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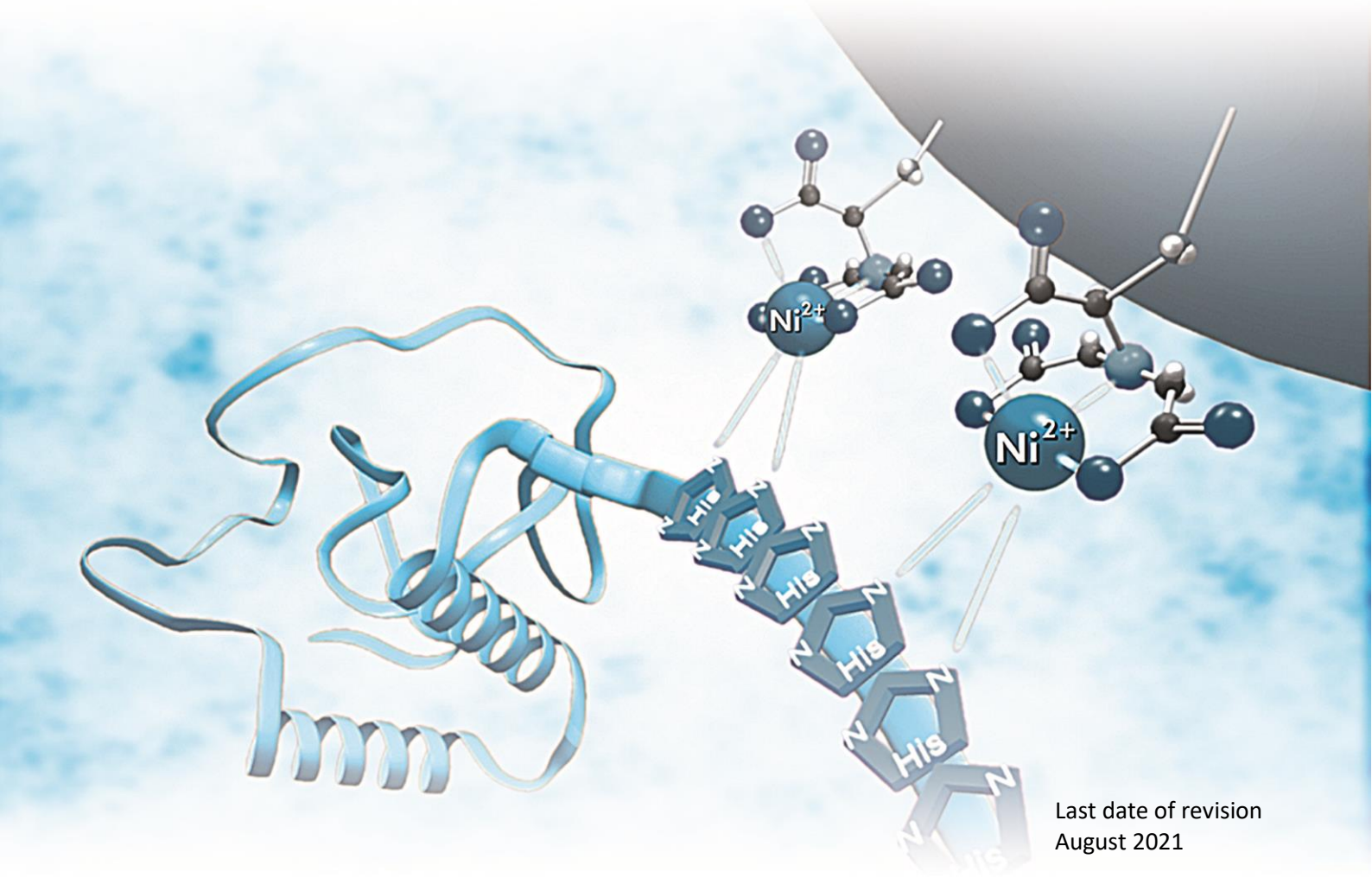
# Expression and purification of proteins using 6x*Histidine*-tag

## A comprehensive manual

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# 1 Introduction

## 1.1 His-tag/Ni-NTA system

The interaction between His-tag and Ni-NTA is based on the selectivity of Ni-NTA (nickel-nitrilotriacetic acid) resin for proteins containing an affinity tag of, e.g., six consecutive Histidine residues [1,2]. NTA, which has four chelating sites for nickel ions, binds nickel more tightly than metal-chelating purification systems like IDA which have only three sites available for interaction with metal ions.

## 1.2 Ni-NTA Resins

Recombinant proteins containing a His-tag can be purified by **Ni-NTA** chromatography which is based on the interaction between a transition $Ni^{2+}$ immobilized on a matrix and the histidine side chains.


**Abb. 1.** Capture of His-tag proteins by metal-chelate affinity matrices relies on two interactions. Both are important for affinity chromatophraphy based protein purification. The first interaction (A) is between the His-tag and a metal ion, e.g., $Ni^{2+}$, and is necessary for the capture of the His-tagged target protein from cell lysates. The second (B) is between the metal io chelating matrix and the metal ion

The extra chelating site prevents nickel-ion leaching, thus providing greater binding capacity and yield. In addition, the His-tag:Ni-NTA affinity chromatography system tolerates high concentrations of urea and guanidine allowing protein purification under denaturing conditions (see Table 1).

**Under physiological conditions host proteins with histidine stretches or host proteins containing metal ions may contaminate the protein preparation. Working with 20 mM imidazole can reduce this problem. However, we recommend the Strep-tag® technology for protein purification under physiological conditions.**

The highly specific interaction of the Strep-tag®II/Twin-Strep-tag® with Strep-Tactin®/Strep-Tactin®XT (biotin binding pocket) leads to protein purification results with outstanding purity (> 95%) without the need for buffer optimizations (see Abb. 2). Furthermore, the Strep-tag® technology tolerates the use of various buffers, detergents and additives making it to a very robust purification system for a large subset of proteins from different protein classes and expression hosts.

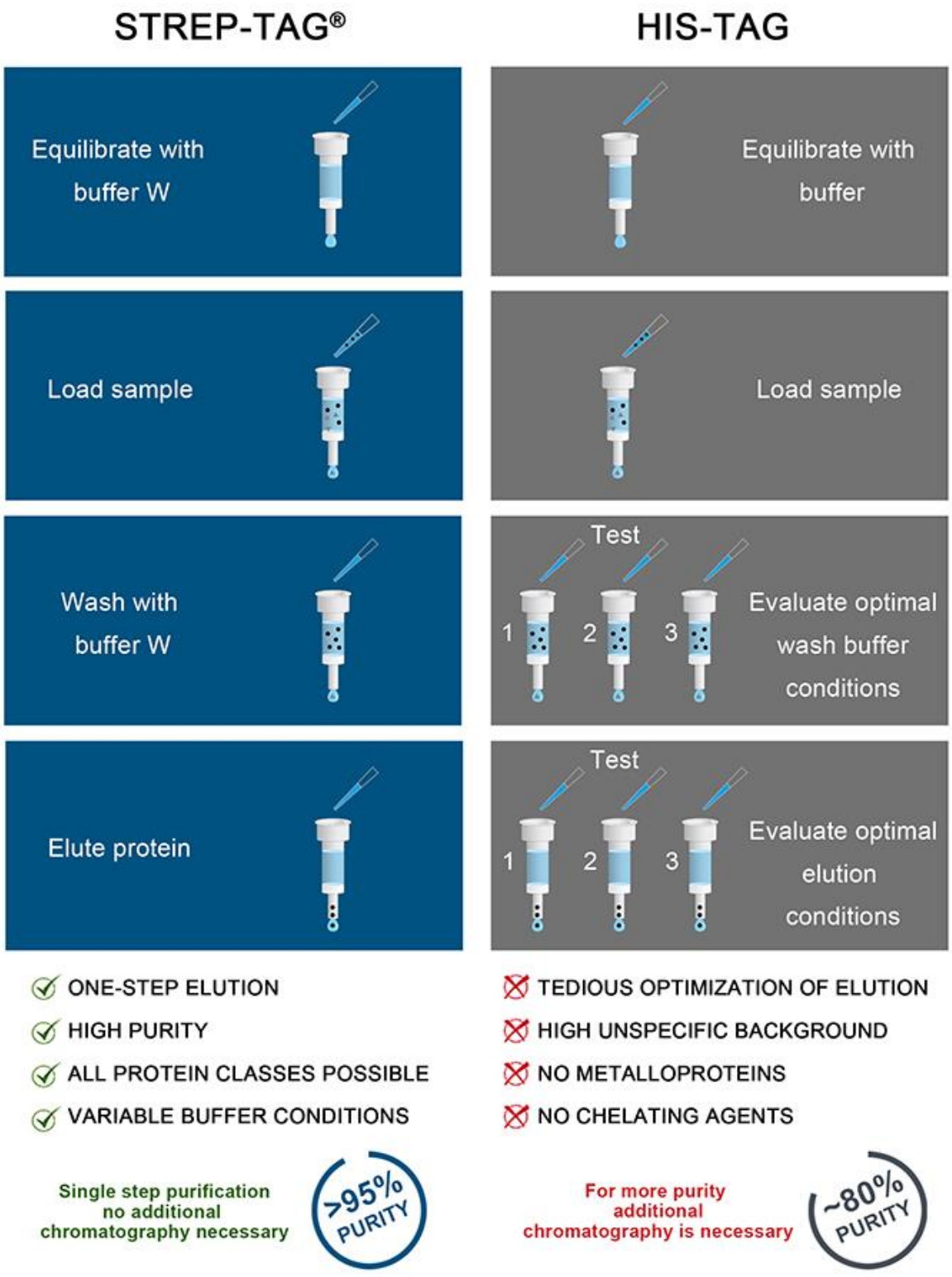



**Abb. 2.** Comparison of the His-tag vs. Strep-tag® purification procedure under physiological conditions regarding ease of use and purity.

**Table 1**. Reagents compatible with His-tag/Ni-NTA interaction.**

| Reagent | Effect | Comments |
|---|---|---|
| **Buffer reagents** | | |
| Tris, HEPES, MOPS | Buffers with secondary or tertiary amines may reduce nickel ions. | Up to 100 mM can be used, however sodium phosphate or phosphate-citrate buffer is recommended. |
| **Chelating reagents** | | |
| EDTA, EGTA | Strips nickel ions from resin. | Up to 1 mM has been used successfully in some cases, but care must be taken. |
| **Sulfhydril reagents** | | |
| β-mercaptoethanol | Prevents disulfide cross-linkages. Can reduce nickel ions at higher concentration. | Up to 20 mM can be used. Do not store resin under reducing conditions. |
| DTT, DTE | At high concentrations (>1 mM) resin may turn reversibly brown due to nickel reduction. Up to 10 mM has been tested and shown not to compromise purification or increase nickel leaching. | Up to 10 mM DTT has been used successfully. Do not store resin under reducing conditions. |
| TCEP | Prevents disulfide cross-linkages. | Up to 1 mM tested successfully. Do not store resin under reducing conditions. |
| **Nonionic detergents** | | |
| n-Hexadecyl-β-Dmaltoside | Removes background proteins and nucleic acids, resolubilizes membrane proteins from membrane compartments. | 0.0003 %* |
| n-Tetradecyl-β-Dmaltopyranoside | | 0.005 %* |
| n-Tridecyl-β-Dmaltopyranoside | | 0.016 %* |
| Brij 35 | | 0.1 %* |
| Digitonin | | 0.6 %* |
| Cymal 6, n-Nonyl-β-Dglucopyranoside, n-Decyl-β-Dmaltopyranoside, | | 1 %* |
| n-Octyl-β-Dglucopyranoside | | 1.5 %* |
| Triton X-100®, Tween-20®, NP-40 | | Up to 2 % can be used. |
| **Zwitterionic detergents** | | |
| Fos-Choline 16 | | 0.05 %* |
| Dodecyldimethylphosph ineoxide | | 0.15 %* |

**Table 1, continued**. Reagents compatible with His-tag/Ni-NTA interaction.**

| Reagent | Effect | Comments |
|---|---|---|
| **Cationic detergents** | | |
| CHAPS | | Up to 1 % can be used. |
| **Anionic detergents** | | |
| **(SDS, sarkosyl)** | | **Not recommended, but up to 0.3 % has been used successfully in some cases.** |
| Triton X-114 | Removes endotoxins | Up to 2 % can be used. |
| **Denaturants** | | |
| GuHCl | Solubilizes proteins | Up to 6 M. |
| Urea | | Up to 8 M. |
| **Amino acids** | | |
| Glycine | | Not recommended. |
| Glutamine | | Not recommended. |
| Arginine | | Not recommended. |
| Histidine | Binds to Ni-NTA and competes with histidine | Can be used at low concentrations (1–2 mM) to inhibit non specific binding and at higher concentrations (>20 mM) to elute the His-tagged protein from the Ni-NTA matrix. |
| **Other additives** | | |
| NaCl | Prevents ionic interactions. | Up to 2 M can be used, at least 300 mM should be used. |
| $MgCl_2$ | | Up to 4 M. |
| $CaCl_2$ | | Up to 5 mM. |
| Glycerol | Prevents hydrophobic interaction between proteins, stabilizes proteins. | Up to 50 %. |
| Ethanol | Prevents hydrophobic interactions between proteins. | Up to 20 %. |
| BugBuster® Protein Extraction Reagent | | Use as recommended. |
| Imidazole | Binds to Ni-NTA and competes with histidine residues in the His-tag. | Can be used at low concentrations (20 mM) to inhibit non specific binding and at higher concentrations (>100 mM) to elute the His-tagged protein from the Ni-NTA matrix. |
| Sodium bicarbonate | | Not recommended. |
| Hemoglobin | | Not recommended. |
| Ammonium | | Not recommended. |
| Citrate | Buffer | Up to 60 mM has been used successfully. |

Note: These reagents have been successfully used in concentrations up to those given.
* Highest concentration tested at QIAGEN. Maximum concentration compatible with Ni-NTA may be higher.
**Data according to references from Qiagen: http://www.qiagen.com/literature/resources/Protein/CompatibilityTable.pdf.

# 2 Expression

## 2.1 Expression in *E. coli* with the *tet*-system (pASG-IBA vectors)

The production of a heterologous protein is often accompanied by an impaired growth of *E. coli* cells. Consequently, regulation of heterologous biosynthesis is generally recommended by the use of a promoter whose activity can be blocked by a repressor. If the foreign protein is cytotoxic, even the production of minute quantities can result in a dramatic selection against the *E. coli* cells which harbor the expression plasmid. In such cases, tight repression of the promoter is required. Synthesis of the gene product is then switched on in a controlled manner simply by adding a chemical inducer. The pASG His-tag expression vectors that carry the promoter/operator region from the *tet*A resistance gene are the state-of-the-art solution for such an inducible expression system [3,4]. The strength of the *tet*A promoter is comparable with that of the *lac*-UV5 promoter (nearly 25% activity of the T7 promoter).
It can be fully induced by adding anhydrotetracycline at a concentration that is not antibiotically effective (200 ng/ml). The constitutive expression of the *tet* repressor gene, which is also encoded on the expression plasmids, guarantees the repression of the promoter in the absence of the inducer. In a Western blot, no expression is detectable under these conditions [3,5]. In contrast to the *lac* promoter, which is susceptible to catabolite repression (cAMP-level, metabolic state) and chromosomally encoded repressor molecules, the *tet*A promoter/operator is not coupled to any cellular regulation mechanisms. Therefore, when using the *tet*-system, there are basically no restrictions in the choice of culture medium or *E. coli* expression strain. For example, glucose minimal media and even the bacterial strain XL1-Blue, which carries an episomal copy of the tetracycline resistance gene, can be used for expression.

| Recommended Buffers/Solutions | Concentration of ingredients | Notes |
|---|---|---|
| Ampicillin | stock solution 100 mg/ml in $H_2O$, sterile filtered | Store in aliquots at -20°C |
| Anhydrotetracycline | stock solution: 2 mg/ml in Dimethylformamid (DMF) | Store at -20°C |
| LB medium | 10 g/l trypton<br>5 g/l yeast extract<br>5 g/l NaCl | |
| Buffer W | 100 mM Tris/HCl<br>150 mM NaCl<br>1 mM EDTA<br>Adjust pH to 8 | |
| 5x SDS-PAGE sample buffer | 250 mM Tris/HCl<br>25% glycerol<br>7.5% SDS<br>0.25 mg/ml bromophenolblue<br>12.5% v/v mercaptoethanol<br>Adjust pH to 8 | |

**Important notes**

The *tet* promoter system is independent of the *E. coli* strain. Following strains were successfully tested: JM83, WK6, B, BL21, MG1655, W3110, XL1-Blue, BL21-CodonPlus™
→ We recommend JM83 or W3110 for periplasmic secretion.

**Protocol**

1. **Preculture: Inoculate 2 ml of LB medium containing 100 µg/ml ampicillin (pASG-IBA plasmids except 2C to 7C) or 30 µg/ml chloramphenicol (pASK-IBA2C to 7C) with a fresh colony harboring the pASG-IBA expression plasmid and shake overnight (200 rpm) at 37 °C.**

   The colony should not be older than 1 week.
   Do not inoculate from glycerol stocks.

   The yield of soluble, functional protein can be substantially increased in most cases by lowering the preculture growth temperature to 22 °C-30 °C. Take care that cells do not reach the stationary phase for extended periods prior to inoculating the production culture.

2. **Culture for expression: Inoculate 100 ml of LB medium containing 100 µg/ml ampicillin (or 30 µg/ml chloramphenicol) with the preculture and shake at 37 °C.**

3. **Monitor the optical density at 550 nm ($OD_{550}$).**
   Cell suspension with an $OD_{550}$ over 1.0 should be diluted with LB medium before measuring.

4. **Take a 1 ml sample immediately before induction.**
   This sample is the non-induced control; pellet cells (microfuge, 30 seconds) and resuspend them in 80 µl Buffer W. Add 20 µl 5x SDS-PAGE sample buffer. Store at -20°C until SDS-PAGE analysis. The whole sample must be incubated in an ultrasonic bath for 15 minutes to shear the chromosomal DNA into small pieces and should be heated to 70°C for 10 minutes prior to SDS-PAGE.

5. **When $OD_{550}$ equals 0.5-0.6, add 10 µl of anhydrotetracycline solution.**
   The yield of soluble, functional protein may be substantially increased, particularly in case of periplasmic expression, by lowering the growth temperature to 22°C - 30°C.

6. **Shake for 3 hours at 200 rpm.**
   Overnight induction may increase protein yields in some cases.

7. **Harvest the cells by centrifugation at 4500 x *g* for 12 minutes (4°C).**

8. **Proceed to "Preparation of Cleared Lysates" (page 15) or store cell pellet at -20°C.**

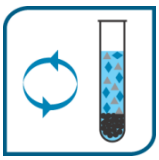
## 2.2 Expression with other systems

Successful bacterial expression has the advantage of obtaining the expression product in a short time at low costs. Nevertheless, there are proteins which cannot be expressed in *E. coli*. Yeast, insect, mammalian or plant cells are alternative expression hosts for such proteins. The His-tag can be expressed in eukaryotic cells. IBA's pESG-IBA line of vectors is designed for mammalian expression via the CMV promoter and they are compatible in many cases with pASG-IBA vectors so that a PCR fragment can be cloned in parallel for both mammalian and bacterial expression. Expression vectors for E. coli, yeast, insect, and mammalian protein expression are provided by IBA's StarGate System (for further information see https://www.iba-lifesciences.com/cloning-strategies.html).

## 2.3 Trouble shooting – Expression

| Problem | Comments and suggestions |
|---|---|
| No or low expression | Check the culture condition (e.g., IPTG, anhydrotetracycline, antibiotics).<br>Check vector (sequence, frame).<br>Check whether the protein is found in the insoluble fraction.<br>Reduction of temperature during cultivation may solve this problem (e.g., 16 °C, 22 °C, 26 °C, 30 °C).<br>Use another expression system (e.g., T7 promoter instead of Tet promoter).<br>Use eukaryotic cells for expression (yeast, insect or mammalian cells). |
| Protein is degraded | Use protease deficient *E. coli* strains.<br>If degradation occurs during cell lysis, add protease inhibitor.<br>If the protein is small (<10 kDa), consider adding a terminal carrier protein.<br>Lower temperature during induction can reduce the problem.<br>Secretion of the recombinant protein to the periplasmic space can reduce the problem. |
| Protein is secreted | Remove all signal sequences from the coding region. |
| Inclusion bodies are formed: protein is insoluble | Reduce expression level by modifying growth and induction conditions, e.g., lower culturing temperature (16 °C, 22 °C, 26 °C, 30 °C).<br>Use another expression system (e.g., Tet promoter instead of T7 promoter). |

# 3 Preparation of *E. coli* cell lysates



## 3.1 Preparation of cleared lysate after cytoplasmic expression of His-tag proteins under native conditions

| Recommended Buffers/Solutions | Concentration of ingredients |
|---|---|
| Ni-NTA Lysis Buffer | 50 mM $NaH_2PO_4$<br>300 mM NaCl<br>10 mM imidazole<br>Adjust pH to 8.0 |
| Lysozyme | |
| 5x SDS-PAGE sample buffer | 0.25 M Tris/HCl<br>25 % glycerol<br>7.5 % SDS<br>0.25 mg/ml bromophenolblue<br>12.5 % v/v mercaptoethanol<br>Adjust pH to 8.0 |

**Protocol**

1. **Thaw the cell pellet for 15 minutes on ice and resuspend the cells in Ni-NTA Lysis Buffer at 2-5 ml per gram wet weight.**
   Ni-NTA Lysis Buffer contains 10 mM imidazole to minimize binding of untagged, histidine rich contaminating proteins and increase purity with fewer wash steps.
   If the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1-5 mM. With His-tag proteins exhibiting high binding affinities, the imidazole concentration can be increased to 20 mM.

2. **Add lysozyme to 1 mg/ml and incubate on ice for 30 minutes.**

3. **Sonicate on ice.**
   Use six 10 second bursts at 200-300 W with a 10 second cooling period between each burst. Use a sonicator equipped with a microtip.

4. **(Optional) If the lysate is very viscous, add RNase A (10 µg/ml) and DNaseI (5 µg/ml) and incubate on ice for 10-15 min.**

5. **Centrifuge lysate at 10,000 x *g* for 20-30 minutes at 4°C to pellet the cellular debris.**
   A certain proportion of the cellular protein, including the His-tag protein, may remain insoluble and will be located in the pellet.

| | |
|---|---|
| **Protocol (continued)** | For more complete recovery of the tagged protein, this material must be solubilized using denaturing conditions.<br><br>**6. Add 5 µl 5x SDS-PAGE sample buffer to 20 µl supernatant and store at -20°C for SDS-PAGE analysis.**<br><br>**7. Proceed to protocols for His-tagged protein purification under native conditions (see protocols 4.1 to 4.4).** |

## 3.2 Preparation of cleared lysate after periplasmic expression of His-tag fusion proteins

Periplasmic proteins are proteins secreted into the periplasmic space located between the outer and inner membrane of *E. coli*. Proper secretion is possible only when the protein of interest has an N-terminal signal peptide which is cleaved after translocation. In order to purify proteins secreted into the periplasmic space [11, 12] using Ni-NTA chromatography resin, the His-tag must be fused to the C-terminus of the target protein. N-terminal His-tags might be processed with the transit signal.

| Recommended Buffers/Solutions | Concentration of ingredients |
|---|---|
| Sucrose buffer | 30 mM Tris/HCl<br>20% sucrose<br>Adjust pH to 8 |
| EDTA | 500 mM solution |
| $MgSO_4$ | 5 mM solution, ice-cold |
| Ni-NTA Lysis Buffer | 50 mM $NaH_2PO_4$<br>300 mM NaCl<br>10 mM imidazole<br>Adjust pH to 8 |

| | |
|---|---|
| **Protocol** | **1. Resuspend cell pellet in sucrose buffer at 80 ml per gram wet weight.**<br><br>**2. Keep the cells on ice and add 500 mM EDTA solution dropwise to 1 mM.**<br><br>**3. Incubate the cells on ice for 5-10 minutes with gentle agitation.**<br><br>**4. Centrifuge the cell suspension at 8000 x *g* for 20 minutes at 4°C.** |

| | |
|---|---|
| **Protocol (continued)** | **5. Remove all the supernatant, and resuspend the pellet in the same volume of ice-cold 5 mM $MgSO_4$ solution.**<br>**6. Shake or stir for 10 minutes in an ice bath.**<br>**7. Centrifuge at 8000 x *g* for 20 minutes at 4°C.**<br>The supernatant is the osmotic shock fluid containing periplasmic proteins.<br>**8. Dialyse supernatant extensively against Ni-NTA Lysis Buffer before continuing with the purification.**<br>**9. Proceed to protocols for His-tag proteins purification under native conditions (see protocols 4.1 to 4.4).** |

## 3.3 Preparation of cleared lysate of His-tag proteins under denaturing conditions

| Recommended Buffers/Solutions | Concentration of ingredients |
|---|---|
| 5x SDS-PAGE sample buffer | 0.25 M Tris/HCl<br>25% glycerol<br>7.5% SDS<br>0.25 mg/ml bromophenolblue<br>12.5% v/v mercaptoethanol<br>Adjust pH to 8 |
| Buffer B | 100 mM $NaH_2PO_4$<br>10 mM Tris/HCl<br>8 M urea<br>Adjust pH to 8 |

| | |
|---|---|
| **Important notes** | Cells can be lysed in either 6 M GuHCl or 8 M urea. It is preferable to lyse the cells in the milder denaturant, urea, so that the cell lysate can be analyzed directly by SDS-PAGE. GuHCl is a more efficient solubilization and cell lysis reagent, however, and may be required to solubilize some proteins. |

| | |
|---|---|
| **Protocol** | **1. Thaw the cell pellet for 15 minutes on ice and resuspend with Buffer B at 5 ml per gram wet weight.**<br>**2. Stir cells for 15-60 minutes at room temperature or lyse them by gently vortexing, taking care to avoid foaming.** Lysis is complete when the solution becomes translucent. |

| | |
|---|---|
| **Protocol, continued** | **3. Centrifuge lysate at 10,000 x g for 20-30 minutes at room temperature to pellet the cellular debris.**<br>Save supernatant (cleared lysate).<br><br>**4. Add 5 µl 5x SDS-PAGE sample buffer to 20 µl supernatant and store at -20°C until SDS-PAGE analysis.**<br><br>**5. Proceed to protocols for His-tag protein purification under denaturing conditions (see protocols 4.3 to 4.8).** |

# 4 Purification of His-tag proteins

## 4.1 Batch purification of His-tag proteins under native conditions

| Recommended Buffers/Solutions | Concentration of ingredients | Notes |
|---|---|---|
| Ni-NTA Superflow® or Ni-NTA Sepharose® | | The binding capacity of Ni-NTA resin is protein dependent and normally lies between 5 and 10 mg/ml (300-500 nmol of a 20 kDa protein) |
| Ni-NTA Lysis Buffer | 50 mM $NaH_2PO_4$<br>300 mM NaCl<br>10 mM imidazole<br>Adjust pH to 8 | The composition of lysis, wash and elution buffers can be modified to suit the particular application, e.g., by adding 0.1% Tween, 5-10 mM β-mercaptoethanol, or 1 mM PMSF, or increasing NaCl or glycerol concentrations.<br>For more information see table 1 on page 6. |
| Ni-NTA Wash Buffer | 50 mM $NaH_2PO_4$<br>300 mM NaCl<br>20 mM imidazole<br>Adjust pH to 8 | |
| Ni-NTA Elution Buffer | 50 mM $NaH_2PO_4$<br>300 mM NaCl<br>250 mM imidazole<br>Adjust pH to 8 | |

**Important notes**

- The amount of cells required depends on the expression level of the His-tag protein and the expression system used.
- The resins are supplied as 50% slurries.

**Protocol**

1. **Add 1 ml of the 50% Ni-NTA slurry to 4 ml cleared lysate (in Ni-NTA Lysis Buffer; see protocol 3.1 or 3.2) and mix gently by shaking (200 rpm on a rotary shaker) at 4 °C for 60 min.**
   10 mM (or 20mM) imidazole in Ni-NTA Lysis Buffer suppresses the binding of non-tagged contaminating proteins and leads to greater purity after fewer washing steps. If the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1-5 mM.

2. **Load the lysate/Ni-NTA mixture onto a column with capped bottom outlet.**

**Protocol (continued)**

3. **Remove bottom cap and collect the column flow-through.**
   Save flow-through for SDS-PAGE analysis.

4. **Wash twice with 4 ml Ni-NTA Wash Buffer; collect wash fractions for SDS-PAGE analysis.**
   20 mM imidazole in the Ni-NTA Wash Buffer elutes non-tagged contaminating proteins. Imidazole concentration can be reduced to 1-5 mM imidazole. This can result in loss of purity, but higher yields of recombinant protein.

5. **Elute the protein 4 times with 0.5 ml Ni-NTA Elution Buffer.**
   Collect the eluate in four tubes and analyse by SDS-PAGE.

## 4.2 Gravity flow purification of His-tag proteins under native conditions

| Recommended Buffers/Solutions | Concentration of ingredients | Notes |
|---|---|---|
| Ni-NTA Superflow or Ni-NTA Sepharose | | The binding capacity of Ni-NTA resin is protein dependent and normally is up to 50 mg/ml (up to 2500 nmol of a 20 kDa protein) |
| Ni-NTA Lysis Buffer | 50 mM $NaH_2PO_4$<br>300 mM NaCl<br>10 mM imidazole<br>Adjust pH to 8 | The composition of lysis, wash and elution buffers can be modified to suit the particular application, e.g., by adding 0.1% Tween, 5-10 mM β-mercaptoethanol, or 1 mM PMSF, or increasing NaCl or glycerol concentrations.<br>For more information see table 1 on page 6. |
| Ni-NTA Wash Buffer | 50 mM $NaH_2PO_4$<br>300 mM NaCl<br>20 mM imidazole<br>Adjust pH to 8 | |
| Ni-NTA Elution Buffer | 50 mM $NaH_2PO_4$<br>300 mM NaCl<br>250 mM imidazole<br>Adjust pH to 8 | |

**Important notes**

Generally, it is recommended to perform chromatography at 4°C. Depending on the individual equipment this is not always possible and chromatography has to be performed at room temperature. If cartridges are stored at 4°C and are transferred to room temperature air bubbles may form due to restricted solubility of air at elevated temperatures. Therefore, it is recommended to equilibrate the cartridges immediately after exposure to higher temperatures with buffer that is equilibrated at such temperatures.

**Protocol**

1. **Equilibrate the Ni-NTA column with 2 CV (column bed volumes) Ni-NTA Lysis Buffer.**
   Storage buffer is removed. The column cannot run dry under gravity flow.

2. **Centrifuge cleared lysate from protocol 3.1 or 3.2 (14,000 rpm, 5 minutes, 4°C, microfuge).**
   Insoluble aggregates are removed which otherwise could clog the column.

3. **Transfer supernatant to the column.**

4. **Once the cell extract has completely entered the column, wash the column 4 times with 2 CV Ni-NTA Wash Buffer.**
   Collect the eluate in 2 CV fractions and apply 2 µl of the first washing fraction and 20 µl of each subsequent fraction to an analytical SDS-PAGE.

5. **Add 6 times 0.5 CV Ni-NTA Elution Buffer and collect the eluate in 0.5 CV fractions.**
   **20 µl samples of each fraction can be used for SDS-PAGE analysis.**
   The purified His-tag fusion protein usually elutes in the 2$^{nd}$ to 5$^{th}$ fraction.

## 4.3 Batch purification of His-tag proteins under denaturing conditions

| Recommended Buffers/Solutions | Concentration of ingredients | Notes |
|---|---|---|
| Buffer B | 100 mM $NaH_2PO_4$<br>10 mM Tris/HCl<br>8 M urea<br>Adjust pH to 8.0 | Cells can be lysed in either 6 M GuHCl or 8 M urea. If GuHCl is used all buffers must include 6 M GuHCl instead of 8 M urea.<br><br>Due to the dissociation of urea, the pH of urea containing buffers should be adjusted immediately prior to use. Do not autoclave. |
| Buffer C<br>(Wash Buffer) | 100 mM $NaH_2PO_4$<br>10 mM Tris/HCl<br>8 M urea<br>Adjust pH to 6.3 | |
| Buffer D1<br>(Elution Buffer1) | 100 mM $NaH_2PO_4$<br>10 mM Tris/HCl<br>8 M urea<br>Adjust pH to 5.9 | |
| Buffer D2<br>(Elution Buffer2) | 100 mM $NaH_2PO_4$<br>10 mM Tris/HCl<br>8 M urea<br>Adjust pH to 4.5 | |

**Important notes**

- The amount of cells required depends on the expression level of the His-tag protein and the expression system used.
- The binding capacity of Ni-NTA resins is protein dependent and normally is up to 50 mg/ml (up to 2500 nmol of a 20 kDa protein).

**Protocol**

1. **Add 1 ml of the 50% Ni-NTA slurry to 4 ml lysate (see protocol 3.3) and mix gently by shaking (e.g., 200 rpm on a rotary shaker) for 15-60 minutes at room temperature.**
   For proteins that are expressed at very high levels (50-100 mg of His-tag protein per liter of cell culture), a 5x concentrated cell lysate (resuspend the pellet from a 20 ml culture in 4 ml Buffer B) can be used. 4 ml of a 5x concentrated cell lysate in Buffer B will contain approximately 1-2 mg of His-tag protein. For much lower expression levels (1-5 mg/liter), 200 ml of cell culture should be used for a 50x concentrated cell lysate (4 ml cell lysate = 0.2-1 mg of His-tag protein).

2. **Load lysate-resin mixture carefully into an empty column with the bottom cap still attached.**

| | |
|---|---|
| **Protocol (continued)** | **3. Remove the bottom cap and collect the flow-through.**<br>Collect flow-through for SDS-PAGE analysis.<br>**4. Wash twice with 4 ml Buffer C.**<br>Keep wash fractions for SDS-PAGE analysis.<br>**5. Elute the recombinant protein 4 times with 0.5 ml Buffer D1, followed by 4 times with 0.5 ml Buffer D2.**<br>Collect fractions and analyze by SDS-PAGE. |

## 4.4 Gravity flow purification of His-tag proteins under denaturing conditions

| Recommended Buffers/Solutions | Concentration of ingredients | Notes |
|---|---|---|
| Buffer B | 100 mM $NaH_2PO_4$<br>10 mM Tris/HCl<br>8 M urea<br>Adjust pH to 8.0 | Cells can be lysed in either 6 M GuHCl or 8 M urea. If GuHCl is used all buffers must include 6 M GuHCl instead of 8 M urea.<br><br>Due to the dissociation of urea, the pH of urea containing buffers should be adjusted immediately prior to use.<br>Do not autoclave. |
| Buffer C<br>(Washing Buffer) | 100 mM $NaH_2PO_4$<br>10 mM Tris/HCl<br>8 M urea<br>Adjust pH to 6.3 | |
| Buffer D1<br>(Elution Buffer1) | 100 mM $NaH_2PO_4$<br>10 mM Tris/HCl<br>8 M urea<br>Adjust pH to 5.9 | |
| Buffer D2<br>(Elution Buffer2) | 100 mM $NaH_2PO_4$<br>10 mM Tris/HCl<br>8 M urea<br>Adjust pH to 4.5 | |

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| **Important notes** | • Ni-NTA Superflow and Ni-NTA Sepharose are available for gravity flow column chromatography.<br>• The binding capacity of Ni-NTA Superflow is protein dependent and normally is up to 50 mg/ml (up to 2500 nmol of a 20 kDa protein). |

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| **Protocol** | **1. Equilibrate the Ni-NTA column with 2 CVs (column bed volumes) of Buffer B.**<br>Storage buffer is removed.<br>The column cannot run dry under gravity flow. |

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| **Protocol (continued)** | **2. Centrifuge cleared lysate from protocol 3.3 (14,000 rpm, 5 minutes, 4°C, microfuge).**<br>Insoluble aggregates which can clog the column are removed.<br><br>**3. Add cleared lysate supernatant to the column.**<br><br>**4. After the cell extract has completely entered the column, wash the column 8 times with 1 CV Buffer C.**<br>Collect the eluate in 1 CV fractions and apply 2 µl of the first fraction and 20 µl of each subsequent fraction to an analytical SDS-PAGE.<br><br>**5. Add 6 times 0.5 CVs Buffer D1 and collect the eluate in 0.5 CV fractions.**<br>Samples of each fraction can be used for SDS-PAGE analysis.<br>If elution is incomplete with Buffer D1, Buffer D2 should be used. |

## 4.5 Troubleshooting

### 4.5.1 "Protein does not bind to Ni-NTA"

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| His-tag is not present. | Sequence ligation junctions to ensure that the reading frame is correct.<br>Check for possible internal translation starts (N-terminal tag) or premature termination sites (C-terminal). |
| His-tag is inaccessible. | Purify protein under denaturing conditions.<br>Move tag to the other terminus of the protein. |
| His-tag has been degraded. | Check that the His-tag is not associated with a portion of the protein that is processed. |
| Binding conditions incorrect. | Check pH and compositions of all buffers and solutions. Dissociation of urea often causes a shift in pH. The pH values should be checked immediately prior to use.<br>Ensure that there are no chelating or reducing agents present, and that the concentration of imidazole is not too high. |

### 4.5.2 "Protein elutes in the Ni-NTA Wash Buffer"

| | |
|---|---|
| Wash stringency is too high. | Lower the concentration of imidazole or increase the pH slightly. |
| His-tag is partially hidden. | Reduce washing stringency. Purify under denaturing conditions. |
| Buffer conditions incorrect. | Check pH and composition of Ni-NTA Wash Buffer.<br>Ensure that there are no chelating or reducing agents present. |

### 4.5.3 "Protein precipitates during purification"

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|---|---|
| Temperature is too low. | Perform purification at room temperature. |
| Protein forms aggregates. | Try adding solubilization reagents such as 0.1% Triton X-100 or Tween-20, up to 20 mM β-mercaptoethanol, up to 2 M NaCl, or stabilizing cofactors such as $Mg^{2+}$. These may be necessary in all buffers to maintain protein solubility. |

### 4.5.4 "Protein does not elute"

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| Elution conditions are too mild (protein may be in an aggregate or multimer form). | Elute with a pH or imidazole step-gradient to determine the optimal elution conditions. |
| Protein has precipitated in the column. | Elute under denaturing conditions.<br>Perform binding and elution in batch format to avoid high local protein concentrations. |

### 4.5.5 "Protein elutes with contaminants"

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| Binding and washing conditions are not stringent enough. | Include 10-20 mM imidazole in the binding and wash buffers. |
| Column is too large. | Reduce the amount of Ni-NTA resin. |
| Contaminants are associated with tagged protein. | Add β-mercaptoethanol to a maximum of 20 mM to reduce disulfide bonds.<br>Increase salt and/or detergent concentrations in the Wash Buffer to disrupt nonspecific interactions. |
| Contaminants are truncated forms of the tagged protein. | Check for possible internal translation starts (C-terminal tag) or premature termination sites (N-terminal tag). Prevent protein degradation during purification by working at 4°C or by including protease inhibitors. Fuse a Strep-tag to the other terminus of the protein to select for full length proteins by a two-step purification by means of both tags. |

### 4.5.6 "Discoloration of resin"

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| Nickel ions are removed or reduced. | Ensure that there are no chelating compounds (resin color turns white) or reducing agents (resin color turns brown) present in all buffers. |

# 5 References

For up-to-date references see www.iba-lifesciences.com

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