Purification

Optimal expression of recombinant proteins in various expression systems including *E. coli* can be easily achieved when the vectors and host cells are carefully chosen, and the growth conditions are properly controlled. Culture conditions and the induction of expression have profound effects on the way the recombinant protein is produced, and in this context directly influence the strategies employed for protein purification. It is therefore advisable to empirically establish optimal conditions with small-scale cultures before purification on a larger scale is attempted. Recombinant proteins expressed in *E. coli* can be produced in a soluble form, but in many cases, especially at high expression levels, they aggregate and form insoluble inclusion bodies. The formation of inclusion bodies is influenced by the nature of the protein, by the host cell, and by the level of expression resulting from the vector choice and the growth and induction conditions. Inclusion bodies invariably limit the utility of standard purification procedures which rely on the protein's native soluble form. Purification of 6xHis-tagged proteins by Ni-NTA affinity chromatography, however, can be performed under native or denaturing conditions and is not affected by problems arising from protein insolubility. Most proteins in inclusion bodies are simply solubilized with detergents or denaturants such as 8 M urea or 6 M GuHCl before the purification steps are initiated.

The basic principles pertaining to the Ni-NTA affinity purification procedure under native or denaturing conditions are outlined in the flowchart (Figure 20); more details appear in the following sections.

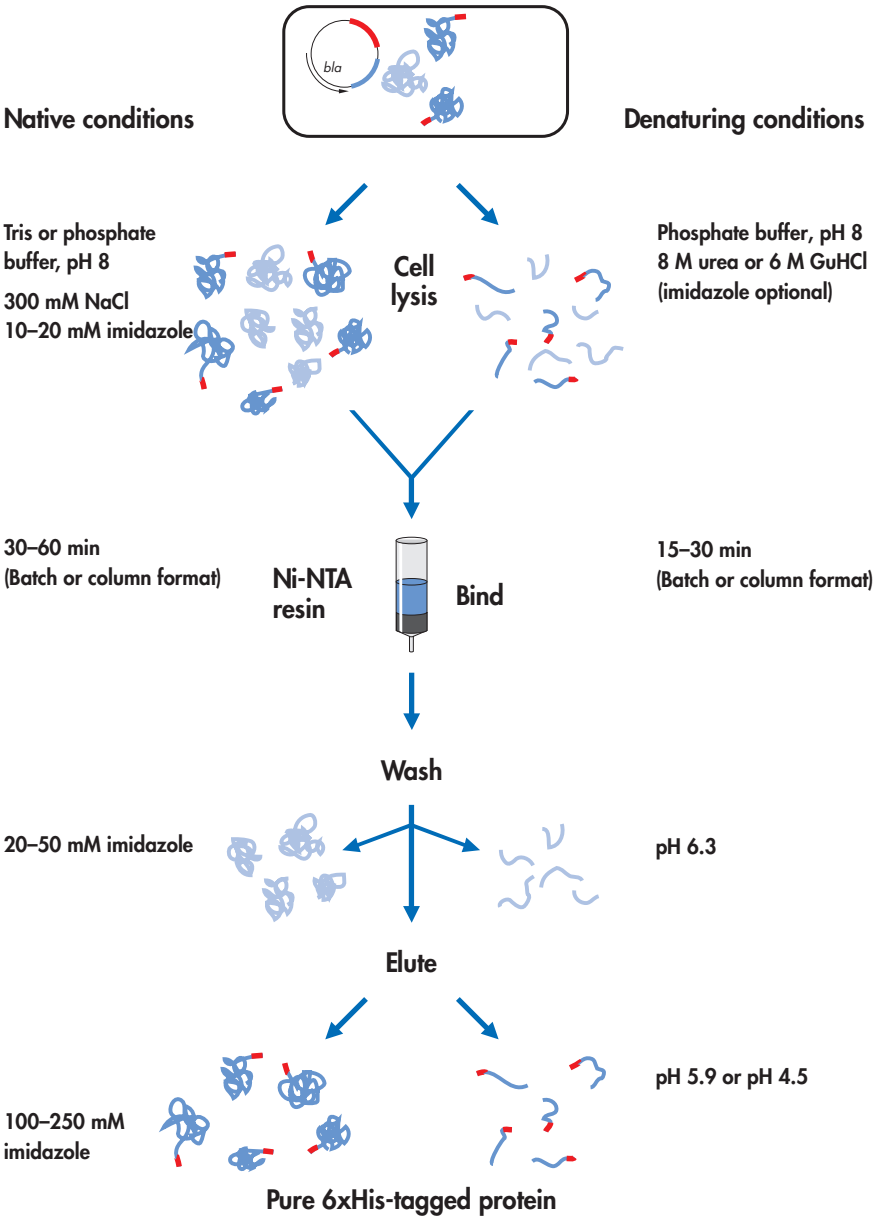

Figure 20. *Purification of 6xHis-tagged proteins using the QIAexpress System.*

Basic principles

Culture size

Optimal purification is dependent on a number of factors, including the amount of 6xHis-tagged protein required and expression level.

The amount of culture required depends on the level at which the protein is expressed, which must be determined empirically for each expression experiment. If the protein is not expressed efficiently, bacteria from a large culture volume must be lysed in a given volume of lysis buffer corresponding to a high "concentration factor". The concentration factor is defined as the ratio of the culture size to the amount of lysis buffer used. Examples of the relationship between expression levels and recommended culture volumes and concentration factors are provided in Table 3.

For example, if a protein that is expressed at 0.1 mg/liter and a miniature batch purification procedure will be used under denaturing conditions (see Protocol 19, page 92), a 100 ml culture should be concentrated 100-fold by resuspending the pellet in 1 ml buffer.

Under native conditions, it is more difficult to predict the amount of soluble protein present in the lysate. Generally, 50- to 100-fold concentration is recommended to purify significant amounts of 6xHis-tagged protein.

Table 3. Determination of cell culture volume requirements

Concentration of 6xHis-tagged protein	Expression level	Culture volume	Amount of 6xHis-tagged protein	Concentration factor*
Denaturing conditions				
50 mg/liter	40%	3 ml	150 µg	3x
10 mg/liter	8%	10 ml	100 µg	10x
2 mg/liter	1.6%	25 ml	50 µg	25x
0.5 mg/liter	0.4%	50 ml	25 µg	50x
0.1 mg/liter	0.08%	100 ml	10 µg	100x
Native conditions				
>1 mg/liter	>1%	50 ml	>50 µg	50x
<1 mg/liter	<1%	100 ml	<100 µg	100x

**After lysis in 1 ml.*

Reducing nonspecific binding

Since there is a higher potential for binding background contaminants under native conditions than under denaturing conditions, low concentrations of imidazole in the lysis and wash buffers (10–20 mM) are recommended. The imidazole ring is part of the structure of histidine (Figure 21). The imidazole rings in the histidine residues of the 6xHis tag bind to the nickel ions immobilized by the NTA groups on the matrix. Imidazole itself can also bind to the nickel ions and disrupt the binding of dispersed histidine residues in nontagged background proteins. At low imidazole concentrations, nonspecific, low-affinity binding of background proteins is prevented, while 6xHis-tagged proteins still bind strongly to the Ni-NTA matrix. Therefore, adding imidazole to the lysis buffer leads to greater purity in fewer steps. For most proteins, up to 20 mM imidazole can be used without affecting the yield. If the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 1–5 mM.

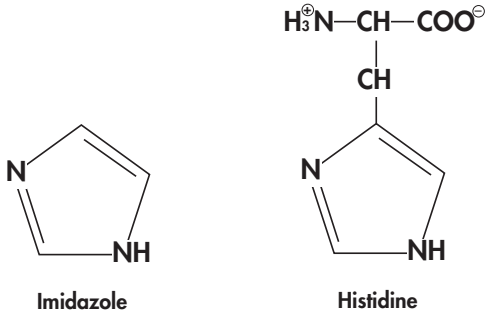

Figure 21. *Chemical structures of histidine and imidazole.*

Binding of tagged proteins to Ni-NTA resin is not conformation-dependent and is not affected by most detergents and denaturants (Table 4, page 74). The stability of the 6xHis–Ni-NTA interaction in the presence of low levels of β-ME (up to 20 mM) in the lysis buffer can be used to prevent the copurification of host proteins that may have formed disulfide bonds with the protein of interest during cell lysis. Detergents such as Triton X-100 and Tween 20 (up to 2%), or high salt concentrations (up to 2 M NaCl) (Table 4, page 74), also have no effect on binding, and may reduce nonspecific binding to the matrix due to nonspecific hydrophobic or ionic interactions. Nucleic acids that might associate with certain DNA and RNA-binding proteins are also removed without affecting the recovery of the 6xHis-tagged protein.

Protein solubility and intracellular location

Since the interaction between Ni-NTA and the 6xHis tag of the recombinant protein does not depend on tertiary structure, proteins can be purified either under native or denaturing conditions. To set up the best purification strategy, it is important to determine whether

the protein is soluble in the cytoplasm or located in cytoplasmic inclusion bodies (see Protocol 6, page 59). Many proteins form inclusion bodies when they are expressed at high levels in bacteria, while others are tolerated well by the cell and remain in the cytoplasm in their native configuration. Proteins that contain appropriate leader peptide sequences may be secreted into the periplasmic space, but this depends on the host cell and on the nature of both the leader peptide and the recombinant protein.

Note: When leader peptides are used, the 6xHis tag cannot be located at the N-terminus, because it would be removed together with the leader sequence following secretion.

Purification under native or denaturing conditions

The decision whether to purify 6xHis-tagged proteins under native or denaturing conditions depends on protein location and solubility, the accessibility of the 6xHis tag, the downstream application, and whether biological activity must be retained. Furthermore, if efficient renaturing procedures are available, denaturing purification and subsequent refolding may be considered.

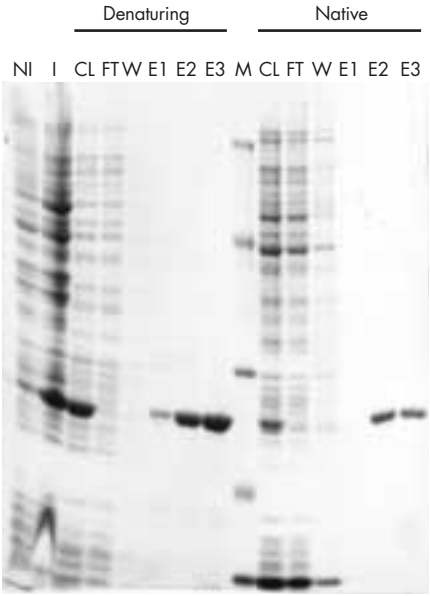

Figure 22. *Denaturing and native purification of heterologously expressed DHFR.* ***NI****: noninduced cells;* ***I****: cells induced with IPTG;* ***CL****: cleared lysate;* ***FT****: flow-through;* ***W****: wash;* ***E1–E3****: eluates. 10% of DHFR is present in soluble form, which can be effectively purified under native conditions.*

Purification under native conditions

If purification under native conditions is preferred or necessary, the 6xHis-tagged protein must be soluble. However, even when most of the protein is present in inclusion bodies, there is generally some soluble material that can be purified in its native form (Figure 22). Purification of tagged proteins under native conditions can be exploited to copurify associated proteins such as enzyme subunits and binding proteins present in the expressing cells (LeGrice *et al.* 1990; Garner *et al.* 1992; Flachmann and Kühlbrandt 1996), added to the lysate prior to purification, or added to the Ni-NTA matrix after the 6xHis-tagged protein is bound.

The potential for unrelated, nontagged proteins to interact with the Ni-NTA resin is usually higher under native than under denaturing conditions. This is reflected in the larger number of proteins that appear in the first wash (Figure 23). Nonspecific binding can be reduced by including a low concentration of imidazole (10–20 mM) in the lysis and wash buffers.

In rare cases the 6xHis tag is hidden by the tertiary structure of the native protein, so that soluble proteins require denaturation before they can be purified on Ni-NTA. As a control, a parallel purification under denaturing conditions should always be carried out. If purification is only possible under denaturing conditions, and this is undesirable, the problem with inaccessible tags can generally be solved by moving the tag to the opposite terminus of the protein.

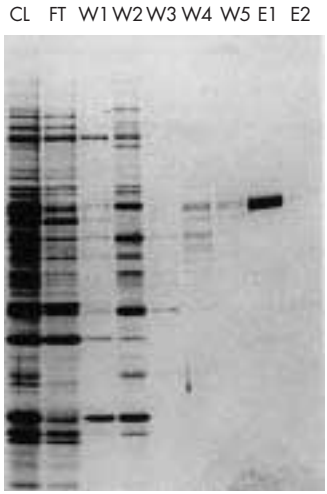

Figure 23. *Purification under native conditions. Human serum response factor (SRF) was expressed from a vaccinia virus vector in HeLa cells and purified using Ni-NTA Agarose with the indicated imidazole concentrations in the wash and elution steps. Proteins were visualized by Coomassie staining.* ***CL****: cell lysate;* ***FT****: flow-through;* ***W1****: 0.8-mM wash;* ***W2 & W3****: 8-mM wash;* ***W4 & W5****: 40-mM wash;* ***E1 & E2****: 80-mM elution. (Reproduced by kind permission of H. Stunnenberg, EMBL, Heidelberg, Germany)*

It is difficult to provide a general protocol for the purification of 6xHis-tagged proteins under native conditions because there is significant variation in protein structure, which can interfere with binding. However there are some general suggestions that may aid in optimizing the native purification procedure:

- Cells can be lysed by sonication or homogenization after treatment with lysozyme. To prevent protein degradation, cells and protein solutions should be kept at 0–4°C at all times; the addition of protease inhibitors may also be necessary.
- Low concentrations of imidazole in the lysis and wash buffers minimize nonspecific binding and reduce the amount of contaminating proteins.
- All buffers should have sufficient ionic strength to prevent nonspecific interactions between proteins and the Ni-NTA matrix. The minimum salt concentration during binding and washing steps should be 300 mM NaCl. The maximal concentration is 2 M NaCl (Table 4, page 74).

Secretion efficiency for proteins containing an export signal sequence can be determined for secreted proteins by purification of the periplasmic extract after osmotic shock (see Protocol 11, page 81).

Purification under denaturing conditions

High levels of expression of recombinant proteins in a variety of expression systems can lead to the formation of insoluble aggregates; in *E. coli*, these are known as inclusion bodies. Strong denaturants such as 6 M GuHCl or 8 M urea completely solubilize inclusion bodies, and 6xHis-tagged proteins. Other denaturants or detergents can be used as well, but the choice of these reagents and the specific concentrations needed must be established empirically. Under denaturing conditions, the 6xHis tag on a protein will be fully exposed so that binding to the Ni-NTA matrix will improve, and the efficiency of the purification procedure will be maximized by reducing the potential for nonspecific binding.

6xHis-tagged proteins purified under denaturing conditions can be used directly, or may have to be renatured and refolded. Protein renaturation and refolding can be carried out on the Ni-NTA column itself prior to elution (Holzinger *et al.* 1996), or in solution (Wingfield *et al.* 1995a); additional suggestions are included in this manual (see "Protein refolding recommendations", page 106).

Batch or column purification

Proteins may be purified on Ni-NTA resins in either a batch or a column procedure. The batch procedure entails binding the protein to the Ni-NTA resin in solution and then packing the protein–resin complex into a column for the washing and elution steps. This strategy promotes efficient binding of the 6xHis-tagged protein especially when the 6xHis tag is not fully accessible or when the protein in the lysate is present at a very low concentration.

In the column procedure, the Ni-NTA resin is first packed into the column and equilibrated with the lysis buffer. The cell lysate is then slowly applied to the column. Washing and elution steps are identical in the batch and column procedure.

Protein binding

Proteins containing one or more 6xHis affinity tags, located at either the amino and/or carboxyl terminus of the protein, can bind to the Ni-NTA groups on the matrix with an affinity far greater than that of antibody–antigen or enzyme–substrate interactions. Binding of the 6xHis tag does not depend on the three-dimensional structure of the protein. Even when the tag is not completely accessible it will bind as long as more than two histidine residues are available to interact with the nickel ion; in general, the smaller the number of accessible histidine residues, the weaker the binding will be. Untagged proteins that have histidine residues in close proximity on their surface will also bind to Ni-NTA, but in most cases this interaction will be much weaker than the binding of the 6xHis tag. Any host proteins that bind nonspecifically to the NTA resin itself can be easily washed away under relatively stringent conditions that do not affect the binding of 6xHis-tagged proteins.

Binding can be carried out in a batch or column mode (see "Batch or column purification", page 69). If the concentration of 6xHis-tagged proteins is low, or if they are expressed at low levels, or secreted into the media, the proteins should be bound to Ni-NTA in a batch procedure, and under conditions in which background proteins do not compete for the binding sites, i.e. at a slightly reduced pH or in the presence of low imidazole concentrations (10–20 mM). At low expression levels under native conditions, binding can be optimized for every protein by adjusting the imidazole concentration and/or pH of the lysis buffer. If high levels of background proteins are still present, equilibrating the Ni-NTA matrix with lysis buffer containing 10–20 mM imidazole prior to binding is recommended. The matrix is thus "shielded", and nonspecific binding of proteins that weakly interact is significantly reduced.

Wash

Endogenous proteins with histidine residues that interact with the Ni-NTA groups can be washed out of the matrix with stringent conditions achieved by lowering the pH to 6.3 or by adding imidazole at a 10–50 mM concentration. In bacterial expression systems, the recombinant proteins are usually expressed at high levels, and the level of copurifying contaminant proteins is relatively low. Therefore it generally is not necessary to wash the bound 6xHis-tagged protein under very stringent conditions. In lysates derived from eukaryotic expression systems the relative abundance of proteins that may contain neighboring histidines is higher; the resulting background problem becomes more critical especially when nondenaturing procedures are employed. In these instances it becomes necessary to increase the stringency of the wash steps considerably. This can be performed most effectively by gradually decreasing the pH of the wash buffer or by slowly increasing the concentration of imidazole in defined steps; step-gradients are preferable because they are much more effective than linear gradients when metal affinity chromatography methods are employed. The optimal pH and/or imidazole concentrations for the washes will vary slightly for each protein and must be determined empirically.

Protein elution

The histidine residues in the 6xHis tag have a pK_a of approximately 6.0 and will become protonated if the pH is reduced (pH 4.5–5.3). Under these conditions the 6xHis-tagged protein can no longer bind to the nickel ions and will dissociate from the Ni-NTA resin. Similarly, if the imidazole concentration is increased to 100–250 mM, the 6xHis-tagged proteins will also dissociate because they can no longer compete for binding sites on the Ni-NTA resin.

Elution conditions are highly reproducible, but must be determined for each 6xHis-tagged protein which is being purified. Monomers generally elute at approximately pH 5.9, whereas aggregates and proteins that contain more than one 6xHis tag elute at approximately pH 4.5.

Reagents such as EDTA or EGTA chelate the nickel ions and remove them from the NTA groups. This causes the 6xHis-tagged protein to elute as a protein–metal complex. NTA resins that have lost their nickel ions become white in color and must be recharged if they are to be reused.

Whereas all elution methods (imidazole, pH, and EDTA) are equally effective, imidazole is mildest and is recommended under native conditions, when the protein would be damaged by a reduction in pH, or when the presence of metal ions in the eluate may have an adverse effect on the purified protein.

Removal of the 6xHis tag

Although it is rarely necessary to remove the short 6xHis affinity tag from the recombinant protein after purification, there are some applications, such as structural analysis by X-ray crystallography or NMR, where the removal of the tag may be desirable. In order to cleave off an N-terminal tag the protease cleavage site must be inserted between the coding sequences of the 6xHis tag and the N-terminus of the protein. Factor Xa Protease recognizes the amino acid sequence Ile-Glu-Gly-Arg and cleaves the peptide bond C-terminal of the arginine residue. The expression vector pQE-30 Xa encodes a Factor Xa Protease recognition site between the N-terminal 6xHis-tag sequence and the multiple cloning site. If the gene of interest is cloned blunt ended at the 5´-end using the *Stu*I restriction site of the vector, Factor Xa Protease cleavage of the purified recombinant protein results in a protein product without any vector-derived amino acids at the N-terminus. After protease digestion, the protein of interest can be repurified in two steps. Xa Removal Resin binds Factor Xa Protease in a batch procedure, and is removed by centrifugation. Subsequently, cleaved 6xHis-tag peptides and undigested 6xHis-tagged protein can be captured by Ni-NTA affinity chromatography (see Protocol 20, page 93).

Specific considerations

Contaminating proteins

Proteins that contain neighboring histidines are not common in bacteria, but are quite abundant in eukaryotic cells. These proteins bind to the Ni-NTA resin much more weakly than proteins with a 6xHis tag, and can thus be easily washed away, even when they are much more abundant than the tagged protein (Janknecht *et al.* 1991). The addition of low concentrations of imidazole in the binding and wash buffers is very effective in preventing contaminating proteins from binding to the Ni-NTA matrix in the first place. This is especially important when purifying 6xHis-tagged proteins under native conditions.

The addition of 20 mM β-mercaptoethanol (β-ME) to the lysis buffer will reduce disulfide bonds that may have formed between contaminating proteins and the 6xHis-tagged protein. Dithiothreitol (DTT) should not be used (see Table 4, page 74).

Proteins that copurify because they are linked to the 6xHis-tagged protein, proteins that associate nonspecifically with the tagged protein, and nucleic acids that associate with the tagged protein can appear as contaminants in the eluate. All of these contaminants can be easily removed by washing the resin under more stringent conditions, by adding low concentrations of a detergent (0.1–1% Triton X-100 or 0.3% Sarkosyl), by increasing the salt concentration to up to 2 M NaCl, or by including ethanol or glycerol to reduce hydrophobic interactions. The optimal amounts of any of these reagents should be determined empirically for each purification protocol.

In some rare cases nontagged, cellular proteins may bind to the carbohydrate resin of Ni-NTA Agarose or Ni-NTA Superflow itself.

Truncated 6xHis-tagged proteins are common contaminants that are copurified by Ni-NTA affinity chromatography. These contaminants result from internal initiation of translation (C-terminally tagged proteins), from premature translation termination (N-terminally tagged proteins), or from protein degradation during protein expression or purification. This can be assessed by monitoring the size of 6xHis-tagged proteins using 6xHis detection reagents (Anti·His Antibodies or Ni-NTA Conjugates) in western blots. Changing the location of the tag to the amino or carboxyl terminus may be necessary to eliminate or minimize the appearance of these truncated forms. Protease inhibitors may also have to be added before and during the lysis steps to minimize protein degradation.

It is preferable to choose column size and total binding capacity to approximately match the amount of 6xHis-tagged protein to be purified (Schmitt *et al.* 1993a). 6xHis-tagged proteins have a higher affinity for the Ni-NTA resin than background proteins. Consequently very few nontagged proteins will be retained on the resin if nearly all available binding sites are occupied by the tagged protein. If too much Ni-NTA matrix is used, other proteins may nonspecifically bind to unoccupied sites and elute as contaminants.

Limitations

Ni-NTA matrices should not be exposed to high concentration of strong reducing agents such as DTT or DTE; these reagents reduce the nickel ions and thereby prevent them from binding 6xHis-tagged proteins. Ni-NTA resins that have been reduced turn brown in color. In most situations, β-mercaptoethanol can be used at concentrations up to 20 mM.

EDTA, EGTA, or any other strong chelating agents bind nickel ions and strip them from the NTA matrices. NTA resins become white in the absence of nickel ions.

Use any reducing or chelating agent with care, and if in doubt, test it on a small amount of Ni-NTA resin. High concentrations of buffer components containing strong electron-donating groups (e.g., NH_4^+), or amino acids such as arginine, glutamine, glycine, or histidine in the lysate should also be avoided.

Cells should be lysed without the use of strong chelating agents such as EDTA, strong reducing agents such as DTT, or ionic detergents such as SDS. Although there are instances in which small amounts of these reagents have been used successfully, we do not recommend their use.

For more detailed information, refer to Table 4, page 74.

Table 4. Compatibility of reagents with Ni-NTA matrices

Reagent	Effect	Comments
Buffer reagents		
Tris, HEPES, MOPS	• Buffers with secondary or tertiary amines will reduce nickel ions	• Up to 100 mM has been used successfully in some cases • Sodium phosphate or phosphate-citrate buffer is recommended
Chelating reagents		
EDTA, EGTA	• Strip 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
DTT, DTE	• Low concentrations will reduce nickel ions	• A maximum of 1 mM may be used, but β-mercaptoethanol is recommended
Detergents		
Nonionic detergents (Triton, Tween, NP-40, etc.)	• Removes background proteins and nucleic acids	• Up to 2% can be used
Cationic detergents		• Up to 1% can be used
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
Denaturants	• Solubilize proteins	
GuHCl		• Up to 6 M
Urea		• Up to 8 M
Amino acids		
Glycine		• Not recommended
Glutamine		• Not recommended

Table continued overleaf

Table 4. Continued

Reagent	Effect	Comments
Arginine		• Not recommended
Histidine	• Binds to Ni-NTA and competes with histidine residues in the 6xHis tag	• Can be used at low concentrations (20 mM) to inhibit non-specific binding and, at higher concentrations (>100 mM), to elute the 6xHis-tagged protein from the Ni-NTA matrix
Other additives		
NaCl	• Prevents ionic interactions	• Up to 2 M can be used, at least 300mM should be used
$MgCl_2$		• 4 M
$CaCl_2$		• 5 mM
Glycerol	• Prevents hydrophobic interaction between proteins	• Up to 50%
Ethanol	• Prevents hydrophobic interactions between proteins	• Up to 20%
Imidazole	• Binds to Ni-NTA and competes with histidine residues in the 6xHis tag	• Can be used at low concentrations (20 mM) to inhibit non-specific binding and, at higher concentrations (>100 mM), to elute the 6xHis-tagged protein from the Ni-NTA matrix
Sodium bicarbonate		• Not recommended
Hemoglobin		• Not recommended
Ammonium		• Not recommended
Citrate		• Up to 60 mM has been used successfully

Purification of 6xHis-tagged proteins produced in other expression systems

Purification of 6xHis-tagged proteins expressed in mammalian cells

Purification of 6xHis-tagged recombinant proteins expressed intracellularly can pose problems. Some of the difficulties of using mammalian cells are that expression levels are typically lower than in bacterial systems and that often only small amounts of cell material are available.

- The total protein content in HeLa cells, for example, is only 3000 µg per 10^7 cells. With recombinant protein expression levels at 0.01 to 1% of total protein, the theoretical maximum protein yield is 0.3 to 30 µg per 10^7 cells.
- Cells can be lysed by sonication, by using freeze-thaw cycles, or by treatment with non-ionic detergents.
- Adjustment of the binding capacity of the Ni-NTA matrix used to the amount of solubilized 6xHis-tagged protein to be captured is crucial for optimal performance and reduced copurification of contaminants.

Ni-NTA Magnetic Agarose Beads are an ideal Ni-NTA matrix for micro-scale protein purification from 10^7–10^8 cells. The total binding capacity of the beads used (3 µg DHFR [24 kDa] binds per 10 µl magnetic bead suspension) can be easily adjusted to the amount of 6xHis-tagged protein expressed by 10^7–10^8 cells allowing efficient purification even from dilute solutions of recombinant protein. Furthermore, proteins can be eluted into very small elution volumes allowing detection of the purified proteins using Coomassie-stained SDS polyacrylamide gels (Wahle *et al.* 1999). A detailed protocol is described on pages 86–87 (Protocol 15). For large-scale purification from larger amounts of cells we recommend using Ni-NTA Agarose or Ni-NTA Superflow which bind 0.5–1 mg of a 24 kDa 6xHis tagged protein per 100 µl resin. Cell lysis can be performed by simply scaling up the micro-scale protocol.

In the hope of increasing the expression rates and of facilitating the purification of the protein of interest without having to resort to cell lysis, it is often attractive to exploit secretion of the 6xHis-tagged protein directly into the medium. Mammalian cell culture media are often supplemented with serum proteins which bind weakly to the Ni-NTA matrix and compete with the 6xHis-tagged protein for binding sites. Amino acids with electron-donating groups such as glutamine or histidine are commonly added to media and have a similar effect.

- First try to purify the 6xHis-tagged protein directly from your medium. Experiments with some widely used media (DMEM, DMEM+10% fetal calf serum, CHO-S-SFMII, and RPMI 1640) showed purification recovery rates of 6xHis-tagged thioredoxin in the range of 45–75% (Wahle *et al.* 1999).

- If the efficiency of 6xHis-tagged protein purification directly from the culture medium is not efficient enough, there are several ways to make the binding conditions more suitable.

We have obtained good results by adding 1/10 volume of a 10x buffer containing, for example, 500 mM NaH_2PO_4, pH 8.0, 1.5 M NaCl, 100 mM imidazole to the medium. This results in appropriate composition and pH (8.0) of the medium for binding 6xHis-tagged proteins. The pH as well as concentrations of NaH_2PO_4, imidazole, and NaCl are then similar to those in the lysis buffer recommended for purification under native conditions. In the example, NaCl is added to increase the final concentration by 150 mM because most media contain NaCl in physiological concentrations resulting in a final concentration of 300 mM. Variations in the concentrations of NaCl and imidazole have to be considered depending on the culture medium used and 6xHis-tagged protein to be purified.
Alternative methods such as dialysis of the medium against a buffer providing optimal binding conditions, size-exclusion chromatography, or ion-exchange chromatography can also be considered (Coligan *et al.* 1995; Deutscher 1990).

Purification of 6xHis-tagged proteins expressed in insect cells

If 6xHis-tagged proteins are expressed intracellularly without virus-mediated lysis of the cells, purification can be performed as described for mammalian systems (see "Purification of 6xHis-tagged proteins expressed in mammalian cells", page 76 and protocol 15, page 86).

Expression of 6xHis-tagged proteins secreted directly into the medium can pose some problems. Media used to culture insect cells usually have an acidic pH (6.0–6.5) or contain electron-donating groups that can prevent binding of the 6xHis-tagged protein to Ni-NTA. Amino acids such as glutamine, glycine, or histidine are present at significantly higher concentrations in media for growing insect cells than in media for growing mammalian cells and compete with the 6xHis-tagged protein for binding sites on Ni-NTA matrices. Grace's medium (Life Technologies), for example, contains approximately 10 mM glutamine, 10 mM glycine, and 15 mM histidine. Table 5 summarizes the results of experiments where we have analyzed recovery rates after purification directly from various media. 6xHis-tagged thioredoxin and 6xHis-tagged chloramphenicol acetyl transferase (CAT) were added to some widely used insect cell media and purified with Ni-NTA Agarose. Recovery rates were between 30 and 100%.

Table 5. Recovery rates of protein purification from insect cell media (as a percentage of the control*)

Medium	Recovery rate for 6xHis-CAT	Recovery rate for 6xHis-thioredoxin
High Five™ Serum-Free Medium	100	100
TMN-FH Serum-containing Medium	75	80
Grace's Serum-containing Medium	60	70
SF-900 Serum-Free Medium	100	95
TC-100 Serum-Free Medium	30	50
BaculoGold™ Serum-Free Medium	100	100

** Control: Purification of CAT and thioredoxin from 50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0*

- First try to purify the 6xHis-tagged protein directly from your media.
- If purification efficiency is not sufficient, several options for optimizing binding conditions can be tested as follows.

Dialysis of the medium against a buffer with the appropriate composition and pH (8.0) similar to the lysis buffer recommended for purification under native conditions usually restores optimal binding conditions. Note that depending on the media used a white precipitate (probably made up of insoluble salts) can occur, but normally the 6xHis-tagged protein remains in solution. This can be tested by either protein quantitation if using a protein-free medium or by monitoring the amount of 6xHis-tagged protein by western-blot analysis using the QIA*express* Detection System (RGS·His, Penta·His, or Tetra·His Antibodies). After centrifugation, 6xHis-tagged protein can be directly purified from the cleared supernatant.

Alternatively, the pH of the medium can be adjusted to 8.0 with a phosphate or Tris·Cl buffer, but again salt precipitation may occur.

Other methods such as size-exclusion chromatography or ion-exchange chromatography can also be considered (Coligan *et al.* 1995; Deutscher 1990).

Purification procedures

Protocol 9. Preparation of cleared *E. coli* lysates under native conditions

Materials

Cell pellet

Lysis buffer

Lysozyme

2x SDS-PAGE sample buffer

Buffer compositions are provided in the appendix on pages 111–114.

1. **Thaw the cell pellet for 15 min on ice and resuspend the cells in lysis buffer at 2–5 ml per gram wet weight.**

   The amount of cells required depends on the expression level of the 6xHis-tagged protein and the expression system used. The binding capacity of Ni-NTA resins is protein-dependent and normally lies between 5–10 mg/ml. For example, Ni-NTA Agarose or Ni-NTA Superflow has a binding capacity of 0.3 µmol/ml (8.0 mg/ml) for 6xHis-tagged DHFR (~26 kDa). **Refer to Table 3 "Determination of cell culture volume requirements" on page 65.**

   Lysis buffer contains 10 mM imidazole to minimize binding of untagged, 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 6xHis-tagged 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 min.**

3. **Sonicate on ice.**

   Use six 10 s bursts at 200–300 W with a 10 s 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 DNase I (5 µg/ml) and incubate on ice for 10–15 min.**

   Alternatively, draw the lysate through a narrow-gauge syringe needle several times.

5. **Centrifuge lysate at 10,000 x *g* for 20–30 min at 4°C to pellet the cellular debris. Save supernatant.**

   A certain proportion of the cellular protein, including the 6xHis-tagged protein, may remain insoluble and will be located in the pellet. For more complete recovery of the tagged protein, this material must be solubilized using denaturing conditions as described in Protocol 10 on page 80 before purification under denaturing conditions.

6. **Add 5 µl 2x SDS-PAGE sample buffer to 5 µl supernatant and store at –20°C for SDS-PAGE analysis.**

7. **Proceed to protocols for purification under native conditions beginning on page 81.**

Protocol 10. Preparation of cleared *E. coli* lysates under denaturing conditions

Materials

Cell pellet

2x SDS-PAGE sample buffer

Buffer B

Buffer compositions are provided in the appendix on pages 111–114.

1. **Thaw the cell pellet for 15 min on ice and resuspend in buffer B at 5 ml per gram wet weight.**

   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. Prior to SDS-PAGE analysis, samples containing guanidine must be treated as described in the appendix on page 115.

   The amount of cells required depends on the expression level of the 6xHis-tagged protein and the expression system used. The binding capacity of Ni-NTA resins is protein-dependent and normally lies between 5–10 mg/ml. For example, Ni-NTA Agarose or Ni-NTA Superflow has a binding capacity of 0.3 µmol/ml (8.0 mg/ml) for 6xHis-tagged DHFR (~26 kDa). **Refer to Table 3 "Determination of cell culture volume requirements" on page 65.**

2. **Stir cells for 15–60 min at room temperature or lyse them by gently vortexing, taking care to avoid foaming.**

   Lysis is complete when the solution becomes translucent.

3. **Centrifuge lysate at 10,000 x *g* for 20–30 min at room temperature to pellet the cellular debris.**

   Save supernatant (cleared lysate).

4. **Add 5 µl 2x SDS-PAGE sample buffer to 5 µl supernatant and store at –20°C for SDS-PAGE analysis.**

5. **Proceed to protocols for purification under denaturing conditions beginning on page 90.**

Protocol 11. Preparation of 6xHis-tagged periplasmic proteins from *E. coli*

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 following translocation. In order to purify proteins secreted into the periplasmic space using Ni-NTA technology, the 6xHis tag must be engineered to the C-terminus of the target protein. N-terminal 6xHis tags will be processed with the transit signal.

Materials

30 mM Tris·Cl, 20% sucrose, pH 8.0

500 mM EDTA

5 mM $MgSO_4$

Lysis buffer

Buffer compositions are provided in the appendix on pages 111–114.

1. **Grow and induce a 1 liter culture as described in Protocol 8, page 61.**
2. **Harvest the cells by centrifugation at 4,000 x *g* for 20 min. Resuspend pellet in 30 mM Tris·Cl, 20% sucrose, pH 8.0, at 80 ml per gram wet weight. Keep the cells on ice and add 500 mM EDTA dropwise to 1 mM. Incubate the cells on ice for 5–10 min with gentle agitation.**
3. **Centrifuge the cell suspension at 8000 x *g* for 20 min at 4°C, remove all the supernatant, and resuspend the pellet in the same volume of ice-cold 5 mM $MgSO_4$. Shake or stir for 10 min in an ice bath.**
4. **Centrifuge at 8000 x *g* for 20 min at 4°C.**

   The supernatant is the osmotic shock fluid containing periplasmic proteins.
5. **Dialyze supernatant extensively against lysis buffer before continuing with the purification.**

For purification under native conditions see below.

Protocols for purification under native conditions

Before purifying proteins under nondenaturing conditions, it is important to check that the protein is soluble (see Protocol 6, page 59). However, even if most of the protein is insoluble, it is often possible to purify traces of soluble material on Ni-NTA resin.

In the absence of strong denaturing agents such as urea, unstable proteins may be subject to degradation during cell harvest and lysis. It is best to work quickly and to keep the cells at 0–4°C at all times. Addition of PMSF or other protease inhibitors may be helpful on a case-by-case basis, but their potential effect on the recombinant protein must be taken into consideration.

Protocol 12. Batch purification of 6xHis-tagged proteins from *E. coli* under native conditions

The amount of cells required depends on the expression level of the 6xHis-tagged protein and the expression system used. The binding capacity of Ni-NTA resins is protein-dependent and normally lies between 5 and 10 mg/ml. For example, Ni-NTA Agarose or Ni-NTA Superflow has a binding capacity of 0.3 µmol/ml resin (8.0 mg/ml) for 6xHis-tagged DHFR (~26 kDa). The resins are supplied as 50% slurries.

For proteins that are expressed at high levels, (10–50 mg of 6xHis-tagged protein per liter of cell culture) a 10x concentrated cell lysate (resuspend the pellet from a 40 ml culture in 4 ml lysis buffer) can be used. Four ml of a 10x concentrated cell lysate in lysis buffer will contain approximately 0.4–2 mg of 6xHis-tagged protein. For much lower expression levels (1–5 mg/liter), 200 ml of cell culture should be used to obtain a 50x concentrated cell lysate (4 ml cell lysate = 0.2–1 mg of 6xHis-tagged protein). See also "Culture size", page 65 for more information.

The lysis buffer contains 10 mM imidazole to minimize binding of untagged, 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 6xHis-tagged proteins exhibiting high binding affinities, the imidazole concentration can be increased to 20 mM.

Materials

Cleared lysate from a 40–200 ml culture (see Protocol 9, page 79)

Ni-NTA matrix

Empty columns

Lysis buffer

Wash buffer

Elution buffer

Buffer compositions are provided in the appendix on page 114.

1. **Add 1 ml of the 50% Ni-NTA slurry to 4 ml cleared lysate and mix gently by shaking (200 rpm on a rotary shaker) at 4°C for 60 min.**

   The 10–20 mM imidazole in the lysis buffer suppresses the binding of nontagged contaminating proteins and leads to greater purity after fewer wash 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 into a column with the bottom outlet capped.**

3. **Remove bottom cap and collect the column flow-through.**

   Save flow-through for SDS-PAGE analysis.

4. **Wash twice with 4 ml wash buffer; collect wash fractions for SDS-PAGE analysis.**

5. **Elute the protein 4 times with 0.5 ml elution buffer. Collect the eluate in four tubes and analyze by SDS-PAGE.**

   The composition of the lysis, wash and elution buffers can be modified to suit the particular application, e.g. by adding 0.1% Tween, 5–10 mM β-ME, or 1 mM PMSF, or increasing NaCl or glycerol concentrations. For more information, see "Purification under native conditions", page 68.

Protocol 13. FPLC purification of 6xHis-tagged proteins from *E. coli* using Ni-NTA Superflow under native conditions

If larger amounts of protein are to be purified or if the purification will be performed using FPLC equipment, a Ni-NTA Superflow column is the method of choice (Figure 24). The physical stability of Ni-NTA Superflow makes it ideal for column chromatography at higher pressures and flow rates.

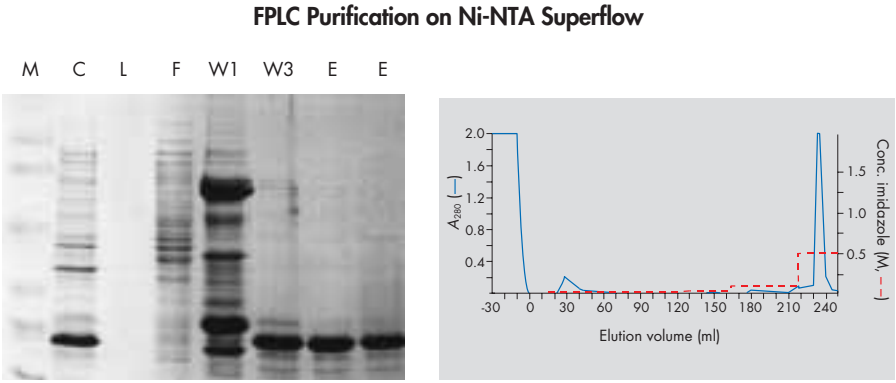

Figure 24. *FPLC purification on Ni-NTA Superflow. 6xHis-tagged phosphatase (~19 kDa) was purified from cleared lysate (1.2 liters) derived from 18 liters induced* E. coli *culture on 6 ml of Ni-NTA Superflow in a 1 cm column at 2 ml/min. Total yield was 38 mg.* ***Left:*** *Coomassie-stained SDS gel;* ***right:*** *elution profile.* ***M:*** *markers;* ***C:*** *induced cells;* ***L:*** *lysate;* ***F:*** *flow-through;* ***W1:*** *20-mM wash;* ***W3:*** *100-mM wash;* ***E:*** *eluates. (Data kindly provided by T. Schäfer, Institute for Biochemistry, University of Lübeck, Germany.)*

Materials

Cleared lysate from a 40–200 ml culture (see Protocol 9, page 79)

Ni-NTA Superflow

Lysis buffer

Wash buffer

Elution buffer

Chromatography column

Buffer compositions are provided in the appendix on page 114.

1. **Assemble the column according to the manufacturer's instructions. Remove the top adapter of the column and cap the bottom outlet.**

2. **Completely resuspend a 50% Ni-NTA Superflow slurry and pour the slurry into the column.**

   Avoid introducing air bubbles. Slowly pour the slurry down a thin glass rod inserted into the empty column. The column and bed size depends on the amount of 6xHis-tagged protein to be purified. Generally, the binding capacity of Ni-NTA Superflow is 5–10 mg protein per ml resin. Ni-NTA Superflow is supplied as a 50% slurry.

3. **Allow the resin to settle.**

   The packing procedure can be accelerated by allowing the buffer to flow through by uncapping the bottom outlet. If desired, a peristaltic pump may be used, but do not exceed flow rates of 2 ml/min.

   Do not allow resin to dry. If this should occur, resuspend resin in lysis buffer and repack the column.

   Before the bed has settled, more slurry may be added to increase bed volume.

4. **Insert top adapter and adjust to top of bed.**

   Do not trap any air bubbles. The column can now be connected to the system.

5. **Equilibrate column with 5 column volumes of lysis buffer**

   The flow rate should not exceed 2 ml/min.

   Monitor elution at 280 nm; the baseline should be stable after washing with 5 column volumes.

6. **Apply lysate to column and wash with lysis buffer until the A_{280} is stable.**

   Usually 5–10 column volumes are sufficient.
   Monitor pressure at this step. If the lysate is very viscous, the pressure may exceed the recommended value (10 bar). Reduce flow rate accordingly.
   Start with a flow rate of 0.5–1 ml/min. If the 6xHis-tagged protein does not bind, the flow rate should be reduced. The flow rate may however be increased for protein elution.
   Collect the flow-through for SDS-PAGE analysis.

7. **Wash with wash buffer until the A_{280} is stable.**

   Usually 5–10 column volumes are sufficient.
   Collect fractions for SDS-PAGE analysis.

8. **Elute the protein with elution buffer.**

   If desired, a step-gradient of elution buffer in wash buffer may be used to elute the protein. Five column volumes at each step are usually sufficient. The 6xHis-tagged protein usually elutes in the second and third column volume.

   Note: Imidazole absorbs at 280 nm, which should be considered when monitoring protein elution. If small amounts of 6xHis-tagged proteins are purified, elution peaks may be poorly visible.

Protocol 14. Protein minipreps of 6xHis-tagged proteins from *E. coli* under native conditions

1. **Transfer 1 ml of bacterial culture to a microcentrifuge tube.**

   The amount of culture used depends on the protein expression level. One ml is sufficient if the protein is expressed at high rates (see Table 3, page 65). If lower expression rates are expected, larger volumes may be necessary.

   If a time course of expression is being performed, take 1 ml samples of a larger culture at 30 min intervals after induction, collect the cell pellets and store them at –20°C until all the samples are ready for processing.

2. **Harvest the cells by centrifugation for 1 min at 15,000 x *g* and discard supernatants.**

   If larger culture volumes are required, refill microcentrifuge tube and centrifuge. Repeat this step until all cells are harvested.

3. **Resuspend cells in 100 µl lysis buffer.**

   If 1 ml culture is used, the concentration factor is ten. This may not be sufficient for some proteins that are to be purified under native conditions (see Table 3, page 65).

4. **Add lysozyme to 1 mg/ml and incubate on ice for 30 min.**

5. **Lyse cells by gently vortexing, taking care to avoid frothing.**

6. **Centrifuge the lysate for 10 min at 15,000 x *g* to remove the cellular debris, and transfer the supernatant to a fresh tube.**

7. **Add 20 µl of a 50% slurry of Ni-NTA resin (10 µl resin has a capacity for 50–100 µg 6xHis-tagged protein) to each tube, and mix gently for 30 min at 4°C.**

8. **Centrifuge for 10 s at 15,000 x *g* to pellet the resin, transfer 10 µl of the supernatant to a fresh tube, and discard the remaining supernatant.**

   Store the supernatant sample on ice.

   Supernatant samples will contain any proteins which have not bound to the resin.

9. **Wash the resin twice with 100 µl of wash buffer.**

   Centrifuge for 10 s at 15,000 x *g* between each elution step and carefully remove the supernatant.

10. **Elute the protein 3 times with 20 µl elution buffer**

    Centrifuge for 10 s at 15,000 x *g* between each elution step and carefully remove the supernatant to a fresh tube.

Protocol 15. Purification of 6xHis-tagged proteins from transfected mammalian cells under native conditions

The following procedure can be used as the starting point for developing a protocol for purification of 6xHis-tagged proteins expressed in mammalian cells. However, further optimization may be necessary. General guidelines for purification of 6xHis-tagged proteins using Ni-NTA Magnetic Agarose Beads are summarized in the *Ni-NTA Magnetic Agarose Beads Handbook*.

Recombinant proteins are often expressed in mammalian cells to allow eukaryotic post-translational processing. Some of the difficulties of using mammalian cells are that expression levels are typically lower than in bacterial systems and that often only small amounts of cell material are available. The total protein content in HeLa cells, for example, is only 3000 µg per 10^7 cells. With recombinant protein expression levels at 0.01 to 1% of total protein, the theoretical maximum protein yield is 0.3 to 30 µg per 10^7 cells.

Ni-NTA Magnetic Agarose Beads are an ideal Ni-NTA matrix for small-scale purification of 6xHis-tagged proteins expressed in mammalian cells (Wahle *et al.* 1999). They allow approximate adjustment of the binding capacity (3 µg DHFR [24 kDa] per 10 µl magnetic bead suspension) to the amount of 6xHis-tagged protein to be captured. This feature is crucial for optimal performance. In addition, the small elution volumes used provide high 6xHis-tagged protein concentrations, even with very small numbers of cells.

Materials

Cell pellet

Ni-NTA Magnetic Agarose Beads

Magnetic separator (QIAGEN 12-Tube Magnet, Cat. No. 36912)

PBS, lysis buffer, wash buffer, and elution buffer

Buffer compositions are provided in the appendix on pages 114–115.

1. **Wash the transfected cells with phosphate-buffered saline (PBS) and collect them by centrifugation for 5 min at 1000 x *g*.**
2. **Resuspend the cells in lysis buffer supplemented with 0.05% Tween® 20 using 500 µl lysis buffer per 10^7 cells.**

   The lysis buffer should always contain imidazole. For most 6xHis-tagged proteins, up to 20 mM imidazole can be used without affecting the binding properties. However, if the tagged protein does not bind under these conditions, the amount of imidazole should be reduced to 5–10 mM.

   If higher concentrations of non-ionic detergent are required to solubilize the 6xHis-tagged protein, use up to 1% detergent in the lysis, wash, and elution buffers. Compatible detergents are Tween 20, Triton® X-100, Igepal® CA-630, and CHAPS.

3. **Lyse the cells by sonication on ice.**

   Use six 15 s bursts at 75 W with a 10 s cooling period between each burst. Use a sonicator equipped with a microtip.

   Optional:

   A Lyse cells by three consecutive freeze/thaw cycles with freezing on dry ice and thawing at room temperature.

   B With 0.5–1% non-ionic detergent in the lysis buffer for solubilization of a hydrophobic 6xHis-tagged protein, it is sufficient to incubate on an end-over-end shaker for 10 min at 4°C. Additional sonication or freeze/thaw cycles are not necessary.

4. **Centrifuge the lysate at 10,000 x *g* for 10 min at 4°C to pellet cellular debris and DNA. Save the supernatant.**

   The supernatant should contain the 6xHis-tagged protein.

5. **Resuspend the Ni-NTA Magnetic Agarose Beads by vortexing for 2 s and then immediately add 10 µl of the 5% Ni-NTA Magnetic Agarose Bead suspension to 500 µl of the cleared lysate containing the 6xHis-tagged protein.**

   Note: Care is necessary to ensure that constant amounts of beads are pipetted. The beads tend to settle out if the suspension is not agitated regularly.

   10 µl magnetic-bead suspension has a binding capacity of 3 µg 6xHis-tagged DHFR (24 kDa). Use of volumes less than 10 µl is not recommended due to the associated handling problems. (Smaller volumes are difficult to pipet and may lead to uneven distribution of beads and reduced reproducibility.)

6. **Mix the suspension gently on an end-over-end shaker for 1–2 h at 4°C.**

7. **Place the tube on the QIAGEN 12-Tube Magnet for 1 min and remove the supernatant from the separated beads using a pipet.**

   To collect suspension droplets from the tube caps, it is helpful to briefly centrifuge the tubes before placing them on the 12-Tube Magnet.

8. **Remove the tube from the magnet, add 1 ml of wash buffer, mix the suspension, place the tube on the 12-Tube Magnet for 1 min, and remove wash buffer from the separated beads using a pipet.**

9. **Repeat step 8 two or three times.**

   After the final washing step, residual buffer should be removed completely.

10. **Add 50 µl of elution buffer, mix the suspension, incubate the tube for 1 min, place on the 12-Tube Magnet for 1 min, and collect the eluate using a pipet.**

    To collect suspension droplets from the tube caps, it is helpful to briefly centrifuge the tubes before placing them on the 12-Tube Magnet.

    If a more concentrated protein solution is required, elute in two aliquots of 25 µl each.

Protocol 16. Purification of 6xHis-tagged proteins from baculovirus-infected insect cells under native conditions

The following procedure can be used as a starting point for developing a protocol for purification of 6xHis-tagged proteins expressed intracellularly in insect cells. However, further optimization may be necessary. General guidelines for purification of 6xHis-tagged proteins using Ni-NTA Agarose or Superflow are summarized on pages 66–72.

Although expression rates are normally higher in insect cells than in mammalian cells, there are some difficulties connected with using baculovirus-infected insect cells for expression. Expressed-protein levels are typically lower than those obtained in bacterial systems, and in general, smaller amounts of cell material are available. The estimated total protein content in insect cells is approximately 20 mg per 10^7 cells. With recombinant protein expression levels ranging between 0.05 % and 50% the theoretical maximum protein yield is 10 µg – 10 mg per 10^7 cells.

Ni-NTA Agarose and Ni-NTA Superflow are ideal matrices for purification of 6xHis-tagged proteins expressed in baculovirus-infected insect cells. Use of an amount of matrix (binding capacity 500–1000 µg protein per 100 µl settled matrix) appropriate for the amount of 6xHis-tagged protein to be captured is crucial for optimal performance of the purification procedure.

Lysis buffer contains 1 % Igepal® CA-630 (Nonidet P40) for lysis of the cells and 10 mM imidazole to minimize binding of non-tagged, contaminating proteins, and to increase purity with fewer wash steps.

Materials

Cell pellet

Ni-NTA matrix

Empty columns

PBS, lysis buffer, wash buffer and elution buffer

Buffer compositions are provided in the appendix on pages 114–115

1. **Wash the transfected cells with phosphate buffered saline (PBS) and collect them by centrifugation for 5 min at 1000 x *g*.**
2. **Lyse the cells in lysis buffer supplemented with 1% Igepal CA-630 using 4 ml lysis buffer per 1–2 x 10^7 cells. Incubate for 10 min on ice.**

   The lysis buffer should always contain imidazole. For most 6xHis-tagged proteins, up to 20 mM imidazole can be used without affecting the binding properties. However, if the tagged protein does not bind under these conditions, the concentration of imidazole should be reduced to 5–10 mM.

3. **Centrifuge the lysate at 10,000 x *g* for 10 min at 4°C to pellet cellular debris and DNA. Save the cleared lysate (supernatant).**

   The supernatant should contain the 6xHis-tagged protein.

4. **Add 200 µl 50% Ni-NTA slurry per 4 ml of the cleared lysate and mix gently by shaking (200 rpm on a rotary shaker) at 4°C for 1–2 h.**

   Equilibrate the Ni-NTA with PBS before adding to the lysate.

5. **Load the lysate-Ni-NTA mixture into a column in which the outlet is capped.**

6. **Remove the outlet cap and collect the column flow-through fraction.**

   Save the flow-through fraction for SDS-PAGE analysis.

7. **Wash twice with 800 µl wash buffer, collecting wash fractions.**

   Save wash fractions for SDS-PAGE analysis

8. **Elute the protein 4 times with 100 µl elution buffer. Collect the eluates in four tubes and analyze by SDS-PAGE.**

   The composition of the lysis, wash and elution buffers can be modified to suit the particular application, e.g. by adding 5-10 mM β-ME, or 1 mM PMSF, or increasing NaCl concentrations. For more information, see "Purification under native conditions", page 68.

Protocol 17. Batch purification of 6xHis-tagged proteins from *E. coli* under denaturing conditions

Materials

Cleared lysate (see Protocol 10, page 80) from 20–200 ml culture

Ni-NTA resin

Empty columns

Buffers A–E

Buffer compositions are provided in the appendix on page 113.

1. **Add 1 ml of the 50% Ni-NTA slurry to 4 ml lysate and mix gently by shaking (e.g., 200 rpm on a rotary shaker) for 15–60 min at room temperature.**

   The amount of lysate required depends on the expression level of the 6xHis-tagged protein and the expression system used. The binding capacity of Ni-NTA resins is protein-dependent and normally lies between 5–10 mg/ml. For example, Ni-NTA Agarose or Ni-NTA Superflow has a binding capacity of 0.3 µmol/ml (8.0 mg/ml) for 6xHis-tagged DHFR (~26 kDa).

   For proteins that are expressed at very high levels (50–100 mg of 6xHis-tagged 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 6xHis-tagged 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 6xHis-tagged protein).

   See also "Culture size" on page 65 for more information.

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

3. **Remove the bottom cap and collect the flow-through.**

   Collect flow-through for SDS-PAGE analysis.

4. **Wash twice with 4 ml buffer C.**

   Keep wash fractions for SDS-PAGE analysis.

5. **Elute the recombinant protein 4 times with 0.5 ml buffer D, followed by 4 times with 0.5 ml buffer E. Collect fractions and analyze by SDS-PAGE.**

   Monomers generally elute in buffer D, while multimers, aggregates, and proteins with two 6xHis tags will generally elute in buffer E.

Protocol 18. FPLC purification of 6xHis-tagged proteins using Ni-NTA Superflow under denaturing conditions

If larger amounts of protein are to be purified or if the purification will be performed using FPLC equipment, a Ni-NTA Superflow column is the method of choice. The physical stability of Ni-NTA Superflow makes it ideal for column chromatography at higher pressures and flow rates.

Materials

Ni-NTA Superflow

Buffers A–E

Chromatography column

1. **Assemble the column according to the manufacturer's instructions. Remove the top adapter of the column and cap the bottom outlet.**
2. **Thoroughly resuspend a 50% Ni-NTA Superflow slurry and pour the slurry into the column.**

   Avoid introducing air bubbles. Slowly pour the slurry down a thin glass rod inserted into the empty column.

   The column and bed size depends on the amount of 6xHis-tagged protein to be purified. Generally, the binding capacity of Ni-NTA Superflow is 5–10 mg protein per ml resin.
3. **Allow the resin to settle.**

   The packing procedure can be accelerated by allowing the buffer to flow through by uncapping the bottom outlet. If desired, a peristaltic pump may be used, but a flow rate of 2 ml/min should not be exceeded.

   Do not allow resin to dry. If this should occur, resuspend resin in buffer B and repack the column.

   Before the bed has settled, more slurry may be added to increase bed volume.
4. **Insert top adapter and adjust to top of bed.**

   Do not trap any air bubbles. The column can now be connected to the system.
5. **Equilibrate column with 5 column volumes of buffer B.**

   Monitor elution at 280 nm; the baseline should be stable after washing with 5 column volumes.
6. **Apply lysate to column and wash with buffer B until the A_{280} is below 0.01.**

   Usually 5–10 column volumes are sufficient.

   Begin with a flow rate of 1 ml/min. Monitor pressure at this step. If the lysate is very viscous, the pressure may exceed the recommended value (10 bar). If necessary reduce flow rate.

   Collect the flow-through for SDS-PAGE analysis.

7. **Wash with buffer C until the A_{280} is below 0.01.**

   Usually 5–10 column volumes are sufficient.

   The buffer C wash removes proteins that bind nonspecifically to the resin. Collect the wash fractions for SDS-PAGE analysis.

8. **Elute protein with buffer D or buffer E.**

   If elution is incomplete with buffer D, buffer E should be used. Monomers usually elute in buffer D, whereas multimers, aggregates, and proteins with two 6xHis tags will generally elute in buffer E. Proteins usually elute in the second and third column volume.

Protocol 19. 6xHis-tagged protein minipreps under denaturing conditions

1. **Transfer 1 ml bacterial culture to a microcentrifuge tube.**

   The amount of culture used depends on the protein expression level. 1 ml is sufficient if the protein is expressed at high rates (see Table 3, page 65). If lower expression rates are expected, larger volumes may be necessary.

   If a time course of expression is being performed, take 1 ml samples of a larger culture at 30 min intervals after induction, collect the cell pellets and store them at –20°C until all the samples are ready for processing.

2. **Harvest the cells by centrifugation for 1 min at 15,000 x *g* and discard supernatants.**

   If larger culture volumes are required, refill microcentrifuge tube and centrifuge. Repeat this step until all cells are harvested.

3. **Resuspend cells in 200 µl buffer B. Lyse cells by gently vortexing, taking care to avoid frothing.**

   The solution should become translucent when lysis is complete. Most proteins are soluble in buffer B. If the solution does not become translucent, lyse cells with buffer A.

4. **Centrifuge the lysate for 10 min at 15,000 x *g* to remove the cellular debris, and transfer the supernatant to a fresh tube.**

5. **Add 50 µl of a 50% slurry of Ni-NTA resin (25 µl resin has a capacity for 125–250 µg 6xHis-tagged protein) to each tube, and mix gently for 30 min at room temperature.**

6. **Centrifuge 10 sec at 15,000 x *g* to pellet the resin, transfer 10 µl of the supernatant to a fresh tube, and discard the remaining supernatant. Store the supernatant samples on ice.**

   The supernatant samples will contain any proteins which have not bound to the resin.

7. **Wash the resin twice with 250 µl of buffer C.**

   Centrifuge for 10 sec at 15,000 x *g* between each wash step and carefully remove the supernatant.

8. **Elute the protein 3 times with 25 µl buffer E.**

   Centrifuge for 10 sec at 15,000 x *g* between each elution step and carefully remove the supernatant to a fresh tube.

Protocol 20. Factor Xa Protease treatment of fusion proteins containing a Factor Xa Protease recognition sequence

Treatment of fusion proteins containing a Factor Xa Protease recognition sequence consists of three steps: a) Factor Xa Protease Cleavage, b) removal of Factor Xa Protease, and c) cleanup of the digested protein. Protocols for all three steps can be found below.

Protocol 20 a. Factor Xa Protease cleavage

Factor Xa Protease is a site-specific endoprotease that preferentially cleaves the C-terminal peptide bond of the recognition sequence Ile-Glu-Gly-Arg. Factor Xa Protease consists of two polypeptides linked by a disulfide bond. The optimal cleavage conditions must be determined individually for each protein to be cleaved. Accessibility of the cleavage site, the adjacent amino acid sequence, and the degree of protein aggregation all affect the cleavage efficiency. Optimization of Factor Xa Protease concentration, temperature (4° to 37°C), and incubation time (2 to 16 h) is recommended. Bear in mind that excess Factor Xa Protease may result in nonspecific proteolysis at secondary sites. Therefore, optimal enzyme specificity is achieved using the lowest amount of protease necessary to achieve complete cleavage.

Optimization of the cleavage conditions should be performed in small-scale reactions using the following protocol as a starting point. We recommend using 20 mM Tris·Cl, pH 6.5; 50 mM NaCl; 1 mM $CaCl_2$ as the reaction buffer.

Materials

Fusion protein containing a Factor Xa Protease recognition sequence

Factor Xa Protease

1x reaction buffer (20 mM Tris·Cl, pH 6.5; 50 mM NaCl; 1 mM $CaCl_2$)

5x SDS-PAGE sample buffer

Buffer compositions are provided in the appendix on pages 111–115.

1. **Prepare four solutions each containing 10 µg of the protein to be cleaved, in 1x reaction buffer. The solutions should have a protein concentration of at least 0.25 µg/µl.**

   Since Factor Xa Protease is sensitive to various buffer constituents we recommend that the protein to be cleaved is prepared in 1x reaction buffer before cleavage. If your individual protein requires other specific buffering conditions, please see the important notes below for the compatibility of some commonly used buffer components with Factor Xa Protease. We also recommend changing the buffer system if you have purified your protein by Ni-NTA affinity chromatography under native conditions, because Factor Xa Protease activity is sensitive to phosphate buffers as well as to high imidazole and NaCl concentrations.

2. **Prepare three serial dilutions of Factor Xa Protease in 1x reaction buffer with concentrations of 1.0, 0.2 and 0.05 Units per µl.**

   It is important to mix the dilutions completely before use to avoid localized differences in enzyme concentration. Diluted protease should be used immediately after preparation.

3. **Add 1 µl of each Factor Xa Protease dilution to one solution of the protein to be cleaved, and adjust the reaction volume to 40 µl using 1x reaction buffer. Adjust the volume of the fourth protein solution to 40 µl using 1x reaction buffer. This sample will serve as a negative control to monitor the progress of cleavage.**

Component	Volume/reaction	Final concentration
10 µg fusion protein	variable	0.25 µg/µl
Factor Xa Protease dilutions	1 µl	1.0, 0.2, and 0.05 U/40 µl
1x reaction buffer	Adjust to 40 µl	

4. **Incubate the reactions at room temperature (15–25°C). Take an 8 µl aliquot from each reaction after 3, 6, 9 and 16 h. Add 2 µl 5x SDS-PAGE sample buffer to each aliquot and mix thoroughly.**

   It is important to mix the aliquots with SDS-PAGE sample buffer immediately to completely quench Factor Xa Protease activity.

5. **Analyze the efficiency of cleavage in each sample by SDS-PAGE.**

   Since the cleaved-off peptide may be very small, the bands corresponding to cleaved and uncleaved protein will run very close together. We therefore recommend using a gradient gel that will give good resolution in the size-range of the protein being analyzed.

Important notes for optimization of cleavage

Protease concentration: Excess Factor Xa Protease may result in nonspecific proteolysis at secondary sites. Optimal enzyme specificity is achieved using the lowest amount of protease necessary to achieve complete cleavage.

Concentration of protein to be cleaved: Factor Xa Protease activity is sensitive to the concentration of protein to be cleaved. A minimum of 10 µg protein per 40 µl reaction (0.25 µg/µl) is recommended.

Incubation temperature: Factor Xa Protease activity increases with increasing incubation temperature from 4°C to 37°C. However, it should be taken into account that reduced incubation temperatures can minimize the accessibility of secondary cleavage sites.

pH: Factor Xa Protease activity decreases with increasing pH from pH 6.5 to pH 9.0. Therefore, we recommend a pH of 6.5 for the reaction buffer. If your individual protein is sensitive to pH, for example, with relation to protein activity or solubility, increase the pH to 7.5.

Buffer system: Use of phosphate buffers will result in reduced Factor Xa Protease activity compared to Tris·Cl or HEPES buffer systems.

Presence of detergents: Up to 1% Triton X-100, Nonidet P-40 and *n*-octylglucoside has no significant effect on Factor Xa Protease specificity or activity. The presence of SDS will significantly reduce enzyme activity.

Presence of denaturants: Significant loss of protease activity is observed at urea concentrations above 100 mM, and guanidine·HCl concentrations above 10 mM.

Presence of reductants: The presence of reductants in the reaction should be avoided, because the subunits of the heterodimeric protease enzyme are linked via a disulfide bridge. However, in some experiments up to 5 mM β-mercaptoethanol has been used with no significant influence on enzyme activity. The use of DTT is not recommended.

NaCl sensitivity: Factor Xa Protease activity decreases with increasing NaCl concentration. Significant inhibition is observed above 100 mM NaCl.

Imidazole sensitivity: Factor Xa Protease is significantly inhibited at or above 100 mM imidazole.

Anionic polymeric sugars: Some experiments have shown that addition of dextran sulfates (5000–10,000 Da) or heparin (5000 or 15,000 Da) to the reaction mixture results in increased Factor Xa Protease activity. 0.5–1 µg polymeric sugar can be added for cleavage of 10 µg recombinant protein in a 40 µl total reaction volume.

Amino acid sequence: Factor Xa Protease preferentially cleaves at the C-terminal side of the recognition sequence Ile-Glu-Gly-Arg↓X. It has been reported that X is in general unspecific, but Ile and Thr are preferred. Where X is a hydrophobic residue, cleavage efficiency is reduced (Owen et al., 1974, He et al., 1993).

Scaleup

Once the optimal cleavage conditions have been found, the reaction can be scaled up proportionally. Following the above protocol, 1 mg recombinant protein would be digested in a total volume of 4 ml. If there is a need to reduce the total reaction volume, perform small scale experiments in which the reaction volume is varied while the protease:recombinant protein ratio and incubation conditions are kept constant.

Protocol 20 b. Removal of Factor Xa Protease

After protease digestion, Factor Xa Protease can be removed by affinity chromatography using Xa Removal Resin. The Xa Removal Resin binds the protease in the reaction mixture while the cleaved recombinant protein remains in solution. After the resin is pelleted by centrifugation, the cleaved recombinant protein is recovered in the supernatant.

Materials

Xa Removal Resin

1x Reaction buffer (20 mM Tris·Cl, pH 6.5; 50 mM NaCl; 1 mM $CaCl_2$)

1. **Calculate the required amount of Xa Removal Resin necessary to capture the Factor Xa Protease present in the cleavage reaction.**

   50 µl bed volume (100 µl slurry) is sufficient to bind 4 Units Factor Xa Protease enzyme in 1x reaction buffer. Use of slurry volumes of less than 25 µl is not recommended due to associated handling problems. If your individual recombinant protein requires cleavage buffer other than the recommended 1x reaction buffer, bear in mind that the protease capture step may be sensitive to the use of other buffers. Binding of Factor Xa Protease to the Xa Removal Resin is unaffected by increasing the pH to 7.5, the presence of 20–100 mM Tris·HCl, and up to 1% Triton X-100 or Nonidet P-40. High salt concentrations will reduce binding capacity. For example, increasing NaCl concentration from 50 mM to 500 mM will result in a 20–40% reduction in binding. The recommended 1x reaction buffer (20 mM Tris·HCl, pH 6.5; 50 mM NaCl; 1 mM $CaCl_2$) supports high-efficiency cleavage and capture.

2. **Resuspend the Xa Removal Resin completely by gentle inversion and then immediately transfer the required amount of slurry into a centrifuge tube of appropriate size.**

   Note: The beads will quickly fall out of suspension. For transfer, use a wide-mouth pipette.

3. **Centrifuge the beads for 5 min at 1000 x *g* and discard the supernatant.**

4. **Resuspend the beads in ten bed-volumes of 1x reaction buffer by gently mixing, centrifuge for 5 min at 1000 x *g*, and discard the supernatant.**

   Equilibration of the beads with 1x reaction buffer is necessary for maximum capture efficiency and prevents contamination of the cleaved recombinant protein with resin storage buffer. Use Xa Removal Resin immediately after equilibration.

5. **Add the cleavage reaction to the equilibrated resin. Mix gently to resuspend the resin and incubate for 10 min at room temperature. Shake on an orbital shaker or place sealed tube on a roller-table to keep beads in suspension.**

   If the cleaved protein is temperature sensitive, binding can be performed at 4°C without any loss of binding efficiency.

6. **Centrifuge the reaction at 1000 x *g* for 5 min to pellet the resin. Collect the supernatant which contains the cleaved protein. Factor Xa Protease remains bound to the resin.**

Protocol 20 c. Removal of undigested 6xHis-tagged protein and cleaved 6xHis peptides after Factor Xa Protease cleavage

After digestion of 6xHis-tagged recombinant proteins with Factor Xa Protease, undigested proteins and released 6xHis-tag/Factor Xa Protease recognition site-peptides are present in the cleavage reaction mixture. Both these species can be removed by Ni-NTA affinity chromatography using a batch procedure, leaving pure, de-tagged recombinant protein in the supernatant. The protocol can be carried out either directly after Factor Xa Protease cleavage or subsequent to the removal of the protease with Xa Removal Resin. The protocol consists of two steps: adjusting the pH to 7.5, and binding of 6xHis-tagged contaminants to Ni-NTA resin.

Materials

1M Tris·HCl pH 8

Ni-NTA Agarose

1. **Adjust the pH of the cleavage reaction mixture to 7.5.**

   This can be accomplished by the addition of ~1/100 volume of 1M Tris·HCl, pH 8.0 when the recommended 1x reaction buffer (20 mM Tris·HCl, pH 6.5; 50 mM NaCl; 1 mM $CaCl_2$) was used for cleavage. It is not necessary to adjust the pH if the protease digestion was performed at pH 7.5. A pH of 7.5 is essential for efficient binding of 6xHis tags to Ni-NTA resin.

2. **Calculate the required amount of Ni-NTA Agarose needed to capture the 6xHis-tagged contaminants.**

   1 ml bed volume (2 ml slurry) is sufficient to bind 5–10 mg 6xHis-tagged protein. For optimal performance, the binding capacity of the Ni-NTA Agarose used for removal should match the total amount of 6xHis-tagged protein that was subjected to Factor Xa Protease cleavage.

3. **Resuspend the Ni-NTA Agarose completely by gently inverting the bottle 4–6 times and then immediately transfer the required amount of slurry into a centrifuge tube of appropriate size.**

4. **Centrifuge the resin for 1 min at 1000 x *g* and discard the supernatant.**

   Optional: To prevent contamination of the recombinant protein with Ni-NTA storage buffer, the pelleted beads can be washed with two bed volumes of 20 mM Tris·HCl, pH 7.5, 50 mM NaCl prior to incubation with the reaction mixture.

5. **Transfer the reaction mixture to the equilibrated resin. Mix gently to resuspend the resin and incubate for 10 min at room temperature. Shake on an orbital shaker or place sealed tube on a roller-table to keep beads in suspension.**

   If the recombinant protein is temperature sensitive, binding can be performed at 4°C without any loss of binding efficiency.

6. **Centrifuge the suspension at 1000 x *g* for 1 min to pellet the resin. Collect the supernatant that contains the pure recombinant protein. 6xHis-tagged contaminants remain bound to the resin.**

Troubleshooting: purification from *E. coli*

	Comments and suggestions
Protein does not bind to the Ni-NTA resin	
6xHis tag is not present.	Sequence ligation junctions to ensure that the reading frame is correct.
	Check for possible internal translation starts (N-terminal tag) or premature termination sites (C-terminal tag).
6xHis tag is inaccessible.	Purify protein under denaturing conditions.
	Move tag to the opposite end of the protein.
6xHis tag has been degraded.	Check that the 6xHis tag is not associated with a portion of the protein that is processed.
Binding conditions incorrect.	Check pH and composition of all buffers and solutions. Dissociation of urea often causes a shift in pH. The pH values should be checked immediately prior to use.
	Ensure that there are no chelating or reducing agents present, and that the concentration of imidazole is not too high.
Protein elutes in the wash buffer	
Wash stringency is too high.	Lower the concentration of imidazole or increase the pH slightly.
6xHis tag is partially hidden.	Reduce wash stringency. Purify under denaturing conditions.
Buffer conditions incorrect.	Check pH and composition of wash buffer.
	Ensure that there are no chelating or reducing agents present.
Protein precipitates during purification	
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-ME, up to 2 M NaCl, or stabilizing cofactors such as Mg^{2+}. These may be necessary in all buffers to maintain protein solubility.

	Comments and suggestions
Protein does not elute	
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.
	Perform binding and elution in batch format to avoid high local protein concentrations.
Protein elutes with contaminants	
Binding and wash conditions 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-ME to a maximum of 20 mM to reduce disulfide bonds.
	Increase salt and/or detergent concentrations, or add ethanol/glycerol to 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.
Discoloration of resin	
Nickel ions are removed or reduced.	Ensure that there are no chelating compounds (resin turns white in color) or reducing agents (resin turns brown in color) present in the buffers.

Troubleshooting: purification from mammalian cells

	Comments and suggestions
No protein band in SDS-PAGE analysis of the eluate.	
Expression is too low.	Check the expression level by western blotting using an Anti·His Antibody or a protein-specific antibody. Alternatively, perform an immunoassay with Ni-NTA Magnetic Agarose Beads (see the *Ni-NTA Magnetic Agarose Beads Handbook*) or ELISA using Ni-NTA HisSorb™ Strips (see the *QIA*express *Detection and Assay Handbook*). If only small amounts of 6xHis-tagged protein are present in the lysate, increase the amount of starting cell material and purify with an equal amount of magnetic beads. Do not exceed lysis volumes of 2 ml — this allows purification in a single 2 ml tube.
6xHis tagged protein has been degraded.	Check that the 6xHis tag is not removed from the protein during post-translational processing. Work at 4°C and add protease inhibitors, such as PMSF.
6xHis-tagged protein partially elutes in the wash buffer or flow-through.	The binding capacity used is too low to bind all of the 6xHis-tagged protein. 10 µl magnetic-bead suspension has a binding capacity of 3 µg 6xHis-tagged DHFR (24 kDa). If significantly larger amounts of 6xHis-tagged protein are present in the lysate, increase the amount of beads accordingly.
Binding of contaminants	
Too much Ni-NTA matrix was used.	Match the total binding capacity of the beads to the amount of 6xHis-tagged protein to be purified by simply adjusting the amount of Ni-NTA Magnetic Agarose Beads suspension used. Proteins that contain neighboring histidines are not common in bacteria, but do occur in eukaryotic cells. These proteins, as well as endogenous proteins with metal-binding sites, normally bind with lower affinity to the Ni-NTA matrix than do 6xHis-tagged proteins. If the binding capacity of the amount of beads used greatly exceeds the amount of 6xHis-tagged protein to be purified, these proteins will bind to the Ni-NTA matrix to a considerably higher extent, and will be subsequently recovered in the eluate.

	Comments and suggestions
Binding and wash conditions are not stringent enough.	Always include 10–20 mM imidazole in the binding buffer and 20 mM imidazole in the wash buffer.
Large amount of nontagged proteins in the lysate when purifying from cells with a very low expression rate.	Perform a second round of purification from the eluate after adjusting the imidazole concentration to 10–20 mM using binding buffer without imidazole. Significantly smaller amounts of background proteins in the binding step reduce the level of contaminants in the final preparation.

Troubleshooting: purification from insect cells

	Comments and suggestions
No protein band in SDS-PAGE analysis of the fractions	
No or low expression.	Check the expression level by western blotting using an Anti·His Antibody or a protein-specific antibody. Alternatively perform an ELISA using Ni-NTA HisSorb™ Strips (see *QIA*express *Detection and Assay Handbook*). If low amounts of 6xHis-tagged protein are present in the lysate, increase the amount of starting cell material and purify with an equal amount of Ni-NTA matrix.
6xHis-tagged protein has been degraded.	Check that the 6xHis tag is not removed from the protein during post-translational processing or by endogenous proteases during the purification procedure. Work at 4°C and add protease inhibitors, such as PMSF.
6xHis-tagged protein partially elutes in the wash buffer or flow-through.	The amount of matrix used is too low to bind all of the 6xHis-tagged protein. 100 µl Ni-NTA agarose has a binding capacity of 500–1000 µg 6xHis-tagged protein. Adjust the amount of matrix used for purification accordingly.
Contaminants bind to resin	
Too much Ni-NTA matrix was used.	Match the total binding capacity of the matrix used to the amount of 6xHis-tagged protein to be purified. Endogenous proteins with metal-binding sites normally bind with lower affinity to the Ni-NTA matrix than do 6xHis-tagged proteins. If the binding capacity of the amount of matrix used greatly exceeds the amount of 6xHis-tagged protein to be purified, these proteins will bind to the Ni-NTA matrix to a considerably higher extent, and subsequently will be recovered in the eluate.
Binding and wash conditions are not stringent enough.	Always include 10–20 mM imidazole in the binding buffer and 20 mM imidazole in the wash buffer.

Troubleshooting: Factor Xa Protease cleavage

	Comments and suggestions
Factor Xa Protease cleavage	
No or incomplete cleavage of the recombinant protein	
Insufficient Factor Xa Protease.	Increase the amount of Factor Xa Protease while keeping the recombinant protein concentration constant.
Recombinant protein concentration is too low.	Increase recombinant protein concentration. A minimum concentration of 0.25 µg/µl is recommended
Buffer components inhibit Factor Xa Protease.	Ensure that there are no buffer components that inhibit Factor Xa Protease activity (see important notes for optimization of cleavage, page 94). Dialyze the 6xHis-tagged protein sample against 20 mM Tris·Cl, pH 6.5; 50 mM NaCl; 1 mM $CaCl_2$ to achieve optimal cleavage conditions.
Incubation time and temperature are insufficient.	Increase incubation temperature up to 37°C and prolong incubation time up to 24 h.
Multiple bands are observed on SDS-gel following cleavage with Factor Xa Protease	
Bands derive from Factor Xa Protease.	If large amounts of Factor Xa Protease are used, two bands (approximately 17–20 kDa and 28–30 kDa) may appear on the gel under reducing conditions. Run Factor Xa Protease alone in an adjacent gel lane as a control.
Protease cleaves within the recombinant protein.	Check that no additional Factor Xa Protease recognition site is present in the recombinant protein. Reduce incubation temperature (RT or 4°C) to minimize exposure of secondary cleavage sites. Reduce amount of Factor Xa Protease used for cleavage. Adjust reaction conditions to obtain partial digestion which may result in selective scission at the desired Factor Xa Protease recognition site.

Factor Xa Removal

Factor Xa Protease not efficiently removed

Amount of Xa Removal Resin used is too low.	Check that you have correctly calculated the amount of resin necessary (see Protocol). Perform a second capture reaction.
Buffer contains components which affect the Factor Xa Protease binding reaction (see protocol).	Dialyze the cleavage reaction mixture against 1x Reaction Buffer and repeat the binding protocol. Try to eliminate incompatible buffer components during the Factor Xa Protease cleavage reaction and repeat the cleavage and capture steps.

Removal of 6xHis-tagged polypeptides

6xHis-tagged peptides bind incompletely or not at all to the Ni-NTA resin.

Binding conditions are incorrect.	Check the pH and composition of reaction mixture. Ensure that there are no chelating or reducing agents present.
Binding capacity of Ni-NTA resin does not match total amount of 6xHis-tagged polypeptides.	Perform a second capture reaction.

Cleaved recombinant protein band intensity is reduced in SDS-PAGE analysis

Cleaved recombinant protein binds to Ni-NTA resin.	Add 20 mM imidazole to the reaction mixture to prevent non-specific binding of the cleaved recombinant protein.

Protein Refolding Recommendations

It is generally preferable to be able to purify recombinant proteins under native conditions, but for many proteins, particularly when large yields are required, this may not always be possible. If proteins are purified under denaturing conditions for use in antibody induction, there is usually no need to renature before injection into the animal. If renaturation is necessary, refolding conditions must be determined empirically for each individual protein. Detailed examples of refolding procedures have been described (Wingfield *et al.* 1995a). The following are a few recommendations that may be helpful in designing refolding experiments.

- Maintain low protein concentration (10–50 µg/ml).
- Include thiol reagents.

  Disulfide bonds contribute to the stability of the native conformation of many proteins. Secondary structure formation may be so favorable that the correct cysteine residues spontaneously bind, i.e., weakly oxidizing conditions are required. If, however, this is not the case, stronger oxidizing conditions may be required. A redox pair of reduced glutathione (GSH) and oxidized glutathione (GSSG) creates the necessary oxidizing potential to make and break disulfide bonds in folding intermediates, thereby allowing the optimal, native conformation to be reached. A GSH:GSSG ratio of 10:1 (at a concentration of 2–5 mM GSH) has been shown to be effective for a number of proteins.
- Remove denaturants slowly by dilution or dialysis.

  Glycine often has remarkable solubilization properties (50 mM, pH 9.0; 5 mM EDTA). If GuHCl must be used as a denaturant, urea included in the renaturation buffer (2 M) may stabilize the protein upon refolding. Very low concentrations of detergents may also be included (0.1–0.5% NP-40 or 0.005% (v/v) Tween 20).
- Include cosolvents.

  Many cosolvents such as glycerol (5–20%) and ethylene glycol, as well as glucose and sucrose (10%) can stabilize proteins. Certain anions (e.g., phosphate and sulfate) and cations (e.g., MES, HEPES) also have positive effects. They exhibit little or no effect on the folding rate constant, but decrease the unfolding rate constant. They act by stabilizing hydrophobic interactions, but care should be taken because they can also stabilize aggregate formation.
- Include salt and maintain neutral pH.

  100 mM KCl, or 150–500 mM NaCl, 2 mM $MgCl_2$

  10–50 mM HEPES-KOH (pH 7.5–7.9)
- Suppress proteolytic degradation with protease inhibitors.

  0.5 mM PMSF, 0.05–2 µg/ml aprotinin, 2 µg/ml pepstatin, or 2–5 µg/ml leupeptin

Many proteins that are insoluble when refolded in solution can be successfully refolded while immobilized on the Ni-NTA matrix. Immobilizing one end of the protein during renaturation appears to prevent intermolecular interactions which lead to aggregate formation. Renature using a linear 6 M – 1 M urea gradient in 500 mM NaCl; 20% glycerol; 20 mM Tris·Cl, pH 7.4, containing protease inhibitors. The gradient should be performed using FPLC equipment to ensure linearity (conventional gradient makers often produce nonlinear gradients), and the renaturation should be performed over a period of 1.5 h. After renaturation, proteins can be eluted by the addition of 250 mM imidazole. See also Holzinger *et al.* (1996).