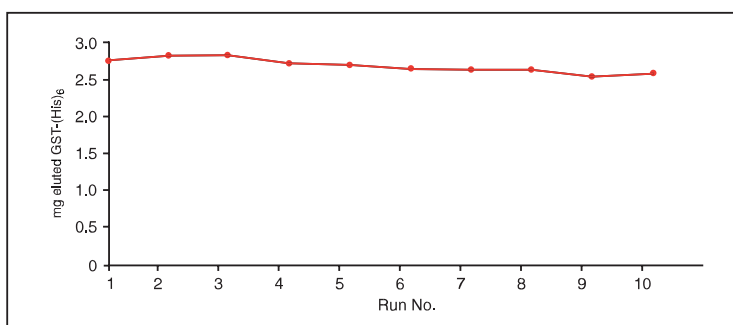
### 10 repetitive purifications of GST-(His)<sub>6</sub>

Samples: 2.5 ml GST-(His)<sub>6</sub> cell extract  
 Binding Buffer: 1 x Binding Buffer, 20 mM imidazole pH 7.4  
 Elution Buffer: 1 x Binding Buffer, 500 mM imidazole pH 7.4  
 Flow rate: 2 ml/min, 312 cm/h  
 Instrumentation: FPLCSystem with FPLCdirector

**Note: No Ni<sup>2+</sup> re-loading of the column between the runs**

#### Result:

Run No.	Eluted GST-(His) <sub>6</sub> , total mg
1	2.76
2	2.82
3	2.83
4	2.72
5	2.71
6	2.65
7	2.64
8	2.63
9	2.54
10	2.59

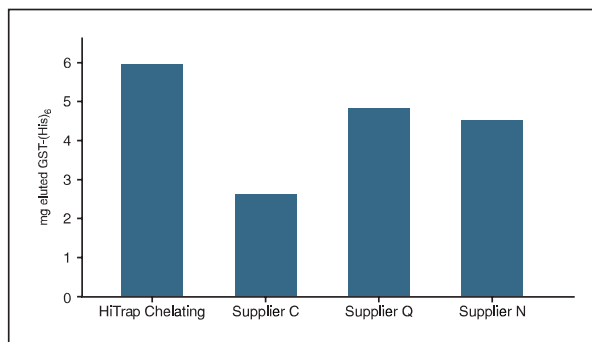


## Media comparison

Samples: 5 ml cytoplasmic cell extract containing GST-(His)<sub>6</sub>  
Columns: All media are packed in HiTrap 1 ml columns  
Binding Buffer: 1 x Binding Buffer, 20 mM imidazole pH 7.4  
Elution Buffer: 1 x Binding Buffer, 500 mM imidazole pH 7.4  
Flow rate: 4 ml/min, 624 cm/h  
Instrumentation: FPLCSystem with FPLCdirector

### Result:

Chromatography media	Eluted ml	Eluted A <sub>280</sub>	Eluted GST-(His) <sub>6</sub> total mg
HiTrap Chelating:	5	1.74	5.88
Supplier C:	5	0.77	2.66
Supplier Q:	5	1.42	4.80
Supplier N:	5	1.35	4.56



## Conclusion

- Using HisTrap, histidine-tagged proteins are purified to high purity in one step.
- Purification of histidine-tagged proteins using HisTrap takes just 10-15 minutes and needs no complicated instrumentation.
- HiTrap Chelating, when charged with Ni<sup>2+</sup> ions will selectively bind histidine-tagged proteins and the proteins are then eluted under mild conditions.
- Purifications performed under both native and denaturing conditions, e.g. in 8 M urea or 6 M guanidine hydrochloride give the same good results.
- The binding capacity for recombinant (His)<sub>6</sub>-tagged proteins is ~12 mg/ml gel.
- The binding of histidine-tagged proteins is relatively insensitive using flow rates in the range 0.5 - 4 ml/min.
- 10 repetitive purifications can be performed on the same column giving the same good results without new metal re-loading of the gel.

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CAT, Cambridge Antibody Technology Ltd., Cambridge, U.K.

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