

Purification of inclusion bodies and refolding of proteins

Basic StrongLab protocol, based on a recipe concocted by Pingwei Li, (cite, if used: Steinle, A., Li, P., Morris, D. L., Groh, V., Lanier, L. L., Strong, R. K. & Spies, T. (2001) 'Interactions of human NKG2D with its ligands MICA, MICB, and homologs of the mouse RAE-1 protein family' *Immunogenetics* **53**, pp. 279-87) a derivative of a recipe originally developed in the Jones lab (O'Callaghan CA, Tormo J, Willcox BE, Blundell CD, Jakobsen BK, Stuart DI, *et al.* (1998) 'Production, crystallization, and preliminary x-ray analysis of the human MHC class Ib molecule HLA-E' *Protein Science* **7**, pp. 1264-1266)

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1. Preparation of inclusion bodies:

a. Harvest bacteria after induction for about 4 hours. (We generally induce expression at OD of about 1.0 for inclusion body preps, this may improve the yield a lot).

Troubleshooting for good protein expression can include the following:

-- Expression at room temp or at 16 degrees rather than 37.

-- Expression from a freshly transfected strain rather than frozen glycerol stock.

b. Lyse bacteria by sonication in the following buffer:*

50mM Tris-HCl, pH 8.0

100mM NaCl

5mM EDTA

0.1% NaN₃

0.5% Triton-X100 (can use a 25% stock)

add 0.1mM PMSF and 1mM DTT immediately before use

Generally we lyse cells from 4 liters of prep in 250ml of lysing buffer and wash with 250ml of the same buffer over the next three steps. 50-mL aliquots work well for sonication.

(* = You can also use a microfluidizer at this step.)

c. After sonication, add 10mM MgSO₄ (use 2.0M stock) to chelate the EDTA, then add DNase (about 0.01mg/ml) and lysozyme to about 0.1mg/ml to the lysate and incubate at RT for 20min.

d. Centrifuge to collect inclusion bodies (for example, 6000 rpm for 15 minutes). Crush the pellet with a spatula, then resuspend it completely by sonication in the lysing buffer. Another portion of DNase and lysozyme can be added at this point to improve the purity of the pellet.

e. Repeat step d two more times without adding DNase and lysozyme.

f. Wash the inclusion body again with lysing buffer without Triton-X100. Resuspend this pellet by sonication as well.

Buffer for final wash (no Triton-X):

50mM Tris-HCl, pH 8.0

100mM NaCl

5mM EDTA

0.1% NaN₃

g. Collect the final inclusion body pellet by centrifugation in 50-mL Falcon tubes.

In most cases this protocol results in inclusion body preps of more than 80% purity.

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2. Dissolve the washed inclusion bodies:

Collect pure inclusion body in 50ml Falcon tubes, add 30-40ml of 100mM Tris buffer at pH 8.0 with 50mM Glycine.

Disperse the pellets completely by sonication and then dissolve the suspension dropwise, stirring vigorously, in 100mM Tris buffer at pH 8.0 with 50mM Glycine and 8.5M urea

(100 mL for every 2 L of culture is adequate for normal-sized pellets; a final inclusion-body concentration of about 1.0mg/ml works well for refolding.)

Then add 5mM GSSH and 0.5mM GSSG and stir overnight at 4 degrees. This will then be ready for refolding by dialysis. (Incubation at 25 degrees often works as well, and a different pH can be tried at this stage.)

(GSSG is oxidized glutathione, GSSH reduced glutathione. If you're having problems with reduction of disulfides, you could substitute a high concentration of DTT at this point, but this adds another few days over which the DTT must be dialyzed away extensively before beginning to lower the urea concentration.)

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3. Refolding

Refolding buffers include

0.1M Tris

0.4M L-Arginine

supplement with 4.0M, 2.0M, 1.0M and 0.00M Urea. (4M urea may require heat as well as vigorous stirring to dissolve)

Set the pH to 8.0 after adding major components of solution.

Then add immediately before use:

1 mM EDTA

1ug/ml Leupeptin and pepstatin (proteinase inhibitors)

0.2mM PMSF

step1. dialyze against RF buffer with 4.0M urea for 24hrs

step2. dialyze against RF buffer with 2.0M urea for 24hrs

step3. dialyze against RF buffer with 1.0M urea for 24hrs

step4. dialyze against RF buffer with 0.0M urea for 24hrs

step5. dilute 0M refolding buffer with water in a 1:4 ratio, dialyze against 1/4*RF buffer for 24hrs

step6. dialyze against PNEA with 0.2mM PMSF for 24hrs

Some steps can continue longer than 1 day if necessary.

If the refolding doesn't work, many factors can be changed in this series of steps to try and improve refolding. These include changing the pH to 6.0, adding 10% glycerol, using 6M guanidinium instead of 8M urea, bringing the protein down to only 0.5M urea then completing the purification and crystallization in 0.5M urea, removing the arginine, and co-refolding with associated protein receptors, etc.

PNEA is 25mM PIPES, 150mM NaCl, 1mM EDTA and 0.02% NaN₃. This is our running buffer for FPLC; it can be replaced with any other buffer you want to use.

Then harvest the refolded protein, centrifuge for 15-20 min. at 12-20,000rpm to remove the precipitant. This supernatant can be concentrated with stir cells or centrifugation concentration, then purified on Superdex 75 column or Ni-NTA column (add MgSO₄ to chelate EDTA in the final refolding buffer before loading on Ni column).

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4. Refolding by rapid dilution

Some people like to do refolding by rapid dilution. This is done by adding solublized inclusion bodies dropwise into a refolding buffer with rapid stirring:

Refolding buffer for dilution:

- Tris 0.1M pH 8.0
- 0.4M L-Arg
- 1ug/ml Leupeptin and Pepstatin
- 0.2mM PMSF
- 0.5mM GSSG(oxidized glutathione) and 5mM GSSH (reduced glutathione)

In our experience, dilution works as well as dialysis for refolding. The problem is that you end up with a large volume of refolded protein with GSSH, which is inconvenient for purification.

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5. Analysis of refolding products

We generally monitor the refolding process with analytical size-exclusion FPLC. In most cases we see a distribution of large aggregates, oligomers, and monomeric forms of our proteins.

Generally a large portion of misfolded aggregates and multimers will crash out when the protein is refolded and/or concentrated. The yield by mass of refolded protein from a pellet for most proteins is about 2-5%, although some proteins refold more easily (20%). A yield of 1% is low but not disastrously so.