

Efficient purification of polyhistidine-tagged proteins under denaturing conditions using MagReSyn® NTA: Inclusion bodies & hidden tags



Isak Gerber; Justin Jordaan, ReSyn Biosciences (Pty) Ltd, Johannesburg, South Africa

Michael Crampton, CSIR Biosciences, Pretoria, South Africa

Abstract

The application note provides a rapid denaturing protocol and adaptable guideline for effective purification of 6xHis-tagged proteins from inclusion bodies, as well as for soluble proteins with a hidden 6xHis-tag. The efficiency of MagReSyn® NTA microparticles for the purification polyhistidine-tagged proteins from *E. coli* under denaturing conditions is demonstrated. Recombinant proteins, which are produced in inclusion bodies, are solubilized in 8 M Urea prior to purification, while soluble proteins with a hidden 6xHis-tag are partially unfolded/denatured in 2-4 M Urea, and applied to the microparticles for affinity purification of the target protein. High-purity tagged proteins are recovered using competitive elution with imidazole.

Introduction

Nickel-NTA-based immobilized metal ion affinity chromatography (IMAC) is one of the most widely used methods for purification of recombinant proteins containing an affinity tag consisting of histidine residues (6xHis). The optimal placement of the affinity tag on the recombinant protein is protein specific and may be on the N-terminal, C-terminal or as a sandwich fusion. Recombinant 6xHis-tagged proteins that are expressed in the cytoplasm or secreted into culture medium remain soluble and can be purified with relative ease using IMAC under mild buffer conditions and with imidazole as the eluant to yield pure, biologically active, protein. However, the expressed recombinant protein may sometimes be expressed in an aggregated form (inclusion bodies), or the expressed tag of a soluble protein may be 'hidden' during protein folding. In both instances these proteins are not suitable for purification using standard IMAC purification. This necessitates protein purification under denaturing conditions involving denaturants such as urea, to first unfold the target protein for interaction of the 6xHis tag with the Ni-NTA activated matrix.

MagReSyn® NTA microparticles are pre-chelated with nickel (Ni^{2+}) ions and have been engineered for exceptional specificity; providing highly pure target proteins. The microparticles were evaluated for the purification 6xHis-tagged proteins expressed in inclusion bodies and for purification of an expressed protein with a hidden 6xHis-tag.

Materials

Purification of Protein from Inclusion Bodies

- *Phosphate Buffered Saline (PBS) Buffer*: 80 mM sodium phosphate pH 7.4, 150 mM NaCl
- *Protein Denaturation Buffer*: 80 mM sodium phosphate pH 7.4, 1 M NaCl, 8 M urea
- *Binding/Wash Buffer*: 80 mM sodium phosphate pH 7.4, 40 mM imidazole, 1 M NaCl, 6 M urea
- *Elution Buffer*: 500 mM Imidazole in 80 mM sodium phosphate pH 7.4, 1 M NaCl, 6 M urea

Purification of Protein Containing Hidden Tag

- *Protein Denaturation Buffer (2X)*: 160 mM sodium phosphate pH 7.4, 2 M NaCl, containing 8, 6 or 4 M urea
- *Binding/Wash Buffer*: 80 mM sodium phosphate pH 7.4, 40 mM imidazole, 1 M NaCl, 4, 3 or 2 M urea
- *Elution Buffer*: 500 mM Imidazole in 80 mM sodium phosphate pH 7.4, 1 M NaCl, 4, 3 or 2 M urea

A magnetic separator will be required to isolate magnetic beads between each step of the protocol.



Methods

Protein Sample Preparation - Inclusion Bodies

E. coli cell culture (1 ml IPTG induced), producing a target 6xHis-tagged protein in inclusion bodies, was harvested by centrifugation (10,000 x g for 5 min). The cell pellet was washed in PBS (2 x 500 µl) to remove excess medium components. The cells were lysed in 1 ml PBS using sonication (Bandelin Sonopuls, 70% power, 2 x 5 min on ice). The inclusion bodies and cell debris were isolated by centrifugation (10,000 x g for 5 min), and the inclusion bodies (in the pellet) were resuspended in *Protein Denaturation Buffer* (500 µl) for protein solubilisation. The sample was incubated at 4°C for 24 h with end-over-end mixing. The sample was clarified by centrifugation (10,000 x g for 5 min). A fraction of the sample supernatant (100 µl aliquot) was diluted to 6 M urea with a combination of PBS, NaCl and imidazole to achieve a final concentration of 80 mM phosphate buffer (pH 7.4), 1 M NaCl, and 40 mM imidazole, before addition to equilibrated MagReSyn® NTA.

Protein Sample Preparation - Hidden Tag

E. coli cell culture (1 ml IPTG induced) expressing a soluble protein with a hidden (inwardly folded) 6xHis-tagged protein was lysed by sonication (as above) and clarified by centrifugation (10,000 x g for 5 min). **Note:** clarification may not be required for automated applications - removal of the clarification step should be verified as necessary. The supernatant containing 6xHis-tagged protein was removed and 100 µl aliquots were diluted with various preparations of double strength *Protein Denaturation Buffer* to obtain final urea concentrations from 2 to 4 M, with 1 M NaCl, and 40 mM imidazole in 80 mM phosphate buffer. The sample was subsequently applied to equilibrated MagReSyn® NTA.

Equilibration of MagReSyn® NTA

MagReSyn® NTA is supplied as a 25 mg.ml⁻¹ suspension in 20% ethanol. The shipping solution needs to be removed and the microparticles equilibrated in *Binding/Wash Buffer* prior to use. The microparticles are recovered by application to a magnetic separator between each step. The MagReSyn® NTA microparticles were thoroughly resuspended by vortex mixing for 3 sec, and an aliquot (10 µl - 250 µg) removed for equilibration. **Note:** the quantity of microparticles may be scaled to match the quantity of expressed protein. The microparticles were pipetted into a 2 ml micro-centrifuge tube, and recovered using a magnetic separator. The shipping solution was removed by aspiration with a pipette and discarded. The microparticles were subsequently equilibrated in 3 x 200 µl *Binding/Wash Buffer* (30 sec a time) with magnetic recovery and aspiration of the *Binding/Wash Buffer* after each magnetic recovery step. The equilibrated MagReSyn® NTA is subsequently suitable for binding of 6xHis-tagged protein.

Protein binding procedure

The sample containing 6xHis-tagged protein was added to the equilibrated MagReSyn® NTA and mixed thoroughly by pipetting. The protein-containing sample was allowed to interact with the microparticles for 15 min at room temperature with gentle agitation to ensure that the microparticles remained in suspension for the duration of the binding procedure. The magnetic particles are then recovered using a magnetic separator, and the supernatant aspirated with a pipette. **Note:** The supernatant can either be discarded, or subsequently used for protein quantification or electrophoresis (e.g. to quantify protein of interest potentially remaining in the supernatant). The microparticles were washed three times by resuspension in 200 µl *Binding/Wash Buffer* for 30 sec each, with recovery by capture on a magnetic separator between each wash. The wash fractions can either be discarded or retained for further analysis.

Protein Elution Procedure

The enriched 6xHis-tagged protein was eluted from the microparticles by adding 20 µl of the appropriate elution buffer (refer Materials) and mixed well by pipetting up and down a few times. The protein was allowed to elute for 1 min at room temperature before placing the tube on the magnetic separator to capture the microparticles. The eluate, containing the protein of interest, was removed by aspiration with a pipette and transferred to a new tube for analysis by gel electrophoresis. To potentially improve recovery of 6xHis-tagged proteins, the elution procedure was repeated with an additional 20 µl elution buffer and pooled for analysis (total of 40 µl).

Gel Electrophoresis

The purity of the enriched 6xHis-tagged proteins was assessed by gel electrophoresis using NUPAGE® 4-12% Bis-Tris precast gels (Life Technologies) according to the manufacturer's instructions. Protein bands were visualized in the gel by staining proteins for 16 h with Colloidal Coomassie Blue stain. The gels were destained in ultrapure water. The gel images were captured on a Syngene G:BOX gel doc system (automatic exposure) and analysed for protein purity using densitometry (GeneSnap software).

Results & Discussion

The efficiency of MagReSyn® NTA for the affinity purification 6xHis-tagged proteins from *E. coli* under denaturing conditions is indicated by the gel electrophoresis images below. **Figure 1** shows the purification of a 6xHis-tagged protein produced in inclusion bodies, while **Figure 2** illustrates purification of protein containing a hidden tag using increasing concentrations of urea.

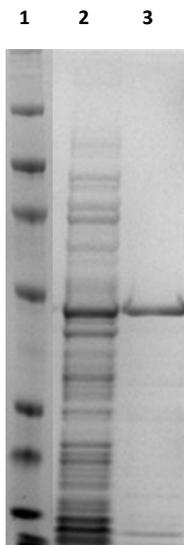


Figure 1. Specific enrichment of 6xHis-tagged target protein from crude *E. coli* inclusion bodies using MagReSyn® NTA under denaturing conditions containing urea. Lane 1 - Molecular weight markers; Lane 2 - Crude protein extract solubilised using 8 M urea; Lane 3 - Purified 6xHis-tagged protein. Protein was purified to $\geq 95\%$ purity using a single chromatography step.

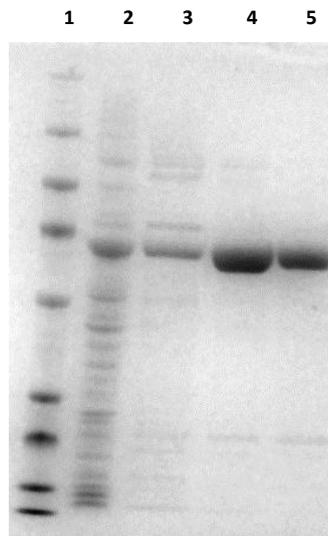


Figure 2: Specific enrichment of soluble 6xHis-tag protein containing a hidden tag. Lane 1 - Molecular weight markers, Lane 2 - Crude protein extract; Lane 3 to 5 - Binding and elution using increasing concentrations of urea, 2, 3 and 4 M using MagReSyn® NTA. The protein was purified to $\geq 95\%$ purity using a single chromatography step, while 3 M urea provided the highest capacity for this protein.

Conclusions

Samples containing histidine tagged proteins, either expressed in inclusion bodies, or with an inaccessible hidden tag, were purified to $\geq 95\%$ purity using MagReSyn® NTA magnetic microparticles under denaturing conditions. The application note provides an adaptable protocol for the rapid purification of 6xHis-tagged proteins from potentially problematic samples. This protocol illustrates the compatibility of the high performance MagReSyn® NTA microparticles under denaturing conditions.

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Ordering Information

Description	Product Code
MagReSyn® NTA 2 ml	MR-NTA002
MagReSyn® NTA 5 ml	MR-NTA005
MagReSyn® NTA 10 ml	MR-NTA010

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