Athena Enzyme Systems Group™

Maximizing Protein Production and Recovery

Protein Refolding Applications Manual

Refolding Kit: Applications Manual

Version 1.0

Athena Enzyme Systems Group™
Athena Environmental Sciences, Inc.
1450 South Rolling Road
Baltimore, MD 21227
Tel.: 410-455-6319
Toll Free: 888-892-8408
Fax.: 410-455-1155
Email: support@athenaes.com
Internet: www.athenaes.com

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Introduction

Reasons for Protein Refolding

For more than 20 years *E. coli* has proved to be a reliable host for the production of heterologous proteins. The well defined genetics, readily available host-vector systems, and established methods has made *E. coli* the first choice for the expression of recombinant proteins. Despite the history of successes, the expression of heterologous proteins the production of soluble functional protein remains unpredictable. Frequently, the over expression of a protein in *E. coli* results in the formation of insoluble inclusion bodies.

The reasons for inclusion body formation are not fully known. Since translation is a slower process than protein folding, it is likely that the misfolding of translation intermediates plays some role. Post-translational modification, such as glycosylation and lyposylation, are known to affect the secondary structure of proteins. In bacteria, these modifications are mostly absent. Further, the chemical environment in which translation occurs in the eukaryotic cell is different than that of the bacterial cell. Each of these factors contributes to varying degrees to how the nascent polypeptide folds, or in the case of recombinant protein expression, misfolds[1].

Several approaches have been used to mitigate misfolding during the over expression of proteins in *E. coli*. These include: 1) fusion of the target protein with a more soluble partner, typically a bacterial protein, 2) co-expression of folding catalysts and chaperones, 3) expression under cultures conditions which reduce the translation rates or effect the intracellular environment, and 4) modification of the protein sequence. Each approach has advantages and disadvantages which must be weighed in light of the intended end-use of the target protein. Further, not all proteins respond favorably to any given approach. Again which approach is best suited to a given protein must be determined empirically and success in producing and recovering soluble active protein is not guaranteed.

Both a bane and blessing, the formation of inclusion bodies renders the expressed protein unusable. The purification of a protein as an inclusion body is relatively simple, easily scalable for commercial applications and in many cases can stabilize the protein until a sufficient degree of purity is obtained. The challenge is that the protein must then be recovered from the insoluble particle. The recovery of soluble active protein from purified inclusion bodies requires the denaturation of the polypeptide and then its refolding to an active form. Many examples of proteins recovered from inclusion bodies are well known and used for both commercial and academic applications. There are well established methods for purifying inclusion bodies and solublizing the aggregated protein by denaturation. There is, however, no reliable method for predicting the conditions needed to refold the protein. Thus, the identification of the conditions needed to properly refold the protein remains an empirical science. The purpose of the Protein Refolding Screen Kit is to help simplify the process of identifying the buffer composition and method which is best suited for the refolding of any given protein.

Principals of the Kit

The information for protein folding is coded in the linear sequence of the polypeptide[2]. With rare exception each protein can be denatured and refolded into a native active state under the right conditions. However, predicting the folding pathway for any give protein is a daunting challenge. For a 100 residue polypeptide there are 9^{100} accessible confirmations. If each conformational search requires 10^{-15} seconds to complete it would take approximately 2.9×10^{79} years to examine each possible configuration. This Levinthal paradox is resolved during protein folding by the progressive stabilization of intermediate states. Productive partially folded confirmations are retained while non-productive folds are rearranged. The key appears to be the cooperative formation of stable native-like secondary structures which serve to nucleate the process. In practical terms elucidating the folding pathway for any given protein requires painstaking analysis and significant technical capabilities. Until a more thorough understanding of the relationship between primary protein sequence and structure is developed and the tools become available for in silico prediction of protein structure, the best available method for determining the conditions for protein folding remains empirical testing.

The parameter affecting protein refolding has been extensively reviewed[3,4,5]. The key to successfully refolding a protein is to prevent off-pathway products from accumulating. These unwanted species form aggregates, a process which can be self-nucleating, resulting in poor recoveries of properly folded proteins. Intermediates with hydrophobic patches which are exposed to solvent are believed to play a significant role in the formation of off-pathway products. Thus, to avoid off-pathway products the main tactic is a continuous or discontinuous buffer exchange where the renaturation buffer is designed to minimize these off-pathway products.

The folding of proteins in solution is affected by a number of physiochemical parameters. These parameters include: Ionic strength, pH, temperature, oxidation state and protein concentration as well as the presence of hydrophobic, polar, chaotropic agents and other proteins. A comprehensive list is given by Clark[4]. Thus, the first step to develop a method for refolding proteins purified from inclusion bodies is to determine the composition of the refolding solution. The Protein Refolding Screen Kit contains 15 different buffer compositions which permit the rapid identification of the factors which are having a major effect on protein folding. From this information experiments can be performed to determine the optimum buffer formulation.

Five different techniques are employed to exchange the denaturant buffer with the refolding buffer including dilution, dialysis, diafiltration, gel filtration and immobilization on a solid support. For screening purposes and, in some cases, small to moderate-scale production, dilution is the simplest approach. Its obvious drawback is that this technique leads to dilute protein solutions that would subsequently have to be concentrated; with larger production volumes it would become cumbersome. The other buffer exchange techniques are fully scalable to commercial production and can be performed under higher protein concentrations. Care must be taken to define the conditions which prevent aggregation under high protein concentrations. Several variations on the basic theme of buffer exchange have been noted for various proteins. For example, a temperature leap in which the target protein is refolded at low temperature followed by a rapid increase in temperature to complete the process has been applied to the refolding of carbonic anhydrase II[6]. During the low temperature incubation, folding intermediates which do not aggregate accumulate and upon a rapid temperature increase the final product is formed with minimal misfolding. Another approach is to expose the protein to intermediate denaturant concentrations that prevent the formation of aggregates but allow refolding to occur. This can be done by rapid dilution followed by slow dialysis into the final buffer (example: lysozyme) or by gradually removing the denaturant by dilution during dialysis (example: immunoglobulin G[7]). A general rule is that if a protein forms aggregates at intermediate concentrations of denaturant, that a fast or slow dilution of denatured protein into renaturation buffer is best. If the protein does not form aggregates at intermediate denaturant concentrations, then slow dialysis with a gradual removal of the denaturant is best.

Components

Buffer 1:	50 mM Tris-maleate pH 6.0, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.75 M Guanidine HCl, 0.5% Triton X-100, 1 mM DTT
Buffer 2:	50 mM Tris-maleate pH 6.0, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH
Buffer 3:	50 mM Tris-maleate pH 6.0, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA, 0.4 M sucrose, 0.75 M Guanidine HCl, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM DTT
Buffer 4:	50 mM Tris-maleate pH 6.0, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.5% Triton X-100, 1 mM GSH, 0.1 mM GSSH
Buffer 5:	50 mM Tris-maleate pH 6.0, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.4 M sucrose, 0.75 M Guanidine HC, 1 mM DTT
Buffer 6:	50 mM Tris-maleate pH 6.0, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.4 M sucrose, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH
Buffer 7:	50 mM Tris-maleate pH 6.0, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.75 M Guanidine HCl, 0.05% polyethylene glycol 3,550, 1 mM DTT
Buffer 8:	50 mM Tris-maleate pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.4 M sucrose, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH
Buffer 9:	50 mM Tris-maleate pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.75 M Guanidine HCl, 0.05% polyethylene glycol 3,550, 1 mM DTT
Buffer 10:	50 mM Tris-maleate pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.4 M sucrose, 0.75 M Guanidine HCl, 1 mM GSH, 0.1 mM GSSH
Buffer 11:	50 mM Tris-maleate pH 8.5, 9.6 mM NaCl, 0.4 mM KCl, 1 mM EDTA, 0.5% Triton X-100, 1 mM DTT
Buffer 12:	50 mM Tris-maleate pH 8.5, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH
Buffer 13:	50 mM Tris-maleate pH 8.5, 240 mM NaCl, 10 mM KCl, 1 mM EDTA, 0.5 M arginine, 0.75 M Guanidine HCl, 0.5% Triton X-100, 1 mM DTT
Buffer 14:	50 mM Tris-maleate pH 8.5, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.5 M arginine, 0.4 M sucrose, 0.75 M Guanidine HCl, 0.5% Triton X-100, 0.05% polyethylene glycol 3,550, 1 mM GSH, 0.1 mM GSSH
Buffer 15:	50 mM Tris-maleate pH 8.5, 240 mM NaCl, 10 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 0.4 M sucrose, 1 mM DTT
DTT	Dissolve contents of vial in 1 ml of deionized water. Store at 4°C.
Glutathione, reduced	Dissolve contents of vial in 1 ml of deionized water. Store at -20°C.
Glutathione, oxidized	Dissolve contents of vial in 1 ml of deionized water. Store at -20°C.

Buffer Preparation: Before using the Buffers add 1 µl DTT, GSH or GSSH solutions to 950 µl of the respective buffer as follows:

DTT Solution	Buffers #1, 3, 5, 7, 9, 11, 13, and 15
GSH Solution	Buffers #2, 4, 6, 8, 10, 12, and 14
GSSH Solution	Buffers #2, 4, 6, 8, 10, 12, and 14

Protocols

Protocol 1: Screening for the basic buffer composition.

Because there are a myriad of chemical and physical conditions which can affect protein refolding, the systematic survey of each factor would be a time consuming process. The screen employed by this kit is based on a fractional factorial experimental design. This allows the researcher to determine the critical factors affecting protein refolding and thus to quickly define a suitable refolding regime.

1. Purify the protein as inclusion bodies and solublize in neutral buffered 8M urea. Supplement the buffer with DTT as required by the protein. Protocol 3 gives methods for purifying and preparing inclusion bodies.
2. Adjust the protein concentration to 1 mg / ml.
3. Dispense 950 µl of each buffer into each of 15 1.5 ml polypropylene microfuge tubes. Dispense 950 µl of the denaturation buffer into tube #16.
4. Slowly add 50 µl of the protein solution to each tube while vortexing the solution gently.
5. Incubate at 4°C or 22°C for 1 hour.
6. Microfuge for 5 min.
7. Carefully pipet the liquid into a clean tube. This should contain refolded, soluble protein. Reserve the pellet.
8. Assess successful refolding as follows:
   - 8.1. By Functional Assay:
     - 8.1.1. It is best to perform a functional assay to determine if any active protein is present.
   - 8.2. By Immunoassay:
     - 8.2.1. Perform an immunoblot, slot / dot blot or microplate assay. Apply 2.0 µg of protein per well. It is important for slot / dot blot and microplate assays that appropriate controls are used to ensure that the signal obtained is due to the target protein and not non-specific antibody binding. Extracts prepared from an isogenic parent strain is best.
     - 8.2.2. Use a quantitative densitometery scan to determine the relative amount of protein recovered.
   - 8.3. By SDS-PAGE:
     - 8.3.1. Mix 40 µl of the soluble fraction with 10 µl 5x SDS-PAGE Loading Dye.
     - 8.3.2. Heat at 100°C for 5 min.
     - 8.3.3. Load 10-20 µl per lane of a gel. This will give 0.4 to 0.8 µg of the target protein per lane. A Tris-glycine SDS gradient acrylamide gel of 4-20% is recommended.
     - 8.3.4. After electrophoresis, stain with Coomassie Blue.
     - 8.3.5. Successful refolding is evidenced by the presence of the target protein in the liquid fraction.
     - 8.3.6. Perform a quantitative densitometery scan to determine the relative amount of protein recovered.

8.4. By Size Exclusion Chromatography:

8.4.1. Inject 0.1 ml of the solution containing the refolded protein into a calibrated 300 x 7.8 ID mm SEC column and chromatograph. (A 5 µm resin with a 300-Å pore size is recommended. See Gooding and Freiser, 1991[8] and Engelhardt, 1991[9] for a general discussion of analytical SEC on proteins.)

8.4.2. Misfolded or aggregated protein will have a different retention time than the correctly folded protein.

9. Interpret the data.

9.1. Successful refolding is achieved when >30% of the input protein or activity is recovered in the soluble fraction. (Yield = Amount of properly folded protein recovered / Amount of protein input.)

9.2. Determine the factors which are having a major effect on protein refolding.

9.2.1. Prepare a spreadsheet with 15 rows corresponding to reactions 1 to 15 and 14 columns corresponding to each of the factors tested (13) and the solutions as shown in the figure below.

9.2.2. Enter the value (i.e., enzyme activity, mass, etc.) obtained for each solution into each cell in the row. For any given solution each factor will have the same value entered. (Note: Numeric descriptors for qualitative assessments will also work, but with less accuracy.)

9.2.3. Calculate the sum of protein recovered for each factor when the factor was present in the solution. $Sum_{Present}$

9.2.4. Calculate the sum of protein recovered for each factor when the factor was absent from the solution. Sum_{Absent}

9.2.5. Calculate the difference between the Present and Absent and divide by 7.5 for each factor. Relative Effect = $Sum_{Present}$ - Sum_{Absent} / 7.5.

9.2.6. Compare the Relative Effect numbers obtained.

9.2.6.1. A positive number indicates a positive effect on refolding.

9.2.6.2. A negative number indicates no effect on refolding.

9.2.6.3. The larger the positive number the greater the effect of the given factor.

9.2.7. Apply this same set of calculations to any other parameters used to test protein refolding such as temperature, protein concentration, etc.

Example analysis table used to determine the critical factors to refolding.

(An Excel spreadsheet file is available at www.athenaes.com/technical_support.htm)

Solution	pH 6.0	pH 8.0	NaCl/KCl	Mg/Ca	… …	GSH/GSSH
1					… …	
2						
. .	. . .	. . .	. . .	. . .	. . .	. . .
14					… …	
15					… …	
$Sum_{Preseent}$					… …	
Sum_{Absent}					… …	
Relative Effect	= $Sum_{Present}$ - Sum_{Absent} / 7.5				… …	

Protocol 2: Optimization of the buffer composition.

Once the critical parameters have been identified, the refolding conditions should be optimized. The extent to which the optimal conditions must be defined depends on the intended use of the protein and whether or not additional purification steps are needed. The following is a general scheme for optimizing the refolding procedures.

1. Determine the optimal buffer.
   - 1.1. Test for protein refolding as in Protocol 1 Step 8 using three levels of each of the critical factors. Select the maximum, minimum and median values for each factor. The experimental design can be to vary each factor individually or employ a statistical design with a three level analysis.[10]
   - 1.2. Refine the optimum factor levels by titering the factor levels within the ranges defined in step 1.1.
   - 1.3. For preparations with more than 20 mg of the target protein, test for refolding at high protein concentrations, i.e., >1 mg/ml. This can be done using the dilution technique or dialysis.
   - 1.4. Scale the refolding to the desired level.
     - 1.4.1. For 1-20 mg protein, the dilution method will suffice. After performing the refolding step, concentrate the protein by ultrafiltration or chromatography and exchange the buffer to one suitable for the intended use of the protein. It may be possible to using the dilution method for up to 100 mg of protein if the refolding can occur at protein concentrations above 0.1 mg/ml.
     - 1.4.2. For refolding more than 20 mg of protein, an alternative method should be employed which does not dilute the protein. The following is a simple approach which most laboratories can readily use and is generally applicable. Alternative techniques are discussed elsewhere.[3,4,5]
       - 1.4.2.1. Prepare 2 liters of Solubilization Buffer (see Protocol 3 Step 1.3)
       - 1.4.2.2. Prepare 4 liters of Refolding Buffer (as determined above).
       - 1.4.2.3. Fill a 3,500 NMCO dialysis membrane with the denatured protein solution. The protein concentration should be at the maximum possible as determined during the refolding optimization process.
       - 1.4.2.4. Dialyze against 2 liters of Solubilization Buffer for 2 hours.
       - 1.4.2.5. Continuously add the Refolding Buffer at the rate of 1 ml/min while removing the Solubilization Buffer at the same rate.
       - 1.4.2.6. After 48 hours, remove the dialysis bag from the solution and dialyze against 2 liters Refolding Buffer at 4°C for 2-4 hours.
       - 1.4.2.7. Dialyze against the buffer needed for the intended use of the protein.
       - 1.4.2.8. Remove any precipitated material by centrifugation at 20,000 xg for 20 min. at 4°C.

Protocol 3: Purification of Inclusion Bodies

There are a myriad of approaches for purifying inclusion bodies. The most common technique for bench-scale applications is centrifugation, with diafiltration and continuous flow centrifugation being used for commercial-scale operations. It is most often best to purify the inclusion bodies as insoluble products and the dissolve them in denaturant before refolding. This will remove unwanted contaminants, especially proteases. Once the inclusion bodies are relatively pure, they are solublized with 6 M guanidine HCl or 8 M Urea. The solubilization is protein-dependent and the conditions needed with regard to the concentration of denaturant, ratio of denaturant to protein, pH, ionic strength, time of exposure to denaturant, temperature,

redox agents or derivatization of thiol groups should be determined empirically. The method below is a general scheme which will work for most proteins. Alternative approaches and more extensive discussions on inclusion body purification can be found in the literature.[3,4,5,11]

1. Materials:
   - 1.1. Cell pellet of the strain in which the target protein was expressed.
   - 1.2. Wash Buffer: 4 M Urea, 0.5 M NaCl, 1 mM EDTA, 1 mg/ml deoxycholate, 50 mM Tris-Cl pH 8.0. (Note: The optimal urea and salt concentration should be determined in a pilot experiment. This is done by suspending the insoluble material from an extract in buffer with different levels of urea. Select the highest urea and salt concentration that does *not* solubilize the target protein. A nonionic detergent may be included in the buffer to improve the purity. Its optimal concentration should be determined as for the urea and salt.)
   - 1.3. Solubilization Buffer: 6 M Guanidine-HCl (or 8 M Urea), 50 mM Tris-Cl pH 8.0, 10 mM DTT.
2. Method
   - 2.1. Prepare a cell-free extract and clarify by centrifugation at 20,000 xg for 30 min. at 4°C.
   - 2.2. Wash the pellet twice with 5 ml/g Wash Buffer. Centrifuge at 20,000 xg for 15 min. at 15°C.
   - 2.3. Suspend the pellet in Solubilization Buffer at 2 ml/g. Heat at 50°C for 10-20 min. to facilitate dissolution.
   - 2.4. Clarify the solution by centrifuging at 20,000 xg for 30 min. Reserve the supernatant.
   - 2.5. Analyze by SDS-PAGE for purity and fractionate by size exclusion chromatography as needed.

Supplement Protocol 1: Methods for increasing soluble protein accumulation.

Before embarking on experiments to define a protein refolding regime, it is advisable to first determine whether or not the target protein can be recovered in a soluble and therefore presumably native state. Two relatively simple and quick tests are recommended; a media screen and induction of chaperone proteins. It is known that medium composition can effect the accumulation of recombinant proteins. Likewise, the relative fraction of soluble protein of an otherwise insoluble product is affected by an as yet undetermined mechanism. To determine whether medium composition affects a particular protein, several media formulations should be screened for accumulation of the target protein in soluble extracts.

The following protocol is for use with Athena's Media Optimization Kit™ or APF Media Optimization Kit™. The method can be adopted for use with any set of media formulations desired.

Prepare the media as per the kit instructions

1. Dissolve the contents of each of the media packets in 1 liter of deionized water.
2. Add 4 ml of glycerol to the Turbo Broth™ and Power Broth™ solutions.
3. Dispense at desired volume into appropriate bottles or flasks.
4. Autoclave at 121°C for 15-20 min, depending on the volume per container, and allow to cool.
5. Dissolve the contents of the Glucose-Nutrient Mix in 100 ml deionized water.
6. Filter sterilize the Glucose-Nutrient Mix using a 0.2 µm filter.
7. Add 50 ml of the sterile Glucose-Nutrient Mix to 1 liter of Hyper Broth™ and 20 ml to 1 liter of Glucose M9Y using aseptic technique.
8. Add sterile antibiotics as needed.

Perform the media screen as follows:

1. Materials
   - 1.1. 50 ml of each of the six different culture media in 250 ml baffle bottomed flasks.
   - 1.2. Wash Buffer: 50 mM sodium phosphate pH 7.5, 150 mM NaCl
   - 1.3. Lysis Buffer: 50 mM Tris-Cl, 0.2 M NaCl, 2 mM EDTA, protease inhibitors as needed
   - 1.4. Enzyme Stock Solution: 10 mg / ml lysozyme, 1.0 mg / ml DNaseI in Lysis Buffer
   - 1.5. Urea Buffer: 8 M urea, 100 mM Na_2HPO_4, 10 mM Tris-Cl pH 7.5 (or as determined for solubilization of the target protein)
   - 1.6. 2x SDS-PAGE Loading Dye: 125 mM Tris-Cl pH 6.8, 4% SDS (w / v), 0.005% bromphenol blue (w / v), 20% glycerol (v / v), 5% β-mercaptoethanol (v / v)
   - 1.7. Tris-Glycine SDS-polyacrylamide gel of appropriate composition
2. Methods
   - 2.1. Inoculate a single colony of the recombinant strain into 10 ml of LB Broth in a shake flask with baffle bottoms. Incubate at 37°C overnight.
   - 2.2. Inoculate 50 ml of each of the six media with 5 ml of the overnight culture. Incubate the cultures at 37°C until the OD_{600} reaches 0.6-0.8.
   - 2.3. Remove a 15 ml sample ("pre-induction"), harvest the cells, wash once with Wash Buffer and collect in a pre-weighed centrifuge tube, and process as in step 2.7.
   - 2.4. Add inducer and continue incubating for 3 hours.
   - 2.5. Remove a 15 ml sample ("post-induction"), harvest the cells in a pre-weighed centrifuge tube, wash once with Wash Buffer, determine the cell pellet mass and process as in step 2.7.
   - 2.6. Harvest the remainder of the culture, wash with 10 ml of wash buffer, determine the mass of the cell pellet, and store the cell pellets at –80°C.
   - 2.7. Analyze for expression of the target protein as follows:
     - 2.7.1. Prepare cell extracts as follows:
       - 2.7.1.1. Suspend the cell pellets from the pre- and post-induction samples in 2 ml of Lysis Buffer per gram of cells.
       - 2.7.1.2. Add lysozyme and DNaseI to 1.0 and 0.1 mg / ml, respectively.
       - 2.7.1.3. Incubate on ice for 60 min.
       - 2.7.1.4. Remove a 100 µl sample and reserve. Label "whole cell extract."
       - 2.7.1.5. Lyse the remaining cells with three cycles of freezing (dry ice-ethanol bath, 5 min.) and thawing (37°C, 5 min.).
       - 2.7.1.6. Clarify the extract by centrifuging at 30,000 xg for 30 min. at 4°C.
       - 2.7.1.7. Reserve the supernatant, "soluble fraction," and suspend the pellet, "insoluble fraction," in 0.5 ml Urea Buffer or other solubilization buffer.
       - 2.7.1.8. Determine the protein concentration in each of the fractions.
     - 2.7.2. Determine the presence of the target protein in the soluble fractions by one of the following means:
       - 2.7.2.1. Functional Assay – Perform a functional assay using equal amounts of protein in the assay.
       - 2.7.2.2. Immunoblot or Microplate Assay – Load equal protein per lane of a gel, well of a slot / dot blot or microplate well. Detect the target protein using a primary antibody to an affinity tag or to the target protein.
       - 2.7.2.3. SDS-PAGE – Load equal amounts of protein per lane. Stain the gel with Coomassie Blue, colloidal Coomassie Blue or silver stain.

3. Interpretation

3.1. Compare the level of target protein obtained from cells grown in each of the six media. ***Select the medium which produces the highest level of soluble target protein per ml of culture.***

Supplemental Protocol 2: Increased soluble protein accumulation using chaperone induction.

Other factors which can increase the accumulation of soluble protein are chaperonin proteins. Chaperones are a class of proteins found in all organisms which play a role in folding of protein or the refolding of mis-folded proteins. Several studies have shown that the co-expression of selected chaperones increases the accumulation of soluble protein during hyper-expression[1]. However, in vitro studies have found that not all proteins are acted on by chaperones uniformly[12]. In other words, while the accumulation of soluble forms of some proteins can be increased by chaperones, other proteins are unaffected. At the present time no classification scheme is available to allow one to predict which proteins are likely to be acted on by a give chaperone or set of chaperonins. Therefore, trial and error testing would be needed to identify a suitable chaperone(s) for a given protein. Complicating this is that not all chaperone proteins are available in sufficient quantities for refolding work.

As an alternative, Athena's scientists have developed a medium additive, Augmedium™, which induces the expression of chaperones. Rather than co-expression one specific chaperone, Augmedium™, causes a sublethal chemical and oxidative stress which results in the expression of a range of chaperone proteins. In this way, prior knowledge of which family of chaperones that act on the target protein is not needed.

Protocol

1. Inoculate 10 ml Turbo Broth™, Turbo *Prime* Broth™ (or other medium known to give good yields of the target protein) supplemented with the appropriate antibiotics, with a single colony of the expression strain and incubate overnight at 37°C.
2. Use the overnight culture to inoculate six 250 ml baffle bottom flasks filled with 50 ml medium each. Incubate at 30°C until the density reaches an OD_{600} of 0.9.
3. Add 0.5, 0.25, 0.125, 0.0625, and 0.03125 ml 50x Augmedium™ to each of five flasks. The sixth flask is the untreated control. Incubate 20 min.
4. Add IPTG (or other inducer as per the expression system) to 1 mM and incubate for 3 hours.
5. Harvest the cultures by centrifugation at 3,000 xg for 20 min. Store the pellets at -20°C or -80°C until processing.
6. Prepare cell-free extract by mechanical, chemical or enzymatic disruption. Clarify the extract by centrifuging at 30,000 xg for 30 min. Reserve the supernatant.
7. Determine the amount of soluble protein in the supernatant by one of the following means:
   - 7.1. Functional Assay – Perform a functional assay using equal amounts of protein in the assay.
   - 7.2. Immunoblot or Microplate Assay– Load equal protein per lane of a gel, well of a slot / dot blot, or microplate well. Detect the target protein with a primary antibody to an affinity tag or to the target protein.
   - 7.3. SDS-PAGE with Coomassie or silver stain – Load equal amounts of protein in each lane. Compare the relative level of target protein accumulated.
8. Select the level of Augmedium™ which yields the highest level of target protein.

Further information is available in the Augmedium™ Protocol Sheet.[13]

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