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About the Kit

Protein Refolding Kit

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Description

The Protein Refolding Kit provides all the necessary reagents to solubilize recombinant proteins that accumulate as inclusion bodies in *E. coli*. The provided buffers and simple protocol enable isolated inclusion bodies to be solubilized under mildly denaturing conditions. Following clarification, the solubilized fraction is dialyzed against a neutral pH buffer containing a reducing agent to encourage correct disulfide bond formation. A second dialysis step then removes excess reducing agent and transfers the protein into the buffer of choice. This procedure has been shown to allow refolding of CBD•Tag™ fusion proteins and other recombinant proteins isolated from inclusion bodies (1, 2).

The components provided in the Protein Refolding Kit have been successfully used at Novagen with a variety of fusion proteins including bacterial alkaline phosphatase, β -galactosidase, green fluorescent protein, β -glucuronidase, and CBD•Tag domains.

Components

The Protein Refolding Kit includes sufficient reagents to process up to 2 g of inclusion bodies.

- 100 ml 10X IB Wash Buffer (200 mM Tris-HCl, pH 7.5, 100 mM EDTA, 10% Triton X-100)
- 50 ml 10X IB Solubilization Buffer (500 mM CAPS, pH 11.0)
- 10 ml 30% N-lauroylsarcosine
- 200 ml 50X Dialysis Buffer (1 M Tris-HCl, pH 8.5)
- 3.2 ml 1 M DTT

Preparation of Inclusion Bodies

This protocol begins with an induced cell culture expressing a recombinant protein; for example, one obtained using the pET System. Detailed protocols for constructing recombinants and optimizing growth and induction parameters are included in the pET System Manual, TB055, available on request from Novagen and also on Novagen's web site at www.novagen.com.

Inclusion bodies are isolated from the crude cell lysate by centrifugation and washed twice with 1X IB Wash Buffer to remove loosely associated contaminants. You will need to prepare approximately 0.5 culture volume of 1X IB Wash Buffer from the 10X stock included in the kit.

1. Harvest the induced culture by centrifugation at $6,500 \times g$ for 15 min at 4°C. Remove and discard the supernatant.
2. Thoroughly resuspend the cell pellet in 0.1 culture volume of 1X IB Wash Buffer (20 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% Triton X-100). Mixing may be necessary for full resuspension.
3. Cool the suspension on ice to 4°C to prevent heating during cell breakage. Lyse the cells by one of the following protocols. The two most commonly used methods for lysis of *E. coli* are sonication and high pressure (French Press) homogenization. Sonication is a more widely used technique, but two disadvantages are lower lysis efficiency and difficulty of scale-up beyond 50 g of cells (unless continuous flow sonication is used). Regardless of the lysis method used, keep samples cold to prevent proteolytic degradation of the target protein.
 - a. **French Press:** Load 40 ml samples of suspended cells in a 50 ml capacity French pressure cell and break at 15,000 psi. Chill the pressure cell on ice between runs. One passage of the sample through the cell is usually sufficient to ensure adequate breakage. At this step, if the extract is viscous, the DNA will need to be sheared either by sonication or an additional pass through the French Press (after adding the protease inhibitors as described in Step 4).
 - b. **Lysozyme plus Sonication:** Add lysozyme to a final concentration of 100 $\mu\text{g}/\text{ml}$ from a freshly prepared 10 mg/ml stock in water. Incubate at 30°C for 15 min. Mix by swirling and sonicate on ice with an appropriate tip until cells are lysed and the solution is no longer viscous. It is important to keep the sample cold during sonication to avoid heat denaturation of proteins. The above settings are general recommendations and may need to be adjusted depending on the energy output of a given sonicator. Consult sonicator manufacturer for recommended settings for your equipment.



Note: Optimal conditions for a given sonicator may be quickly determined by performing a time course analysis. Remove samples at various times during the sonication, centrifuge at 12,000 x g for 5 min, and then determine the protein concentration in the supernatant by a standard assay, e.g., Bradford, BCA, etc. When the protein concentration in the supernatant reaches a plateau, proceed to the next step.

4. **Optional.** Immediately following cell lysis, add protease inhibitors. To each 40 ml of homogenate, add 5 ml Protease Inhibitor Cocktail Set II with EDTA (Cat. No. 539132) or 1 ml Protease Inhibitor Cocktail Set III without EDTA (Cat. No. 539134).
5. Collect the inclusion bodies by centrifugation at 10,000 × g for 10 min.
6. Remove the supernatant and thoroughly resuspend the pellet in 0.1 culture volume of 1X IB Wash Buffer.
7. Repeat centrifugation as in Step 5 and save the pellet.
8. Again, thoroughly resuspend the pellet in 0.1 culture volume of 1X IB Wash Buffer. Transfer the suspension to a clean centrifuge tube with known tare weight.
9. Collect the inclusion bodies by centrifugation at 10,000 × g for 10 min. Decant the supernatant and remove the last traces of liquid by tapping the inverted tube on a paper towel.
10. Weigh the tube and subtract the tare weight to obtain the wet weight of the inclusion bodies. A typical wet weight for inclusion bodies is approximately 1–4 mg/ml of culture when the insoluble protein constitutes 10–40% of the total cell protein and the OD₆₀₀ of the harvest is approximately 3.0.

Solubilization and Refolding

Many methods have been published describing refolding of insoluble proteins (3–6). Most protocols describe the isolation of insoluble inclusion bodies by centrifugation followed by solubilization under denaturing conditions. The protein is then dialyzed or diluted into a non-denaturing buffer where refolding occurs. Because every protein possesses unique folding properties, the optimal refolding protocol for any given protein must be empirically determined. Optimal refolding conditions can be rapidly determined on a small scale by a matrix approach, in which variables such as protein concentration, reducing agent, redox treatment, divalent cations, etc., are tested. Once the optimal concentrations are found, they can be applied to a larger scale solubilization with refolding of the target protein. See Appendix A for further discussion.

This Protein Refolding Kit uses a CAPS buffer at alkaline pH in combination with N-lauroylsarcosine (included as a separate reagent) to achieve solubility of the inclusion bodies. If your target protein is sensitive to detergent, you have the option of using other denaturants. The 10X IB Solubilization Buffer has also been used in combination with 4–4.5 M urea for solubilization and refolding of inclusion bodies with both CBD_{clos} and CBD_{cex} fusion proteins with good success. Other reported denaturants include guanidine hydrochloride, SDS (3), and cetyltrimethylammonium chloride (7).

It is important to note that urea solutions must be used promptly and not stored for long periods because urea decomposes to form cyanate ions (especially at high concentration and temperature, and at alkaline pH), which can covalently modify primary amines on the target protein. The cyanate problem can be minimized by the addition of Tris or lysine to the urea buffer to act as a cyanate scavenger.

N-lauroylsarcosine does not impair refolding and purification, but it can be removed using an anion exchange resin (4) and the protocol on p. 5. The amount of detergent present and the effectiveness of removal can be determined using a turbidimetric detergent assay (4), which is described on p. 5.

1. From the wet weight of the inclusion bodies to be processed, calculate the amount of 1X IB Solubilization Buffer necessary to resuspend the inclusion bodies at a concentration of 10–20 mg/ml.
2. At room temperature, prepare the calculated volume of 1X IB Solubilization Buffer supplemented with 0.3% N-lauroylsarcosine (1/100 dilution of the stock provided; up to 2% N-lauroylsarcosine may be used, as needed). For 50 ml of 1X IB Solubilization Buffer/N-lauroylsarcosine, 5 ml of 10X IB Solubilization Buffer is diluted to 49.45 ml with deionized water, then supplemented with 0.5 ml of 30% N-lauroylsarcosine and 50 μl of 1M DTT (if desired).



3. Add the calculated amount of 1X Solubilization Buffer/N-lauroylsarcosine from step 2 to the inclusion bodies and gently mix. Large debris can be broken up by repeated pipetting.

Note:

Not all material present in the inclusion bodies will be solubilized and some stringy precipitates of cell debris will be visible.

4. Incubate at room temperature for 15 min.
5. Clarify by centrifugation at $10,000 \times g$ for 10 min at room temperature. Transfer the supernatant containing the solubilized protein into a clean tube, avoiding pelleted debris. Use caution because the pellet may be soft and/or loose.

Dialysis Protocol for Protein Refolding

The kit includes a 50X Dialysis Buffer and 1 M DTT for convenient dialysis of solubilized inclusion bodies.

1. Prepare the required volume of buffer for dialysis of your solubilized protein. In general, dialysis should be performed with at least two buffer changes of greater than 50 times the volume of the sample. For example, if your sample volume is 15 ml, the dialysis volume is 750 ml and a total of 1.5 L of dialysis buffer will be required. For this example, dilute 30 ml of 50X Dialysis Buffer in 1.5L of deionized water and supplement with 150 μ l 1 M DTT (0.1 mM DTT final concentration).

Note:

When working with CBD_{clos} fusions, it is recommended that dialysis buffers be supplemented with NaCl at 100 mM (use a 5 M stock solution). In some cases, CBD_{clos} fusions appear to be sensitive to low salt buffers.

2. Dialyze for at least 3 h at 4°C. Change the buffer and continue. Dialyze for an additional 3 or more hours.
3. Prepare additional dialysis buffer as determined in step 1, but omit DTT.
4. Continue the dialysis through two additional changes (3 h each) with the dialysis buffer lacking DTT.
5. If any visible insoluble material is evident following dialysis, centrifuge the dialyzed protein solution at $10,000 \times g$ for 10 min at 4°C. Functional assays of the target protein should also be done to determine whether additional thiol reagent treatments are necessary for disulfide oxidation and isomerization.

Redox Refolding Buffer to Promote Disulfide Bond Formation

To promote disulfide bond formation, additional dialysis in the presence of a redox pair (not provided in the kit) is sometimes performed. A commonly used redox pair is oxidized and reduced glutathione (Calbiochem #3542 and #3541, respectively). Due to the instability of the glutathione reagents in solution, these solutions should be used promptly. Other redox pairs that are potentially suitable for this technique include DTT, and cystamine/cysteamine.

Another more economical method for disulfide bond formation is the use of $CuSO_4$ or $NaSeO_3$ catalyzed air oxidation (5). The reaction can be monitored by titrating free thiol groups with Ellman's reagent, 5, 5'-dithio-bis-(2-nitrobenzoic) acid (Pierce Chemical Co.). The disadvantage of this approach is that improperly formed disulfides are not reduced and reformed as would occur with a redox approach.

The following dialysis step is designed to begin after the DTT has been removed by dialysis in step 4 of the refolding protocol above.

1. Prepare a dialysis buffer containing 1 mM reduced glutathione and 0.2 mM oxidized glutathione in 1X Dialysis Buffer. The volume should be 25 times greater than the volume of the solubilized protein sample. Chill to 4°C.
2. Dialyze the refolded protein overnight at 4°C.
3. Assay a sample of the dialyzed protein for target protein activity.
4. If necessary, continue dialysis at room temperature or 37°C to enhance disulfide exchange rates.

Note:

An alternative to this method is to prepare concentrated stock solutions of oxidized and reduced glutathione and add them directly to the target protein, incubate (with periodic assay for activity of target) and then dialyze to remove the excess redox reagent.



Removal of N-lauroylsarcosine with Anion Exchange Resin

This treatment may not be necessary for purified or dialyzed samples. In our laboratory, the residual detergent level following dialysis is often 100-fold lower than the concentration in the solubilization step, typically less than 0.003%. It is advisable to assay the detergent level in the sample, as described below, before proceeding with Dowex™ treatment. Dowex resin (or equivalent anion exchange resin) can be used for the removal of residual N-lauroylsarcosine from protein samples. Before using this method, confirm that your target protein does not bind to the anion exchange resin under the described conditions. The method can also be performed in the presence of 6 M urea by adding solid urea to both the equilibrated Dowex resin and protein sample and has been reported to reduce the level of protein binding to the resin (4).

1. Prepare a stock of Dowex 1 anion exchange resin (AG 1-X8, 200-400 mesh, Cl-form; Bio-Rad Laboratories) by washing sequentially with 1 M NaOH, deionized water, 4 M acetic acid, deionized water and sterile 50 mM Tris-HCl, pH 7.5–8.0. Store in 50 mM Tris-HCl buffer as a 50% slurry at 4°C until ready for use.
2. Calculate the amount of Dowex necessary based on the level of detergent present in the sample. For example, if the sample were solubilized with 0.3% N-lauroylsarcosine, then the amount required is calculated: $0.3\% \text{ N-lauroylsarcosine} \times (5\% \text{ Dowex}/X\% \text{ N-lauroylsarcosine}) = 1.5\% \text{ Dowex}$. Based on this calculation, you would add 1.5 ml Dowex/100 ml protein sample or 3 ml of a 50% slurry of Dowex.
3. Add the calculated amount of resin to your sample, remembering to take into account that your resin stock is 50%.
4. Stir the sample for 15 min at room temperature.
5. Remove the resin by filtration through a 0.45–1 µm nylon membrane. (Cellulose acetate can be used as a substitute but some minor losses can occur with CBD fusions due to functional CBD binding to the filter.)
6. Assay for detergent and repeat Dowex treatment, if necessary.

Turbidimetric Assay for N-lauroylsarcosine

N-lauroylsarcosine concentration in protein samples can be assayed by the protocol below, but be aware that the precipitation of the target proteins under the conditions used can lead to erroneously high readings. Protein samples solubilized in urea can be used for controls in this assay to determine if your target protein is precipitating. The turbidimetric assay can detect detergent at concentrations of 0.025 mg/ml (0.0025%) or higher (4) and has a linear range from 0.0025% to 0.025%. For samples with detergent concentration values above this range, either a higher dilution of sample can be used, or an exponential regression analysis can be performed for the standard curve to allow interpolation above the linear range of the assay.

1. Prepare a stock solution of 0.3% N-lauroylsarcosine and dilute serially in deionized water to prepare a series of standards of known detergent concentration.
2. Dilute the protein test samples in deionized water in a similar manner. Also include a deionized water control sample. The volume of each dilution should be 0.5–1 ml.
3. Add 100 µl 1 M HCl/ml dilution.
4. Vortex briefly and incubate at room temperature for 5 min.
5. Read OD₄₀₅ of the standards and prepare a standard curve of OD₄₀₅ vs. N-lauroylsarcosine concentration. Using the curve, interpolate the detergent concentration for the test samples.



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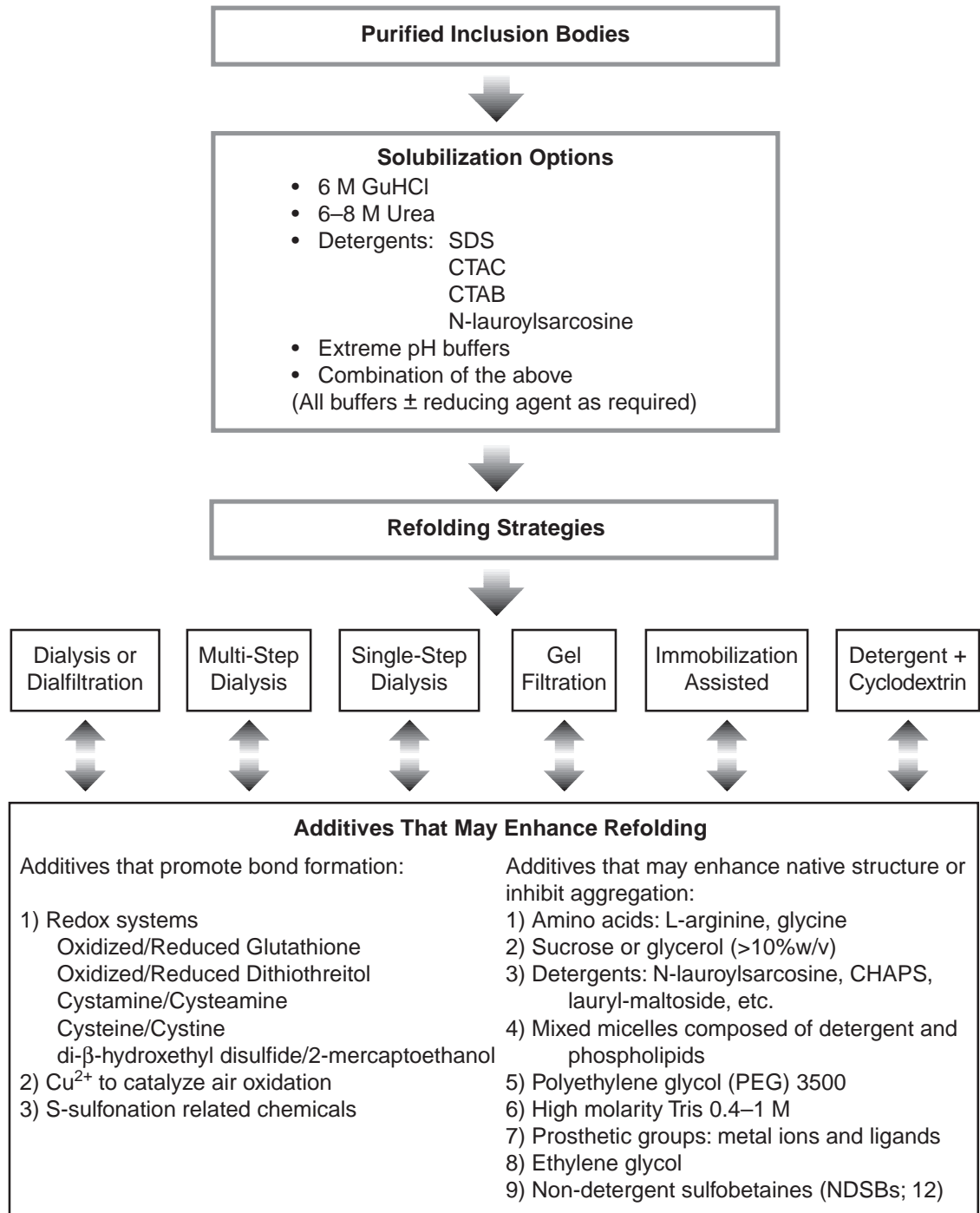
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Appendix A: Additional Information on Protein Refolding

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 (1, 3–12; 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.





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 3.

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 (11). One advantage realized by this method is the ability to refold and elute at relatively high protein concentrations (5 mg/ml in reference 11). 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 (10).

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 (9). 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 (3, 8), 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 (3). 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 disulfides. 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 (1). 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 (1, 3). A unique set of compounds, the non-detergent sulfobetaines (NDSBs, available from Calbiochem) appear to be particularly useful in preventing aggregation during refolding (12). 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. However, we have achieved quantitative recovery of CBD fusion protein from 1 µg/ml solutions using CBIND™ cartridges. 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 Zn²⁺ for proper refolding and Mg²⁺ for full enzymatic activity. When dialyzing solubilized inclusion bodies against a buffer containing these two cations, enzymatically active protein was recovered (8). 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) (3). 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 3, 5). 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.