# Rapid and efficient purification and refolding of a (histidine)$_6$-tagged recombinant protein produced in *E. coli* as inclusion bodies



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## Summary

This Application Note describes the purification and refolding of a recombinant protein tagged with a (histidine)$_6$-tag at its N-terminus. Using a simple but efficient purification and refolding procedure, a protein initially produced as intracellular inclusion body material in *Escherichia coli* is converted to soluble protein exhibiting the desired activity. This protocol has been used successfully for several different (histidine)$_6$-tagged recombinant proteins.

## Introduction

Heterologous expression of foreign genes in *E. coli* can be engineered to lead to either intracellular accumulation of recombinant protein, or to secretion and accumulation in the periplasmic space. While the latter mode of expression is sometimes advantageous in terms of protein folding, solubility, and cysteine oxidation, the magnitude of protein production is generally much higher when intracellular expression is used (1).

However, recombinant protein accumulated intracellularly is frequently laid down in the form of inclusion bodies, insoluble aggregates of misfolded protein lacking biological activity (2,3,4,5). The high buoyant density of inclusion bodies facilitates their separation from soluble *E. coli* proteins and cell debris by differential centrifugation (4,6,7).

Conventional methods for refolding of insoluble recombinant proteins include slow dialysis or dilution into a buffer of near-neutral pH (8). Gel filtration, ion exchange, or hydrophobic interaction chromatography have been used (9,10,11) to facilitate the refolding step.

Affinity tagging of the recombinant protein, for example by the addition of several consecutive histidine residues, makes the efficient purification and refolding in a single chromatographic step possible. Since binding of the histidine tract to immobilized divalent metal ions can occur in the presence of a chaotropic agent (such as urea or guanidine hydrochloride) at high concentration, (histidine)$_6$-tagged inclusion body protein can be solubilized by chaotropic extraction and directly bound to an affinity matrix. Removal of contaminating proteins and refolding by exchange to non-denaturing buffer conditions can then be performed before elution of the protein from the column (12).

A general protocol for the purification and refolding of a (histidine)$_6$-tagged recombinant protein produced in *E. coli* is shown in Figure 1.

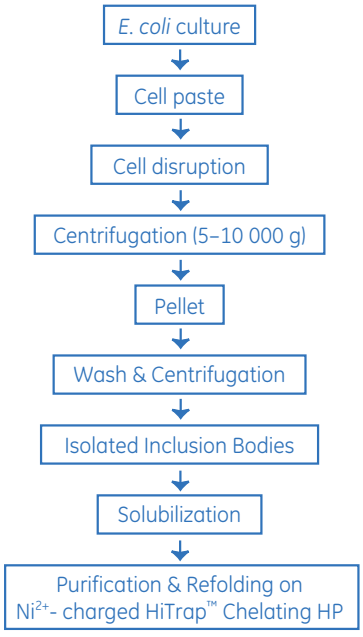



**Fig 1.** General scheme for the extraction, solubilization, and refolding of (histidine)$_6$-tagged recombinant proteins produced as inclusion bodies in *Escherichia coli* cells.

## Disruption, wash, and isolation of inclusion bodies

Resuspend the cell paste from a 100 ml culture of *E. coli* expressing (histidine)$_6$-tagged recombinant protein in 4 ml 20 mM Tris-HCl pH 8.0. Disrupt the cells with sonication on ice (e.g., 4 × 10 sec.) and centrifuge at high speed for 10 min at 4°C. The pellet, containing the inclusion bodies, is resuspended in 3 ml cold 2 M urea, 20 mM Tris-HCl, 0.5 M NaCl, 2% Triton™ X-100 pH 8.0 and sonicated as above. Centrifuge at high speed for 10 min at 4°C. Subject the pellet to a second round of urea wash. At this stage the pellet material can be washed once in buffer lacking urea, and then stored frozen for later processing.

## Solubilization and sample preparation

Resuspend the pellet in 5 ml 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol pH 8.0. Stir for 30–60 min in room temperature and centrifugate 15 min at high speed, 4°C. Remove remaining particles by passing the sample through a 0.22 µm or 0.45 µm filter.

The optimal concentration of reducing 2-mercaptoethanol (0–5 mM) must be determined experimentally for each individual protein.

Proceed directly with the purification and refolding steps.

## Preparation of the column

HiTrap™ Chelating HP 1 ml column is washed with 5 ml distilled water using a 5 ml syringe. Load 0.5 ml 0.1 M $NiSO_4$ and continue to wash with 5 ml distilled water. Equilibrate the column with 5–10 ml 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol pH 8.0.

## Purification and refolding

### *Loading and washing*

Load the sample and wash the column with 10 ml 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercapto-ethanol pH 8.0. Change the buffer to 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol, 6 M urea pH 8.0 and wash with 10 ml.

### *Refolding*

Refolding of the bound protein is performed using a linear 6–0 M urea gradient, starting with the wash buffer above and finishing at one without urea. A gradient volume of 30 ml or higher and a flow rate of 0.1–1 ml/min can be used, while the optimal renaturation rate should be determined experimentally for each protein. Continue to wash with 5 ml of buffer without urea after the gradient has come to its endpoint.

### *Elution*

Elute the refolded recombinant protein using a 10–20 ml linear gradient starting with 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol pH 8.0 and ending with the same buffer including 500 mM imidazole (Fig 2).

Fractions containing the eluted protein are pooled and subjected to buffer exchange using a HiTrap Desalting or PD-10 column, in order to remove imidazole. The refolded (histidine)$_6$-tagged protein is now ready for analysis of biological activity.

The choice of HiTrap column size depends on the amount of expressed protein.

While in this example a HiTrap Chelating HP 1 ml column is used, a HiTrap Chelating HP 5 ml is also available and should be used if the expected amount of recombinant protein exceeds 10 mg. For further scaling-up, Chelating Sepharose™ Fast Flow is available.

| | |
|---|---|
| *Column:* | $Ni^{2+}$-loaded HiTrap Chelating HP 1 ml |
| *Sample:* | N-terminal (histidine)$_6$-tagged recombinant protein produced in *E.coli* |
| *Flow rates:* | 0.1–1 ml/min, sample loading and refolding 1 ml/min, wash and elution |
| *Binding Buffer:* | 20 mM Tris-HCl, 0.5 M NaCl, 5 mM imidazole, 6 M guanidine hydrochloride, 1 mM 2-mercaptoethanol pH 8.0 |
| *Washing buffer:* | 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 6 M urea, 1 mM 2-mercaptoethanol pH 8.0 |
| *Refolding buffer:* | 20 mM Tris-HCl, 0.5 M NaCl, 20 mM imidazole, 1 mM 2-mercaptoethanol pH 8.0 |
| *Refolding gradient:* | 30 ml |
| *Elution Buffer:* | 20 mM Tris-HCl, 0.5 M NaCl, 500 mM imidazole, 1 mM 2-mercaptoethanol pH 8.0 |
| *Elution gradient:* | 10 ml |
| *Fraction volumes:* | 3 ml sample loading, wash and refolding 1 ml elution |

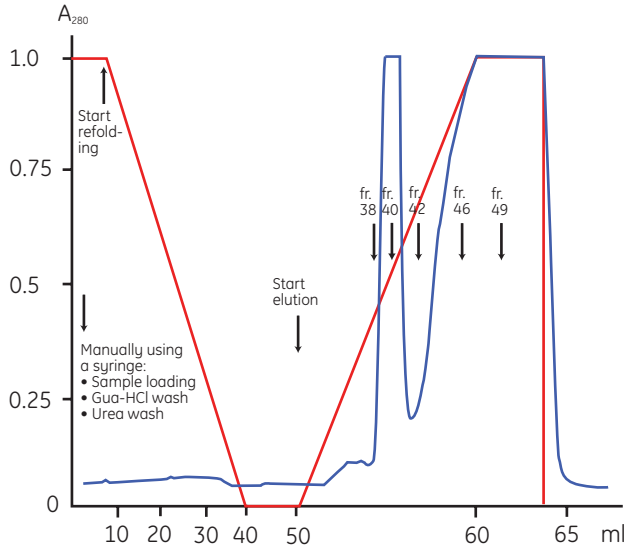



**Fig 2.** On-column refolding and purification of a (histidine)$_6$-tagged protein from inclusion bodies on $Ni^{2+}$-charged HiTrap Chelating HP.

| | |
|---|---|
| *Column:* | Superdex™ 75 HR 10/30 ($V_T$: 24 ml) |
| *Sample:* | 0.2 ml purified and refolded N-terminal (histidine)$_6$-tagged recombinant protein eluted from HiTrap Chelating HP 1 ml |
| *Buffer:* | 0.15 M NaCl |
| *Flow rate:* | 0.5 ml/min |
| *Fraction volume:* | 1 ml |

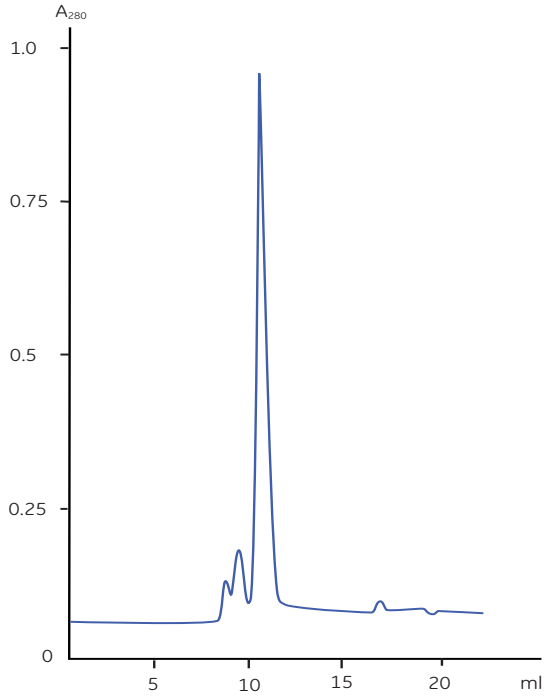
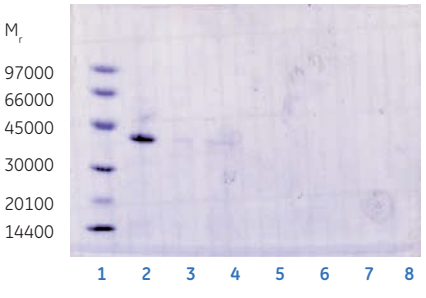
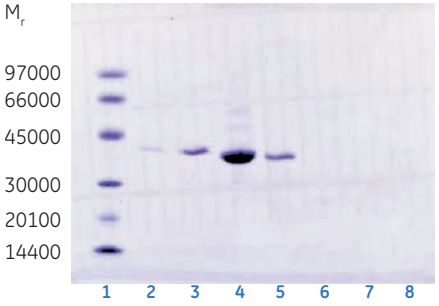



**Fig 3.** Analysis using gel filtration of refolded (histidine)$_6$-tagged protein.

| | |
|---|---|
| *Gel:* | PhastGel™ Gradient 10–15 |
| *Sample pretreatment:* | Dilution 1:5 with 15% SDS, 30% 2-mercaptoethanol, 10 mM Tris, 1 mM EDTA |
| *Sample volume:* | 1 µl |
| *Molecular weight standard :* | Low Molecular Markers |
| *Staining:* | Coomassie™, according to the manufacturer's standard protocol |
| *Instrument:* | PhastSystem™ |




**Lane 1:** Low Molecular Markers
**Lane 2:** Starting material for HiTrap Chelating 1 ml
**Lane 3:** Fraction 1 Gua-HCl wash (manually)
**Lane 4:** Fraction 2 Gua-HCl wash (manually)
**Lane 5:** Fraction 3 Gua-HCl wash (manually)
**Lane 6:** Fraction 4 Gua-HCl wash (manually)
**Lane 7:** Fraction 1 Urea wash (manually)
**Lane 8:** Fraction 2 Urea wash (manually)




**Lane 1:** Low Molecular Markers
**Lane 2:** Fraction 38
**Lane 3:** Fraction 39
**Lane 4:** Fraction 40
**Lane 5:** Fraction 41
**Lane 6:** Fraction 42
**Lane 7:** Fraction 46
**Lane 8:** Fraction 49

**Fig 4.** SDS-PAGE analysis.

## Analysis

The aggregation state and purity of the refolded (histidine)$_6$-tagged recombinant protein eluted from HiTrap Chelating HP is checked by gelfiltration on Superdex 75 HR 10/30 (Figure 3) and SDS-PAGE (Figure 4).

## Regeneration and storage

Regenerate the column with 5 ml 6 M guanidine hydrochloride, 20 mM Tris-HCl, 0.5 M NaCl, 50 mM EDTA, pH 8.0. Wash with 10 ml distilled water followed by 10 ml 20% ethanol. Store the column in 20% ethanol.

## References: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# Ordering information

| Product | Quantity | Code No. |
|---|---|---|
| HiTrap Chelating HP | 5 × 1 ml | 17-0408-01 |
| HiTrap Chelating HP | 1 × 5 ml | 17-0409-01 |
| HiTrap Chelating HP | 5 × 5 ml | 17-0409-03 |

| Related literature | Code No. |
|---|---|
| Recombinant Protein Purification Handbook, Principles and methods | 18-1142-75 |
| Affinity Chromatography, Columns and Media Selection Guide | 18-1121-86 |
| Affinity Chromatography Handbook, Principles and Methods | 18-1022-29 |
| HiTrap Column Guide | 18-1129-81 |

| Related Products | Quantity | Code No. |
|---|---|---|
| HiTrap Desalting | 5 × 5 ml | 17-1408-01 |
| HiPrep™ 26/10 Desalting | 1 × 53 ml | 17-5087-01 |
| HiPrep 26/10 Desalting | 4 × 53 ml | 17-5087-02 |
| PD-10 Column | 30 | 17-0851-01 |
| Chelating Sepharose Fast Flow | 50 ml | 17-0575-01 |
| Superdex 75 10/300 GL | 1 | 17-5174-01 |
| Superdex 200 10/300 GL | 1 | 17-5175-01 |
| HiLoad™ 16/60 Superdex 30 pg | 1 | 17-1139-01 |
| HiLoad 26/60 Superdex 30 pg | 1 | 17-1140-01 |
| HiLoad 16/60 Superdex 75 pg | 1 | 17-1068-01 |
| HiLoad 26/60 Superdex 75 pg | 1 | 17-1170-01 |
| HiLoad 16/60 Superdex 200 pg | 1 | 17-1069-01 |
| HiLoad 26/60 Superdex 200 pg | 1 | 17-1171-01 |
| XK 16/20 column | 1 | 18-8773-01 |
| XK 16/40 column | 1 | 18-8774-01 |
| XK 26/20 column | 1 | 18-1000-72 |
| XK 26/40 column | 1 | 18-8768-01 |

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