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Description

Ni-NTA His•Bind Resins

Store Ni-NTA His•Bind Resin and Ni-NTA His•Bind Superflow at 4°C.

Ni-NTA His•Bind Resin is used for rapid one-step purification of proteins containing a His•Tag® sequence by metal chelation chromatography. The His•Tag sequence binds to Ni^{2+} cations, which are immobilized on the Ni-NTA His•Bind Resin. After unbound proteins are washed away, the target protein is recovered by elution with imidazole. The versatile system allows proteins to be purified under gentle, non-denaturing conditions or in the presence of either 6 M guanidine or urea.

With the His•Tag/His•Bind technology, purification is based on the affinity between the 6–10 neighboring histidines of the His•Tag sequence and an immobilized metal ion (usually Ni^{2+}). The metal is held by chelation with reactive groups covalently attached to a solid support. The Ni-NTA His•Bind Resins use nitriloacetic acid (NTA) as the chelator, which has four sites available for interaction with metal ions. NTA chemistry minimizes leaching of the metal during purification and is compatible with up to 20 mM β-mercaptoethanol for reduction of disulfide bonds.

Both Ni-NTA His•Bind Resin and Ni-NTA His•Bind Superflow have a binding capacity of 5–10 mg protein per ml resin. Ni-NTA His•Bind Resin is intended for gravity flow columns only. Ni-NTA His•Bind Superflow is compatible with FPLC or gravity flow applications.

Ni-NTA Buffer Kit

- 2 × 125 ml 4X Ni-NTA Bind Buffer
- 125 ml 4X Ni-NTA Wash Buffer
- 50 ml 4X Ni-NTA Elute Buffer

Store the unopened Ni-NTA Buffer Kit at room temperature.

The Ni-NTA Buffer Kit contains 4X concentrated solutions for protein binding, washing and elution under native (non-denaturing) conditions. The concentrated solutions should be diluted to 1X with sterile deionized water shortly before use. 1X solutions may be stored at 4°C for short periods.

BugBuster Ni-NTA His•Bind Purification Kit

- 2 × 100 ml BugBuster Protein Extraction Reagent
- 10,000 U Benzonase Nuclease
- 10 ml Ni-NTA His•Bind Resin
- pkg/4 Chromatography Columns

Store BugBuster reagent at room temperature. Store Benzonase at –20°C.

The BugBuster Ni-NTA His•Bind Purification Kit combines the Ni-NTA His•Bind Resin and BugBuster Protein Extraction Reagent for convenient preparation of soluble cell extracts and affinity purification of His•Tag fusion proteins. BugBuster Protein Extraction Reagent is a ready-to-use solution formulated for the gentle disruption of the cell wall of *E. coli*, resulting in the liberation of soluble protein. The kit includes Benzonase® Nuclease for viscosity reduction and removal of nucleic acids from protein preparations. Cells are harvested by centrifugation as usual, followed by suspension in BugBuster reagent. During a brief incubation, soluble proteins are released without denaturation. The extract is clarified by centrifugation, which removes cell debris and insoluble proteins. The clarified extract is ready to apply to the Ni-NTA His•Bind Resin.

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Overview

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 His•Tag® fusion proteins by Ni-NTA His•Bind 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 Gu-HCl before the purification steps are initiated.

Basic principles

Lysis and binding buffers

In general, bacterial cells can be lysed directly in the same buffer used for binding to the Ni-NTA His•Bind Resin. In the following protocols, 1X Ni-NTA Binding Buffer should be used for lysis and binding under native conditions, and is referred to as "lysis/binding buffer". For purification under denaturing conditions, use the denaturing lysis/binding buffer recipe given on page 21.

Culture size

Optimal purification is dependent on a number of factors, including the amount of His•Tag fusion 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 in a larger amount of culture must be lysed in a given volume of lysis/binding buffer corresponding to a high “concentration factor”. The concentration factor is defined as the ratio of the culture size to the amount of lysis/binding buffer used. Examples of the relationship between expression levels and recommended culture volumes and concentration factors are provided in Table 1.

For example, if a protein is expressed at 0.1 mg/ml and a miniature batch purification procedure will be used under denaturing conditions (see “Batch purification under denaturing conditions”, page 19), a 100 ml culture should be concentrated 100 fold by resuspending the pellet in 1 ml of denaturing lysis/binding buffer.

Under native conditions, it is more difficult to predict the amount of soluble protein present in the lysate. Generally, 50–100 fold concentration is recommended to purify significant amounts of His•Tag fusion protein.

Table 1. Determination of cell culture volume requirements

Concentration of His•Tag fusion protein	Expression level	Culture volume	Amount of His•Tag fusion protein	Concentration factor*
Denaturing conditions				
50 mg/liter	40%	3 ml	150 µg	3×
10 mg/liter	8%	10 ml	100 µg	10×
2 mg/liter	1.6%	25 ml	50 µg	25×
0.5 mg/liter	0.4%	50 ml	25 µg	50×
0.1 mg/liter	0.8%	100 ml	10 µg	100×
Native conditions				
> 1 mg/liter	> 1%	50 ml	> 50 µg	50×
< 1 mg/liter	< 1%	100 m	< 100 µg	100×

*After lysis in 1 ml.

Reducing nonspecific binding

Since there is a higher potential for binding background contaminants under native conditions, low concentrations of imidazole in the lysis/binding and washing buffers (10–20 mM) are recommended. The imidazole ring is part of the structure of histidine. The imidazole rings in the histidine residues of the His•Tag® sequence 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 background proteins. At low imidazole concentrations, the affinity of the 6–10 histidine residues in the His•Tag sequence is strong, allowing efficient binding of the His•Tag fusion proteins, whereas nonspecific binding of background proteins is prevented. Therefore, adding imidazole to the lysis/binding 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 His•Tag fusion protein does not bind under these conditions, the amount of imidazole should be reduced to 1–5 mM.

Binding of His•Tag fusion proteins to Ni-NTA His•Bind Resin is not conformation-dependent and is not affected by most detergents and denaturants (Table 2, page 8). The stability of the His•Tag-Ni-NTA interaction in the presence of low levels of β-ME (up to 20 mM) in the lysis/binding 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 2, page 8), 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 His•Tag fusion protein.

Protein solubility and intracellular location

Since the interaction between Ni-NTA and the His•Tag sequence 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. 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 secretion signal peptides are used, the His•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 condiflons

The decision whether to purify His•Tag fusion proteins under denaturing or nondenaturing conditions depends on protein location and solubility, the accessibility of the His•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.

Purification under native conditions

If purification under native conditions is preferred or necessary, the His•Tag fusion 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. Purification of His•Tag fusion proteins under native conditions can be exploited to copurify associated proteins such as enzyme subunits and binding proteins present in the expressing cells (2, 3), added to the lysate prior to purification, or added to the Ni-NTA His•Bind matrix after the His•Tag fusion protein is bound.

The potential for unrelated, untagged proteins to interact with the Ni-NTA His•Bind 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. Nonspecific binding can be reduced by including a low concentration of imidazole (10–20 mM) in the lysis/binding and wash buffers (1X Ni-NTA Wash Buffer No. 70925 contains 20 mM imidazole).

In rare cases the His•Tag sequence is hidden by the tertiary structure of the native protein, so that soluble proteins require denaturation before they can be purified on Ni-NTA His•Bind Resin. As a

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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.

It is difficult to provide a general protocol for the purification of His•Tag® fusion proteins under native conditions because there is significant variation in protein structure that can interfere with binding. However there are some general suggestions that may aid in optimizing the native purification procedure:

- Cells can be lysed gently by using BugBuster™ Protein Extraction Reagent. Inclusion of Benzonase® Nuclease reduces viscosity and removes nucleic acids.
- Alternatively, 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/binding 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 His•Bind matrix. The minimum salt concentration during binding and washing steps should be 300 mM NaCl. The maximal concentration is 2 M NaCl (Table 2, page 8).

Secretion efficiency for proteins containing a secretion signal sequence can be determined for secreted proteins by purification of the periplasmic extract after osmotic shock (see "Preparation of periplasmic proteins", page 13)

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 Gu-HCl or 8 M urea completely solubilize cells, inclusion bodies, and His•Tag fusion 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 His•Tag on a protein will be fully exposed so that binding to the Ni-NTA His•Bind matrix will improve, and the efficiency of the purification procedure will be maximized by reducing the potential for nonspecific binding.

His•Tag fusion 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 His•Bind column itself prior to elution (4), or in solution (5); additional suggestions are included in this manual (see "Protein Refolding Methods", page 22).

Batch or column purification

Proteins may be purified on Ni-NTA His•Bind Resins in either a batch or a column procedure. The batch procedure entails binding the protein to the Ni-NTA His•Bind 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 His•Tag fusion protein especially when the His•Tag sequence 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 His•Bind Resin is first packed into the column and equilibrated with 1X Ni-NTA Bind 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 His•Tag sequences, 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 His•Tag sequence does not depend on the three-dimensional structure of the protein. Even when the sequence 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 His•Tag. Any host proteins that bind nonspecifically to the Ni-NTA His•Bind

Resin itself can be easily washed away under relatively stringent conditions that do not affect the binding of His•Tag fusion proteins.

Binding can be carried out in a batch or column mode (see "Batch or column purification", page 5). If the concentration of His•Tag® fusion protein is low, or if it is expressed at low levels, or secreted into the media, the protein should be bound to Ni-NTA His•Bind Resin in a batch procedure, and under conditions in which background proteins do not compete for the binding sites, e.g. 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 bind buffer. If high background levels are still present, equilibrating the Ni-NTA His•Bind matrix with a binding buffer containing up to 20 mM imidazole prior to binding is recommended (1X Ni-NTA Bind Buffer contains 10 mM imidazole). 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 His•Bind Resin 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. (1X Ni-NTA Wash Buffer contains 20 mM imidazole). 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 His•Tag fusion 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

Although several elution methods are effective(imidazole, pH, and EDTA), 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. Elution can be effected by increasing the imidazole concentration to 100–250 mM. The His•Tag fusion proteins will dissociate from the Ni-NTA His•Bind Resin because they can no longer compete for binding sites. 1X Ni-NTA Elute Buffer contains 250 mM imidazole for elution of proteins under native conditions; see page 21 for additional buffer recipes.

The histidine residues in the His•Tag sequence 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 His•Tag fusion protein can no longer bind to the nickel ions and will dissociate from the Ni-NTA His•Bind Resin.

Elution conditions are highly reproducible, but optimum conditions must be determined for each His•Tag fusion protein which is being purified. Monomers generally elute at approximately pH 5.9, whereas aggregates and proteins that contain more than one His•Tag sequence 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 His•Tag fusion protein to elute as a protein-metal complex. NTA His•Bind Resins that have lost their nickel ions become white in color and must be recharged if they are to be reused.

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 His•Bind Resin much more weakly than proteins with a His•Tag sequence, and can thus be easily washed away, even when they are much

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more abundant than the His•Tag fusion protein (6). 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 His•Bind matrix in the first place. This is especially important when purifying His•Tag fusion 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 His•Tag® fusion protein. Dithiothreitol (DTT) should not be used (see Table 2, page 8).

Proteins that copurify because they are linked to the His•Tag fusion protein, proteins that associate nonspecifically with the His•Tag fusion protein, and nucleic acids that associate with the His•Tag fusion 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 0.5% Sarkosyl), by increasing the salt concentration to up to 2 M NaCl, or by including ethanol or glycerol (up to 30%) to reduce hydrophobic interactions. The optimal amounts of any of these reagents should be determined empirically for each purification protocol. Nucleic acids may also be removed with Benzonase treatment prior to binding.

In some rare cases untagged, cellular proteins may bind to the carbohydrate matrix of Ni-NTA His•Bind Resin or Ni-NTA His•Bind Superflow™ itself. This situation might be remedied by using a different matrix such as His•Bind Quick Resin or His•Bind Fractogel® for the purification procedure.

Truncated His•Tag fusion proteins are common contaminants that are copurified by Ni-NTA His•Bind affinity chromatography. These contaminants result from internal initiation of translation (C-terminal His•Tag fusion proteins), from premature translation termination (N-terminal His•Tag fusion proteins), or from protein degradation during protein expression or purification. This can be assessed by monitoring the size of His•Tag fusion proteins using other detection methods (e.g. S•Tag™ Western Blot Kit or HSV•Tag® Monoclonal Antibody for the appropriate vectors). Changing the location of the tag to the amino or carboxyl terminus may have to be carried out 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 His•Tag fusion protein to be purified (7). His•Tag fusion proteins have a higher affinity for the Ni-NTA His•Bind Resin than the background proteins. Consequently very few nontagged proteins will be retained on the resin if nearly all available binding sites are occupied by the His•Tag fusion protein. If too much Ni-NTA His•Bind matrix is used, other proteins may nonspecifically bind to unoccupied sites and elute as contaminants.

Limitations

Ni-NTA His•Bind 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 His•Tag fusion proteins. Ni-NTA His•Bind 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 strips them from the matrix. NTA His•Bind 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 His•Bind 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, please refer to Table 2, page 8.

Table 2. Compatibility of reagents with Ni-NTA His•Bind 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
Sulfhydryl 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)	Removes background proteins and nucleic acids	Up to 2%
Cationic detergents		Up to 1 % can be used
Anionic detergents (SDS, Sarkosyl)		Not recommended, but up to 0.3% has been used successfully in some cases
BugBuster Reagent	Releases soluble proteins	Use as supplied
Denaturants		
Gu-HCl	Solubilize 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 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•Tag® fusion protein from the Ni-NTA His•Bind matrix
Other additives		
NaCl	Prevents ionic interactions	Up to 2 M Should use at least 150 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 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•Tag fusion protein from the Ni-NTA His•Bind matrix
Sodium bicarbonate		Not recommended
Hemoglobin		Not recommended
Ammonium		Not recommended
Citrate		Up to 60 mM has been used successfully

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Purification of His•Tag® fusion proteins produced in eukaryotes

Purification of His•Tag fusion proteins expressed in eukaryotic cells can pose a few problems. In the hope 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 His•Tag fusion protein directly into the medium. Media used to culture yeast or insect cells usually have an acidic pH or contain electron-donating groups that could preclude binding of the His•Tag fusion protein to Ni-NTA His•Bind Resins. The pH must be adjusted, and any interfering components may need to be removed by ultrafiltration or dialysis prior to purification with Ni-NTA His•Bind matrices. Dialysis of a sample prior to purification on Ni-NTA His•Bind is recommended as a safeguard.

Mammalian cell culture media are often supplemented with serum proteins which bind weakly to the Ni-NTA His•Bind matrix and compete with the His•Tag fusion protein for binding sites. Amino acids such as glutamine or histidine are commonly added to media and have a similar effect. Lysates of yeast, insect, or mammalian cells can also contain contaminants that interfere with the binding step. In these instances the presence of the His•Tag fusion proteins in the crude lysate or medium can often be verified by enzymatic assay using the S•Tag™ Rapid Assay Kit or the FRETWorks™ S•Tag Assay Kit, or detected on Western blots using the S•Tag Western Blot or LumiBlot™ Kits, the T7•Tag or HSV•Tag® Monoclonal Antibodies (using the appropriate vectors). However, purification can only be carried out after a dialysis or filtration step preceding Ni-NTA His•Bind affinity chromatography. Dialysis of the medium against a buffer with the appropriate composition and pH (8.0) usually restores optimal binding conditions. Alternative methods such as size-exclusion chromatography or ion-exchange chromatography can also be considered (8, 9).

Removal of the His•Tag sequence

It is rarely necessary to remove the His•Tag sequence from the recombinant protein after purification. If the tag must be removed (from very small proteins, for example, where the tag represents a large portion of sequence), a vector should be chosen that encodes a protease cleavage site. Most Novagen vectors containing the His•Tag sequence also encode protease cleavage sites (thrombin, Factor Xa, or enterokinase).

Carboxypeptidase A (Calbiochem Cat. No. 217285) may be useful for the removal of C-terminal His•Tag sequences. The enzyme efficiently removes aromatic C-terminal residues (10, 11) until it encounters a basic residue, at which point degradation is terminated.

Purification Procedures

Preparation of cleared lysates using BugBuster™/Benzonase®

Materials

- Cell culture
- BugBuster™ Protein Extraction Reagent
- Benzonase® Nuclease
- 2X SDS-PAGE sample buffer

Note: *This fraction will consist of soluble protein present in both the periplasm and cytoplasm. If a separate periplasmic fraction is desired, follow the osmotic shock procedure given in "Preparation of periplasmic proteins" on page 13, or refer to the pET System Manual (TB055) or other suitable method. The final pellet from the osmotic shock procedure can then be used in this protocol.*

1. Harvest cells from liquid culture by centrifugation at 10,000 × g for 10 min using a pre-weighed centrifuge tube. Decant and allow the pellet to drain, removing as much liquid as possible. Determine the wet weight of the pellet.
2. Completely resuspend the cell pellet in room temperature BugBuster reagent by pipetting or gentle vortexing, using 5 ml reagent per gram of wet cell paste. At this point protease inhibitors may be added (see notes below).

Optional: *Add 1 µl (25 units) of Benzonase per ml of BugBuster reagent used for resuspension.*

3. Incubate the cell suspension on a shaking platform or rotating mixer at a slow setting for 10–20 min at room temperature. If Benzonase was added, the extract should not be viscous at the end of the incubation.
4. Remove insoluble cell debris by centrifugation at 16,000 × g for 20 min at 4°C. If desired, save the pellet for inclusion body purification as described below.
5. Transfer the supernatant to a fresh tube. The soluble extract can be loaded directly onto Ni-NTA His•Bind Resin equilibrated with 1X Ni-NTA Bind Buffer. Maintain clarified extracts on ice for short term storage (a few h) or freeze at –20°C until needed (see notes below).

Inclusion body purification

1. Process the induced culture according to steps 1–4 above for the soluble protein fraction.
2. Resuspend the pellet from step 4 above in the same volume of BugBuster reagent that was used to suspend the cell pellet. Pipet up and down and vortex to obtain an even suspension (see note *i* below).
3. Add lysozyme to a final concentration of 200 µg/ml (use 1/50 volume of a freshly prepared 10 mg/ml stock in water). Vortex to mix and incubate at room temperature for 5 min.
4. Add 6 volumes of 1:10 diluted BugBuster reagent (in deionized water) to the suspension and vortex for 1 min.
5. Centrifuge the suspension at 16,000 × g for 15 min at 4°C to collect the inclusion bodies. Remove the supernatant with a pipet.
6. Resuspend the inclusion bodies in ½ the original culture volume of 1:10 diluted BugBuster, mix by vortexing, and centrifuge as in step 5. Repeat this wash step 2 more times.
7. Resuspend the final pellet of purified inclusion bodies in your buffer of choice. Inclusion bodies prepared in this manner are compatible with resuspension in 1X IB Solubilization Buffer provided in Novagen's Protein Refolding Kit.

Notes

a) The recommended amount of BugBuster to use (5 ml/g cells) corresponds to about 3 ml for a 50 ml culture, which usually produces about 0.5–0.7 g cell pellet under the conditions described here. For small cultures use up to 1/5 culture volume for resuspension (e.g., use 300 µl BugBuster for 1.5 ml cultures). There are no adverse effects to using larger volumes of BugBuster, as required, to simplify handling.

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b) For small scale extractions (1.5 ml or less), centrifugation to remove cell debris and to collect inclusion bodies can be performed at top speed in a microcentrifuge (14,000–16,000 × g).

c) Extraction and Benzonase digestion are most efficient when performed at room temperature. Protease inhibitors (e.g., Protease Inhibitor Cocktail Set III) can be added along with the BugBuster reagent if desired. It should also be noted that serine protease inhibitors should be used with caution if the target protein is to be treated with thrombin, Factor Xa or enterokinase, because any active inhibitor carried though the purification may affect cleavage reactions. Although it is likely that the inhibitors will be removed and/or inactivated during purification, we recommend including a dialysis or gel filtration step prior to proteolytic cleavage with rEK, Factor Xa or thrombin.

d) BugBuster can be used for frozen or fresh cell pellets. There are instances where the processing of frozen cell pellets may be required, especially when monitoring expression levels over an extended time course or where the number of samples is large. BugBuster is fully compatible with frozen cell pellets; in fact, more protein may be released if cells are frozen prior to processing. When performing comparisons of target protein from multiple samples, all samples should be processed in a similar manner (i.e. all fresh or all frozen) to prevent erroneous results. Superior extraction efficiencies may be obtained when pelleted cells are frozen prior to resuspension in BugBuster and processing. Extraction efficiency is somewhat strain-dependent, and appears to be especially efficient with BL21 and derivative strains (e.g., Tuner™ strains).

e) BugBuster has known compatibility with Tris and phosphate-based buffer systems in the near-neutral pH range. The detergents in BugBuster will precipitate at or above 1 M NaCl. When using high salt buffer compositions or acid or alkaline pH ranges for chromatography, BugBuster should be evaluated on a small-scale prior to scale-up of purification procedures. Attention should be paid to any observed precipitation of the BugBuster reagent or target protein, and compatibility of the BugBuster reagent with buffers used for equilibration of chromatography columns.

f) BugBuster reagent is compatible with reducing agents such as 2-mercaptoethanol and DTT, as well as with EDTA and many other compounds. Note, however, that reducing agents may activate certain proteases, and reducing agents and EDTA will interfere with protein binding to His•Bind Resin. Up to 20 mM 2-mercaptoethanol can be used with Ni-NTA His•Bind Resin. Benzonase is minimally inhibited by EDTA concentrations < 1 mM. However, an EDTA concentration of 5 mM results in a 90% loss of activity due to chelation of essential Mg^{2+} ions.

g) Extracts should be stored at a temperature that is compatible with target protein activity; some target proteins may be inactivated by freeze-thaw cycles.

h) Storage at temperatures below 4°C may cause precipitation of the detergents in BugBuster reagent. Incubate in a room temperature water bath with gentle swirling to redissolve.

i) Thorough resuspension of pellets is critical to obtaining a high purity inclusion body preparation. The inclusion body pellet must be completely dispersed during BugBuster wash steps in order to solublize and remove contaminating proteins from the pellet.

j) For SDS-PAGE and Western blot analysis, a load volume of approximately 2.4 µl would give a normalized amount of protein, given a concentration factor of 25 and an OD_{600} of 3 at harvest, using a 15 well mini gel. When 1/5 culture volume of BugBuster is used, this would correspond to a load volume of approximately 12 µl (since the concentration factor is 5). Because the optimal amount of material to load will vary with the expression level of the target protein, the efficiency of the extraction, and detection sensitivity of the Western blot method, these amounts should be used as guidelines only.

k) Benzonase can be diluted for ease of handling small quantities with 50 mM Tris-HCl pH 8, 20 mM NaCl, and 2 mM $MgCl_2$. Diluted samples can be stored at 4°C for several days without loss of activity.

l) Although Benzonase requires Mg^{2+} for activation, it does not appear to require additional Mg^{2+} under the conditions described here. The activity is sufficient for effective viscosity reduction and nucleic acid digestion using just the BugBuster reagent for cell resuspension.

m) Benzonase is inhibited (approximately 50% reduction in relative activity) by monovalent cation concentrations > 50 mm, phosphate concentrations > 20 mM, and by ammonium sulfate concentrations > 25 mM.

n) Benzonase treatment is not generally recommended for purification of proteins that must be nuclease free. However, depending on the processing methods, Benzonase may be removed during purification. Residual nuclease activity can be checked by incubation of the purified protein with RNA or DNA markers followed by gel analysis.

o) Benzonase® appears to have no effect on the FRETWorks™ S•Tag™ Assay.

Preparation of cleared lysates under native conditions

Materials

- Cell pellet (fresh or frozen)
- 1X Ni-NTA Bind Buffer (lysis/binding buffer)
- Lysozyme
- 2X SDS-PAGE sample buffer

Buffer compositions are provided on page 21.

1. **Thaw the cell pellet for 15 min on ice (if frozen) and resuspend the cells in 1X Ni-NTA Bind Buffer at 2–5 ml per gram wet weight.**
   The amount of cells required depends on the expression level of the His•Tag® fusion protein and the expression system used. The binding capacity of Ni-NTA His•Bind Resins is protein-dependent and normally lies between 5–10 mg/ml. For example, Ni-NTA His•Bind Resin or Ni-NTA His•Bind Superflow has a binding capacity of 0.3 µmol/ml (8.0 mg/ml) for His•Tag DHFR (~26 kDa). Please refer to Table 1, "Determination of cell culture volume requirements" on page 3. Lysis buffer contains 10 mM imidazole to minimize binding of untagged, contaminating proteins and increase purity with fewer wash steps. If the His•Tag fusion protein does not bind under these conditions, the amount of imidazole should be reduced to 1–5 mM. With His•Tag fusion 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 sec bursts at 200–300 W with a 10 sec 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 × g for 20–30 min at 4°C to pellet the cellular debris. Save supernatant.**
   A certain proportion of the cellular protein, including the His•Tag fusion protein, may remain insoluble and will be located in the pellet. For more complete recovery of the His•Tag fusion protein, this material must be solubilized using denaturing conditions as described in Protocol 3 below 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 14.**

Preparation of cleared lysates under denaturing conditions

Materials

- Cell pellet
- 2X SDS-PAGE sample buffer
- Buffer B (denaturing lysis/binding buffer)

Buffer compositions are provided on page 21.

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 Gu-HCI 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. Gu-HCI 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

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treated as described on page 21.
The amount of cells required depends on the expression level of the His•Tag® fusion protein and the expression system used. The binding capacity of Ni-NTA His•Bind Resins is protein-dependent and normally lies between 5–10 mg/ml. For example, Ni-NTA His•Bind Resin or Ni-NTA His•Bind Superflow™ has a binding capacity of 0.3 µmol/ml (8.0 mg/ml) for His•Tag DHFR (~26 kDa). Please refer to Table 1, "Determination of cell culture volume requirements" on page 3.

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 × 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 16.**

Preparation of periplasmic 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 that is cleaved following translocation. In order to purify proteins secreted into the periplasmic space using Ni-NTA His•Bind technology, the His•Tag sequence must be engineered to the C-terminus of the target protein. N-terminal His•Tag sequences will be proteolytically removed along with the transit signal.

Materials

- 30 mM Tris-Cl, 20% sucrose, pH 8.0
- 500 mM EDTA
- 5 mM $MgSO_4$
- 1X Ni-NTA Bind Buffer

Buffer compositions are provided on page 21.

1. **Grow and induce a 1 liter culture.**
2. **Harvest the cells by centrifugation at 4,000 × 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 × 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 ice bath.**
4. **Centrifuge at 8000 × g for 20 min at 4°C.**
   The supernatant is the osmotic shock fluid containing periplasmic proteins.
5. **Dialyze supernatant extensively against 1X Ni-NTA Bind Buffer before continuing with the purification.**

Protocols for Purification Under Native Conditions

Before purifying proteins under native (non-denaturing) conditions, it is important to check that the protein is soluble. However, even if most of the protein is insoluble, it is often possible to purify traces of soluble material on Ni-NTA His•Bind Resin.

In the absence of strong denaturing agents such as urea, unstable proteins in cell lysates may be subject to degradation. It is best to work quickly and to keep the lysate at 0–4°C at all times. Addition of AEBSF or Protease Inhibitor Cocktail Set III (Cat. Nos. 101500, 539134) may be helpful on a case-by-case basis, but their potential effect on the recombinant protein must be taken into consideration.

Batch purification under native conditions

The amount of lysate required depends on the expression level of the His•Tag® fusion protein and the expression system used. The binding capacity of Ni-NTA His•Bind Resins is protein-dependent and normally lies between 5–10 mg/ml. For example, Ni-NTA His•Bind Resin and Ni-NTA His•Bind Superflow™ have a binding capacity of 0.3 µmol/ml (8.0 mg/ml) for His$^-$ Tag DHFR (~26 kDa). Please refer to Table 1, "Determination of cell culture volume requirements" on page 3.

For proteins that are expressed at high levels, (10–50 mg of His•Tag fusion protein per liter of cell culture) a 10X-concentrated cell lysate can be used. Four ml of a 10X concentrated cell lysate will contain approximately 0.4–2 mg of His•Tag fusion 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 His•Tag fusion protein). See also "Culture size", page 3 for more information.

The 1X Ni-NTA Bind Buffer contains 10 mM imidazole to minimize binding of untagged, contaminating proteins and increase purity with fewer wash steps. If the His•Tag fusion protein does not bind under these conditions, the amount of imidazole should be reduced to 1–5 mM. With His•Tag fusion proteins exhibiting high binding affinities, the imidazole concentration can be increased to 20 mM.

Materials

- Cleared lysate from a 40–200 ml culture
- Ni-NTA His•Bind matrix
- Empty columns
- 1X Ni-NTA Bind Buffer
- 1X Ni-NTA Wash Buffer
- 1X Ni-NTA Elute Buffer

Buffer compositions are provided on page 21. Concentrated stocks of Bind, Wash and Elute buffers are available in the Ni-NTA Buffer Kit (Cat. No. 70899-3).

1. **Add 1 ml of the 50% Ni-NTA His•Bind slurry to 4 ml of 1X Ni-NTA Bind Buffer and mix gently. Allow the resin to settle by gravity, and remove 4 ml of the supernatant with a pipet.**
2. **Add 4 ml of cleared lysate to the Ni-NTA His•Bind slurry and mix gently by shaking (200 rpm on a rotary shaker) at 4°C for 60 min.**
3. **Load the lysate-Ni-NTA His•Bind mixture into a column with the bottom outlet capped.**
4. **Remove bottom cap and collect the column flow-through. Save flow-through for SDS-PAGE analysis.**
5. **Wash with 2 × 4 ml 1X Ni-NTA Wash Buffer, collect wash fractions. Save wash fractions for SDS-PAGE analysis.**
6. **Elute the protein with 4 × 0.5 ml 1X Ni-NTA Elution Buffer. Collect the eluate in four tubes and analyze fractions by SDS-PAGE.**

The composition of 1X Ni-NTA Bind, Wash and Elute Buffers can be modified to suit the particular application, e.g. by adding 0.1% Tween, 5–10 mM β-ME, Protease Inhibitor Cocktail III, or increasing NaCl or glycerol concentrations. For more information, see Table 2, "Compatibility of

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reagents with Ni-NTA His•Bind matrices" on page 8 and "Protocols for Purification Under Native Conditions" on page 14.

FPLC purification 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 His•Bind Superflow column is the method of choice. The physical stability of Ni-NTA His•Bind Superflow makes it ideal for column chromatography at higher pressures and flow rates.

Materials

- Cleared lysate from a 40–200 ml culture
- Ni-NTA His•Bind Superflow™
- 1X Ni-NTA Bind Buffer
- 1X Ni-NTA Wash Buffer
- 1X Ni-NTA Elute Buffer
- FPLC column and hardware

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 His•Bind 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 His•Tag® fusion protein to be purified. Generally, the binding capacity of Ni-NTA His•Bind 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 do not exceed a flow rate of 2 ml/min.
   Do not allow resin to dry. If this should occur, resuspend the 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 FPLC system.
5. **Equilibrate column with 5 column volumes of 1X Ni-NTA Bind 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 1X Ni-NTA Bind 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 His•Tag fusion 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 the column with 1X Ni-NTA 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 1X Ni-NTA Elute Buffer.**
   If desired, a step-gradient of elution buffer in wash buffer may be used to elute the protein. 5 column volumes of each step are usually sufficient. The His•Tag fusion protein usually elutes in the second and third column volume.

Note: *Imidazole also absorbs at 280 nm, which should be considered when monitoring protein elution. If small amounts of His•Tag fusion proteins are purified, elution peaks may be poorly visible.*

Protein minipreps under native conditions

Materials

- Microcentrifuge tubes
- Lysozyme
- Ni-NTA His•Bind Resin
- 1X Ni-NTA Bind Buffer
- 1X Ni-NTA Wash Buffer
- 1X Ni-NTA Elute Buffer

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 1, page 3), 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 × g and discard supematants.**
   If larger culture volumes are required, refill microcentrifuge tube and centrifug Repeat this step until all cells are harvested.
3. **Resuspend cells in 100 µl 1X Ni-NTA Bind 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 1, page 3).
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.**

Optional: *Add 2.5 units of Benzonase per 100 µl of resuspended cells. For ease of handling make a 10-fold diluted stock of Benzonase (2.5 units per microliter; see note k on page 11 for dilution buffer).*

6. **Centrifuge the lysate for 10 min at 15,000 × 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 His•Bind Resin (10 µl resin has a capacity for 50–100 µg His•Tag® fusion protein) to each tube, and mix gently for 30 min at 4°C.**
8. **Centrifuge for 10 sec at 15,000 × 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 with 2 × 100 µl of 1X Ni-NTA Wash Buffer.**
   Centrifuge for 10 sec at 15,000 × g between each elution step and carefully remove the supernatant.
10. **Elute the protein with 3 × 20 µl 1X Ni-NTA Elute Buffer**
    Centrifuge for 10 sec at 15,000 × g between each elution step and carefully remove the supernatant to a fresh tube.
11. **Analyze the supernatant from Step 8 (unbound fraction) and the eluates from Step 10 by SDS-PAGE.**

Protocols for Purification Under Denaturing Conditions

Batch purification under denaturing conditions

Materials

- Cleared lysate in denaturing lysis/binding buffer, from 20–200 ml culture
- Ni-NTA His•Bind Resin
- Empty columns
- Buffers A–E (see page 21)

1. **Add 1 ml of the 50% Ni-NTA His•Bind 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 cells required depends on the expression level of the His•Tag fusion protein

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and the expression system used. The binding capacity of Ni-NTA His•Bind Resins is protein-dependent and normally lies between 5–10 mg/ml. For example, Ni-NTA His•Bind Resin and Ni-NTA His•Bind Superflow have a binding capacity of 0.3 µmol/ml (8.0 mg/ml) for His•Tag DHFR (~26 kDa). Please refer to Table 1, "Determination of cell culture volume requirements" on page 3.

For proteins that are expressed at very high levels (50–100 mg of His•Tag fusion 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 fusion 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 fusion protein).

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. Save flow-through for SDS-PAGE analysis.**
4. **Wash with 2 × 4 ml buffer C.**
   Keep wash fractions for SDS-PAGE analysis.
5. **Elute the recombinant protein with 4 × 0.5 ml buffer D, followed by 4 × 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 His•Tag sequences will generally elute in buffer E.

FPLC purification 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 His•Bind 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

- Cleared lysate in denaturing lysis/binding buffer
- Ni-NTA His•Bind Superflow
- Buffers A–E
- FPLC column and hardware

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 His•Bind 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 His•Tag® fusion protein to be purified. Generally, the binding capacity of Ni-NTA His•Bind 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 His•Tag sequences will generally elute in buffer E. Five column volumes are sufficient. His•Tag fusion proteins usually elute in the second and third column volume.

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Protein minipreps under denaturing conditions

Materials

- Microcentrifuge tubes
- Ni-NTA His•Bind Resin
- Buffers A–C, E (see page 21)

1. **Transfer 1 ml 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 1, page 3), 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 × 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 × 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 His•Bind Resin (25 µl resin has a capacity for 125-250 µg His•Tag fusion protein) to each tube, and mix gently for 30 min at room temperature.**
6. **Centrifuge 10 sec at 15,000 × 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 with 2 × 250 µl of buffer C.**
   Centrifuge for 10 sec at 15,000 × g between each wash step and carefully remove the supernatant.
8. **Elute the protein with 3 × 25 µl buffer E.**
   Centrifuge for 10 sec at 15,000 × g between each elution step and carefully remove the supernatant to a fresh tube.
9. **Analyze the fractions by SDS-PAGE.**
   If Buffer A was used to lyse the cells, refer to “Preparation of guanidine containing samples for SDS-PAGE”, on page 21.

Troubleshooting

Possible explanation	Comments and suggestions
Protein does not bind to the Ni-NTA His•Bind Resin	
His•Tag® sequence 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).
His•Tag sequence is inaccessible.	Purify protein under denaturing conditions. Move tag to the opposite end of the protein.
His•Tag sequence 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 composition of all buffers and solutions.
	Dissociation of urea often causes a shift in pH. 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.
His•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.
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 His•Bind Resin.
Contaminants are associated with His•Tag fusion 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 His•Tag fusion protein.	Check for possible internal translation starts (C-terminal His•Tag) or premature termination sites (N-terminal His•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.

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Solutions

Buffers for purification under native conditions

1X Ni-NTA Bind Buffer (use for lysis and binding):
50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 10 mM imidazole
(optional additions for lysis include 1/10th volume 10X BugBuster or lysozyme; see "Preparation of cleared lysates using BugBuster/Benzonase" on page 10, and "Preparation of cleared lysates under native conditions" on page 12)

1X Ni-NTA Wash Buffer: 50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 20 mM imidazole

1X Ni-NTA Elute Buffer: 50 mM NaH_2PO_4, pH 8.0; 300 mM NaCl; 250 mM imidazole

Buffers for purification under denaturing conditions

Denaturing lysis/bind buffers

Buffer A: 6 M Gu-HCl; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl, pH 8.0

Buffer B: 8 M urea; 0.1 M NaH_2PO_4; 0.01 Tris-Cl, pH 8.0

Denaturing wash buffer

Buffer C: 8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl pH 6.3

Denaturing elution buffers

Buffer D: 8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl pH 5.9

Buffer E: 8 M urea; 0.1 M NaH_2PO_4; 0.01 M Tris-Cl pH 4.5

Note: Due to the dissociation of urea, the pH of Buffers B, C, D, and E should be adjusted immediately prior to use. Do not autoclave.

Preparation of guanidine-containing samples for SDS-PAGE

Since fractions that contain Gu-HCl will form a precipitate when treated with SDS, they must either be diluted with H_2O (1:6), dialyzed before analysis, or separated from the Gu-HCl by TCA precipitation.

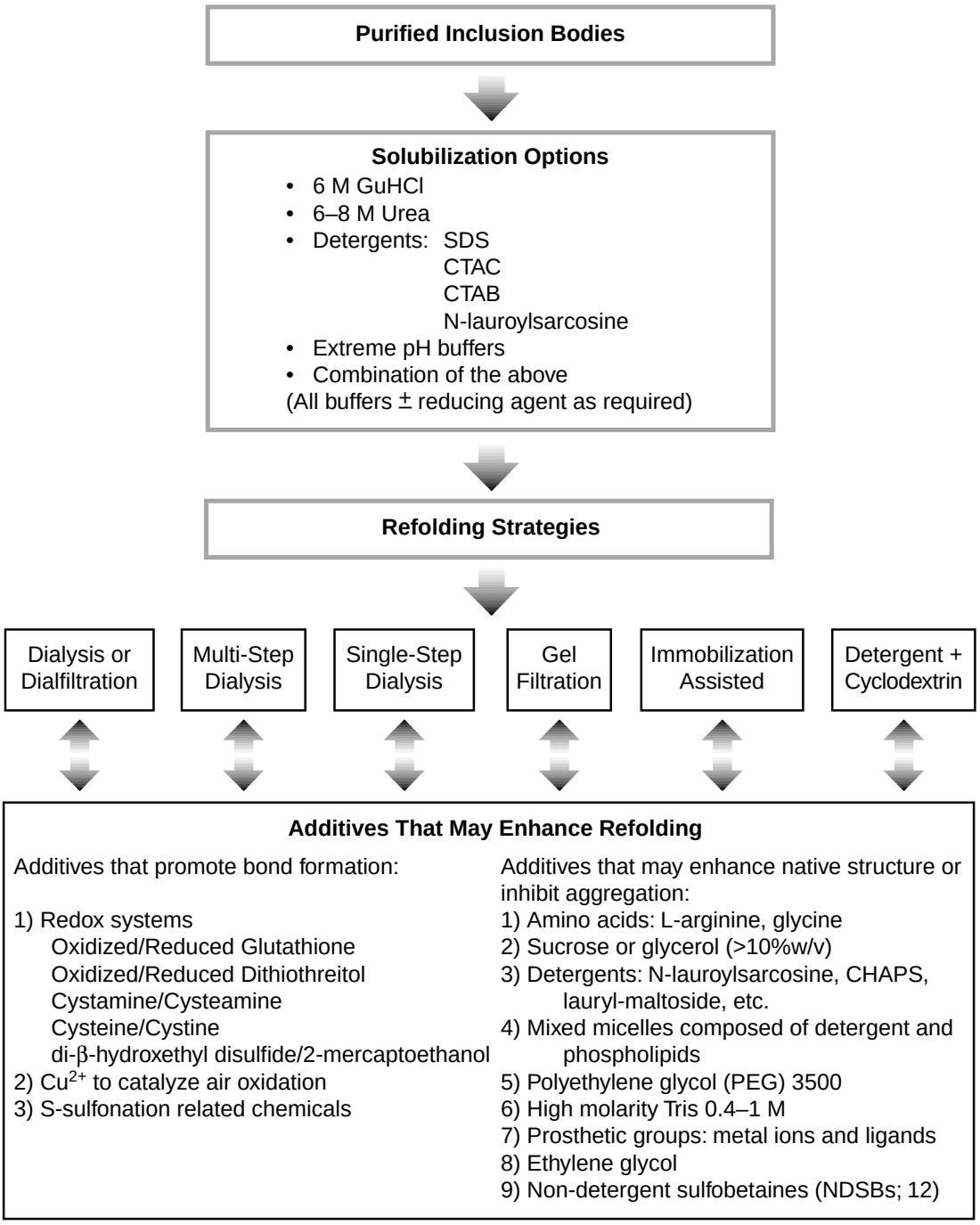
TCA precipitation

1. Dilute 10–25 µl protein samples to 100 µl with dH_2O
2. Add 100 µl of 10% TCA.
3. Leave on ice for 20 min; centrifuge for 15 min in a microcentrifuge.
4. Wash pellet with 100 µl of ice-cold ethanol, dry, and resuspend in sample buffer.
   If there are any traces of Gu-HCl present, samples must be loaded immediately after heating for 7 min at 95°C.

Additional Information on Protein Refolding

Protein refolding methods

In the figure below, a number of refolding strategies are shown that have been used to optimize recovery of functional target protein (12–19); not all are detailed in this bulletin). Inclusion bodies are solubilized by exposure to high molarity denaturants, detergents, acidic or alkali buffers, or through combinations of reagents. In general, the presence of Cys residues in the target protein, or specific requirements for the proper formation of essential disulfides, often dictate the use of a reducing agent in the solubilization and refolding buffers.



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When optimizing refolding conditions, consider three basic factors:

- The choice of refolding method
- The inherent properties of the target protein
- The composition of the refolding buffer

The following is a brief examination of these factors. Depending on the target protein being studied, one or more of these strategies can be incorporated into the protocol used with the Protein Refolding Kit reagents to improve recovery of active, properly folded protein.

Single Step Dialysis or Diafiltration: This method is based on the relatively slow removal of denaturant by buffer exchange through a membrane of defined molecular weight cut-off. Additives that inhibit aggregation are typically included when the target protein is refolded at intermediate to high concentration (0.1 to 10 mg/ml). Refolding under dilute conditions (1–100 µg/ml) can be performed in the absence of additives; however, this is usually impractical for large amounts of protein due to volume considerations.

Multi-Step Dialysis: Instead of proceeding directly to complete denaturant removal as in the Single Step Dialysis Method, denaturant is removed in a step wise fashion. This strategy is beneficial when an intermediate concentration of the denaturant can serve to prevent aggregation and/or destabilize improperly folded protein but no longer denature properly folded protein. The denaturant concentration under which these conditions exist can be approximated by transverse urea gradient gel electrophoresis as summarized in reference (13).

Single-Step Dilution: The denatured protein is diluted many fold directly into refolding buffer to achieve low concentration conditions that promote folding and disfavor aggregation. Slow stepwise addition of the denatured protein to the refolding buffer produces protein concentrations that are effective for refolding because properly refolded protein is not believed to participate in aggregation. In this way, the concentration of properly folded protein and the overall volume can be kept at reasonable levels.

Gel Filtration: The rate of buffer exchange in this method is intermediate between that found in Single Step Dilution and Dialysis. This method is only suitable for proteins that do not produce insoluble intermediates upon refolding.

Immobilization Assisted: This method requires that the target protein possess an affinity tag for an insoluble matrix. Immobilization of the target protein on the matrix should theoretically circumvent protein-protein aggregation due to the spatial separation of the bound protein (18). One advantage realized by this method is the ability to refold and elute at relatively high protein concentrations (5 mg/ml in reference (18)). In particular, this method seems well suited for the development of optimized refolding protocols for CBD fusions.

Detergent + Cyclodextrin: This method was devised in an attempt to simulate the role that chaperones play in the refolding process. Detergents that prevent aggregation of the target protein in the first step are stripped away by exposure to cyclodextrins in the second step thereby allowing the protein to refold (17).

Protein properties that influence refolding conditions

Disulfide Bonds

The presence of (or the requirement for) disulfide bonds in a correctly folded fusion protein help indicate what refolding reagents to use. If analytical amounts of native target protein are available for assay, methods are described for “trapping” reduced disulfides that may be useful in determining whether your target has disulfides that are essential to protein function (16). In general, when disulfide bonds are necessary for correct folding, a reducing agent such as DTT is included in the solubilization step and maintained during the initial refolding to prevent intermolecular disulfide cross-linking. The reducing agent is then removed by dialysis and, when necessary, thiol redox reagents, such as oxidized/reduced glutathione (13, 15), are added to catalyze correct disulfide bond formation and inhibit the formation of non-productive disulfide intermediates. Thiol reagent treatment of solubilized inclusion bodies encourages disulfide bond formation and exchange through the use of a low molecular weight thiol and disulfide mixture. The reaction occurs through a two-step process where thiol groups on the target protein form a mixed disulfide intermediate with the low molecular weight disulfide followed by a disulfide exchange

reaction with a second thiol group residing on the target protein to form an intramolecular disulfide (13). Several factors influence the rate of disulfide exchange, including reaction temperature, pH (pH 8–9 to favor formation of thiolate anions: S^-), conformation and proximity of cysteine residues, and steric hindrance or reactivity of thiols. Exogenous low molecular weight thiol is added to allow reversibility of wrongly paired disufides. The reductant (RSH, e.g., 1 mM reduced glutathione) is typically added in a five to ten-fold molar excess over the oxidant (RSSR, e.g., 0.2 mM oxidized glutathione). Proteins that lack essential disulfides can often be solubilized and refolded without the addition of reducing agents.

Hydrophobicity and Aggregation

The presence of high numbers of aromatic and aliphatic residues (ala, cys, val, ile, leu, met, phe, and trp) are thought to decrease the solubility of proteins during refolding and contribute to the aggregation of protein folding intermediates (12). The simplest way to minimize hydrophobic aggregation is to include a mild detergent in the buffer during solubilization of inclusion bodies. N-lauroylsarcosine has been reported to effectively reduce hydrophobic aggregation and allow the recovery of monomeric, active protein from inclusion bodies (12, 13). A unique set of compounds, the non-detergent sulfobetaines (NDSBs) appear to be particularly useful in preventing aggregation during refolding (19). Another frequently used approach, although less convenient, is to dilute solubilized protein to < 1 µg/ml to decrease intermolecular aggregation. This approach has the disadvantage of dramatically increasing the required solution volumes for even small amounts of protein. Alternatively, proteins can be concentrated prior to purification by precipitation with ammonium sulfate.

Prosthetic Groups

Knowledge of any essential prosthetic groups can be helpful in optimizing buffers for your specific target protein. For example, bacterial alkaline phosphatase is reported to require Zn^{2+} for proper refolding and Mg^{2+} for full enzymatic activity. When dialyzing solubilized inclusion bodies against a buffer containing these two cations, enzymatically active protein was recovered (15). The use of divalent cations in the presence of DTT is not recommended because it can cause oxidation of the reducing agent and precipitation of the reduced salt.

Net Charge

The net charge of a given protein can be used as a guide for the determination of buffer conditions that may enhance protein solubility. The net charge of a protein can be calculated by summing the basic amino acid residues (arginine, lysine) and subtracting the absolute value of the acidic amino acid residues (aspartic and glutamic acid) (13). If the net charge is near zero, refolding at neutral pH may result in aggregation and poor recovery of protein following solubilization. By shifting the pH away from neutral, greater solubility and recovery of functional protein are possible.

Refolding buffer additives

A primary consideration when determining refolding buffer composition is whether or not there is a need for disulfide bond formation as discussed above. In addition, a wide variety of compounds have been demonstrated to enhance the refolding process for specific proteins including amino acids, mild detergents, polymers, prosthetic groups, sugars, ligands and mixed micelles (summarized in (13, 14)). In general, these additives are believed to exert their influence by either decreasing the tendency of mis-folded or partially folded intermediates to aggregate, stabilizing the properly folded product, inhibiting non-productive refolding pathways or a combination of the above.

Determination of optimal refolding conditions for a given target protein is still largely an empirical process. Performing small-scale matrix experiments in which numerous additives are tested under similar conditions is often required. This type of analysis is greatly facilitated by target proteins that have activity that is easily assayed when properly folded. In lieu of this, the presence of detection tags (e.g., S•Tag™ sequence) in the target protein can be used to monitor the success of refolding given the appropriate fractionation of properly and improperly refolded protein.

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References

1. QIAGEN. (1997) The QIAexpressionist A handbook for high-level expression and purification of 6xHis-tagged proteins, QIAGEN GmbH, Hilden, Germany.
2. Le Grice, S. F. and Gruninger-Leitch, F. (1990) *Eur. J. Biochem.* **187**, 307–314.
3. Flachmann, R. and Kuhlbrandt, W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93**, 14966–14971.
4. Holzinger, A., Phillips, K. S. and Weaver, T. E. (1996) *Biotechniques* **20**, 804–808.
5. Wingfield, P. T. (1995) in Current protocols in protein science, vol. 1 (Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W. and Wingfield, P. T., eds.), pp. 6.6.1–6.1.22, John Wiley and Sons, New York.
6. Janknecht, R., de Martynoff, G., Lou, J., Hipskind, R. A., Nordheim, A. and Stunnenberg, H. G. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88**, 8972–8976.
7. Schmitt, J., Hess, H. and Stunnenberg, H. G. (1993) *Mol. Biol. Rep.* **18**, 223–230.
8. Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W. and Wingfield, P. T. (1995) in Current protocols in protein science, vol. 1, John Wiley and Sons, New York.
9. Deutscher, M. P. (1990) *Methods Enzymol.* **182.**
10. Hochuli, E., Bannwarth, W., Dobeli, H., Gentz, R. and Stueber, D. (1988) *Bio/Technique*s **6**, 1321-1325.
11. Hochuli, E. (1990) in Genetic engineering, principles and methods, vol. 12 (Setlow, J. K., ed.), pp. 87–98, Plenum Press, New York.
12. Burgess, R. R. (1996) *Methods Enzymol.* **273**, 145–149.
13. Mukhopadhyay, A. (1997) *Adv. Biochem. Eng. Biotechnol.* **56**, 61–109.
14. Rudolph, R. and Lilie, H. (1996) *FASEB J.* **10**, 49–56.
15. Walker, K. W. and Gilbert, H. F. (1994) *J. Biol. Chem.* **269**, 28487–28493.
16. Darby, N. and Creighton, T. E. (1995) in Protein Stability and folding: Theory and Practice (Shirley, B. A., ed.), Humana Press, Inc.
17. Rozema, D. and Gellman, S. H. (1996) *J. Biol. Chem.* **271**, 3478–3487.
18. Stempfer, G., Holl-Neugebauer, B. and Rudolph, R. (1996) *Nat. Biotechnol.* **14**, 329–334.
19. Vuillard, L., Rabilloud, T. and Goldberg, M. E. (1998) *Eur. J. Biochem.* **256**, 128–135.

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BugBuster™ Protein Extraction Reagent	100 ml	70584-3
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BugBuster (primary amine-free) Protein	100 ml	70923-3
Extraction Reagent	500 ml	70923-4
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