

Purifying Recombinant His-Tagged Proteins

Improving the Process with Use of Genetically Tailored Expression Host NiCo21(DE3)

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Immobilized metal affinity chromatography (IMAC) is a reliable method for isolation of recombinant protein following over-expression in *Escherichia coli*. The polyhistidine tag (6–10 consecutive amino acids) offers high affinity to immobilized metals such as nickel and cobalt. *E. coli* and other expression hosts rely on many essential proteins with metal cofactors, and these endogenous metal-binding proteins may also bind to IMAC resins and complicate isolation of the desired target protein.

The aim of this article is to provide guidance in experimental design and execution

so that a recombinant protein of interest may be purified to the highest level with a minimum number of chromatography steps.

Highly expressed, well-behaved histidine-tagged proteins are routinely purified to greater than 80% purity with a single metal affinity column. However, as expression of the target protein decreases, competitive binding by endogenous host proteins becomes more of an issue.

The most often cited *E. coli* “contaminant” protein is SlyD, a peptidyl-prolyl isomerase. SlyD is also referred to as wonderful histidine-rich protein (WHP) for its ability to bind to IMAC columns, such as those containing nickel-nitrilotriacetic acid (Ni-NTA)

resins, in the presence of 6 M guanidine-HCl.

Other Ni-NTA-binding proteins are not necessarily histidine-rich but do have surface-exposed clusters. For example, GlmS (involved in cell-wall biosynthesis) has a histidine content of only 3.9%, yet we have observed GlmS contamination in many Ni-NTA elution fractions.

Common IMAC Contaminants

Many endogenous proteins display affinity to immobilized metal, and these proteins were classified by Bolanos-Garcia and Davies¹ according to the imidazole concentration required to elute them from Ni-NTA sepharose resin: class I (>80 mM), class II (55–80 mM), and class III (30–50 mM). Class I proteins were listed as S15 (10 kDa), Hfq (11 kDa), Fur (17 kDa), Cu²⁺/Zn²⁺-superoxide dismutase (17 kDa), Yoda (22 kDa), CRP (24 kDa), SlyD (21 kDa), and ArgE (42 kDa).

When our group overexpressed three model proteins in BL21(DE3), we consistently observed DnaK (70 kDa), GlmS (67 kDa) AceE (100 kDa), EF-Tu (43 kDa), ArnA (74 kDa), CRP, and SlyD in elution fractions after extensively washing the Ni-NTA column with buffer containing 25 mM imidazole.

In addition, carbonic anhydrase (Can) was observed as an Ni-NTA contaminant after cultivating cells by high-density fermentation but not as a result of shake flask growth. In fact, Bolanos-Garcia and Davies reported that Can expression is maximal during slow growth, at high cell densities, and during starvation conditions.

Multiple chromatography steps may of course eliminate contaminant proteins, but no single column will offer specific removal. Many of the contaminants are less than 25 kDa, so we recommend size-exclusion chromatography to improve the purity of target

proteins 40 kDa or larger.

A second complementary approach is to use a genetically-tailored expression host to eliminate problematic contaminants. Since many metal-dependent proteins are essential during the stressful action of recombinant protein over-expression, simply deleting the respective genes is not a viable option. Therefore, at New England Biolabs, we chose to engineer a specialized host where the surface residues of GlmS are altered and three other contaminants (SlyD, ArnA and Can) are tagged with an alternative tag to enable specific, single-resin removal.

We developed the NiCo21(DE3) expression strain,² which encodes a *glmS(6His>6Ala)* allele in place of the wt-*glmS* gene. Furthermore, the genomic copies of *slyD*, *arnA* (aka *yfbG*), and *can* (aka *yadF*) are tagged with the CBD open reading frame. The CBD tag, also utilized by the IMPACT expression system, consists of the chitin-binding domain originating from *Bacillus circulans*. The 7kDa CBD tag offers high affinity to chitin resin in various buffer conditions including those commonly used for metal chelate chromatography.

Accordingly, Ni-NTA elution fractions may be applied to chitin resin directly without buffer adjustment in order to remove three primary contaminants. NiCo21(DE3), which is derived from BL21(DE3), was evaluated at each step of its construction. Specifically, the performance of the derived strain was compared with that of the parent strain.

The examination included specific assays to ensure that each modified protein maintained proper function. As shown in Figure 1, the examination showed that the growth rates of NiCo21(DE3) and BL21(DE3) are indistinguishable, and that the protein expression potentials of NiCo21(DE3) and BL21(DE3) are equally robust.

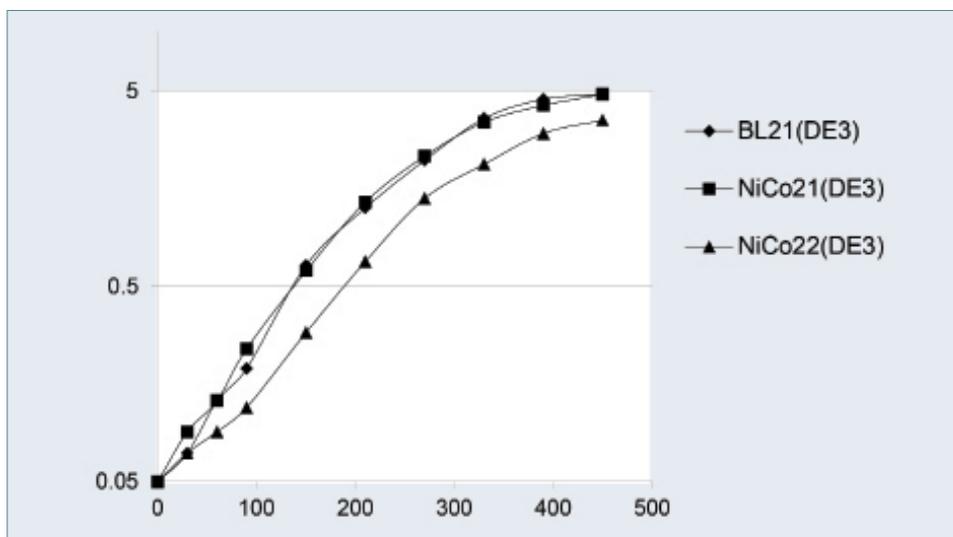


Figure 1. NiCo21(DE3) has the same growth rate as BL21(DE3), as determined by a growth analysis performed at 30°C in LB medium. NiCo22(DE3) carries an additional tag at the C-terminus of pyruvate dehydrogenase subunit AceE, and this modification affects growth.

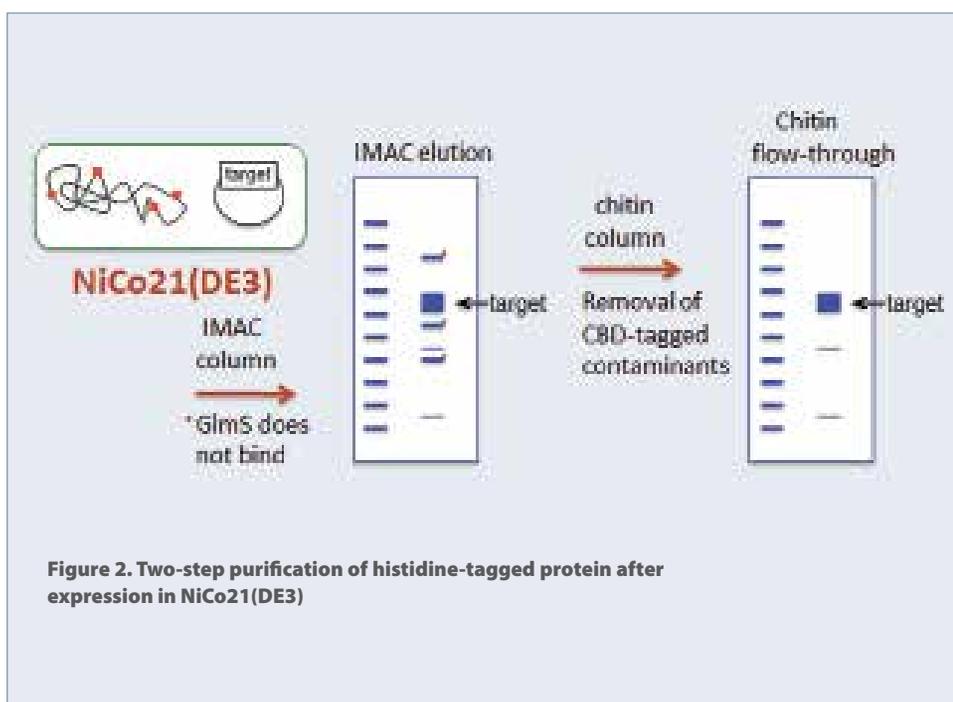


Figure 2. Two-step purification of histidine-tagged protein after expression in NiCo21(DE3)

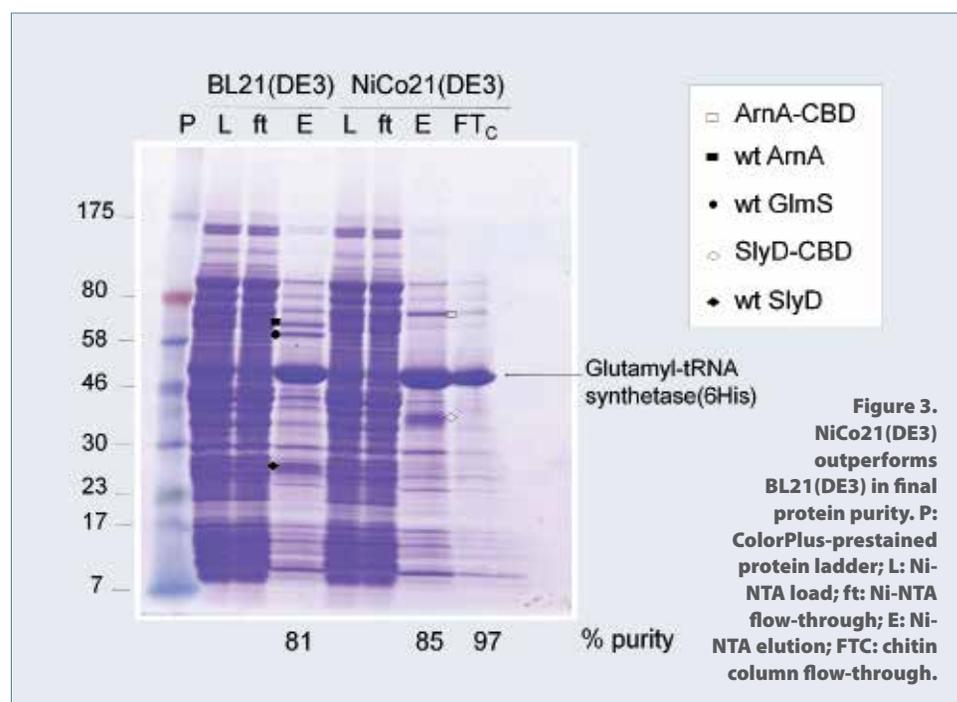


Figure 3. NiCo21(DE3) outperforms BL21(DE3) in final protein purity. P: ColorPlus-prestained protein ladder; L: Ni-NTA load; ft: Ni-NTA flow-through; E: Ni-NTA elution; FTC: chitin column flow-through.

Recommended Purification Protocol (Native Conditions)

Express the recombinant protein within NiCo21(DE3) and begin the purification process with an IMAC column. Cell-breakage buffer/IMAC column buffers should be prepared within the pH range of 7.5–8.0 to ensure that histidine side chains are deprotonated. A minimum of 10 mM imidazole should be added to the lysis/loading buffer, and a minimum of 20 mM imidazole should be used for the wash step.

To obtain the greatest purity, 25–50 mM imidazole may be used in the wash buffer with little effect on target protein yield in many cases. Typically, high salt conditions (300–500 mM NaCl) are used for equilibration, load, wash, and elution steps to minimize nonspecific protein interactions with the IMAC resin. However, we find that cell breakage is more efficient in 100 mM NaCl, and that lower salt also reduces the likelihood of hydrophobic proteins partitioning into the insoluble fraction.

The following cell-breakage buffer is recommended (which is adjusted to 300–500 mM NaCl after isolation of the soluble fraction): 50 mM sodium phosphate buffer, pH 8.0, 100 mM NaCl, 5 mM MgCl₂, 5% glycerol, 20 mM imidazole, and protease inhibitor (lacking EDTA). Before cell breakage, treat the cell suspension with lysozyme (0.3 mg/mL) for 30 min on ice. After breakage, optionally add an enzyme to degrade genomic DNA to reduce lysate viscosity (for example, Benzonase at 10 units/mL for 15 min on ice).

Adjust the salt concentration to 500 mM with solid NaCl, centrifuge the lysate, filter the supernatant (0.45 µm, polyethersulfone membrane), and apply to the IMAC column equilibrated with breakage buffer containing 500 mM NaCl and 10–20 mM imidazole. A small-scale pilot study is advised to determine the IMAC-binding properties of each individual target protein.

Figure 2 shows a theoretical purification result when employing NiCo21(DE3) as the expression host. Furthermore, Figure 3 shows a direct comparison between BL21(DE3) and NiCo21(DE3) when expressing and purifying histidine-tagged glutamyl-tRNA synthetase. With BL21(DE3) as the host, target protein purity was 81% after a single Ni-NTA column, whereas purity was 85% with the NiCo21(DE3) strain.

A significant purity improvement was achieved by then exposing the Ni-NTA elution pool to chitin resin to eliminate SlyD-CBD, ArnA-CBD, and Can-CBD. After chitin exposure, target protein purity was increased to 97% as determined by a Caliper LabChip GXII analysis. The chitin resin contaminant removal step may be accomplished

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by batch-mode, gravity column, or FPLC as long as pressure is maintained at or below 0.5 MPa.

NiCo21(DE3) was designed to be an all-purpose protein expression host, and this strain may be seamlessly substituted for BL21(DE3). Importantly, antibiotic resistance markers are absent from the chromosome to allow for versatility in expression vector selection. As with any DE3 strain, the

T7 RNA polymerase gene is IPTG-inducible, but keep in mind that DE3 strains are also compatible with promoters recognized by the *E. coli* RNA polymerase. NiCo21(DE3) is compatible with expression vectors employing the following promoters: T7, T7-lac, T5-lac, Ptac, Ptrc, Plac, ParaBAD, and PrhaBAD.

In summary, researchers do not need to compromise when choosing NiCo21(DE3)

as the lab workhorse. Researchers may realize specific benefits during nickel- or cobalt-mediated capture of recombinant protein. **GEN**

References

- 1 Bolanos-Garcia V. M., Davies O. R. 2006. *Biochim. Biophys. Acta* 1760: 1304–1313.
- 2 Robichon C., et al. 2011. *Appl. Environ. Microbiol.* 77:4634–4646.

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