Protocol for refolding protein from inclusion body preparations:
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Inclusion body preparation:
Express the protein in *Escherichia coli* and purify it from inclusion bodies.

1. Resuspend the cell pellet from 2 litres of culture in 30ml 1x PBS and sonicate on ice to lyse the cells.
2. Pellet crude inclusion bodies at 12000g for 30 minutes and discard the supernatant.
3. Resuspend the pellet in ~30ml Triton wash solution (0.5% Triton X100, 50mM Tris-HCl pH8, 100mM NaCl and 0.1% sodium azide) using a homogeniser to fully grind up the pellet.
4. Pellet inclusion bodies at 25000g for 10 minutes.
5. Repeat this wash 4 or 5 times - the pellet actually slightly whiter the more washes that are performed.
6. Perform one final wash mainly to remove any remaining Triton. To achieve this, resuspend using the homogeniser again in 50mM Tris pH8, 100mM NaCl and then spin at 25000g for 10 minutes.

It's a good idea to do this thoroughly because the cleaner the inclusion body prep the better the results in the refold and subsequent purification.

7. Dissolve the purified inclusion bodies in 6M Guanidine HCl, 50 mM Tris pH8, 100mM NaCl. 10mM EDTA and 10 mM DTT. Break up the pellet a bit using a pipette tip and then preferably leave at 4°C on a shaker/rocker for at least a few hours to fully dissolve.

The amount of GnHCl buffer needed will vary but start with 3 ml and add a little more if necessary later on to complete the dissolving process. It's often useful to leave this overnight.

8. Once fully dissolved, spin at 25000g for 20 minutes and remove the supernatant.

Often, the pellet at this point is like jelly and is the same colour as the supernatant so it can barely be seen. Carefully pour off the supernatant into a fresh tube and leave the pellet behind.

9. Do a rough protein estimation (e.g. an A_{280} using the calculated extinction coefficient) and store in ~20mg (total protein) aliquots at -20°C.

If preferred, 8M Urea can be used as the denaturant, although GnHCl is a more powerful denaturant. If GnHCl is used and SDS-PAGE is to be used to check purity and presence of protein, make sure to dilute any sample by about 25 fold in 1x SDS loading buffer - the sample may appear to have pale blue precipitate floating in it but after boiling this should not affect the migration on the gel. At this point the protein sample should be very concentrated so only 1 µl or so diluted up to 25 µl should be loaded. Urea samples don't normally have this problem in SDS.

Refolding by rapid dilution:

1. Defrost an aliquot of denatured protein and add a little fresh DTT (to about 5-10mM) and leave on a shaker at 4°C for approximately an hour to ensure all the disulphides are broken. Meanwhile, make up the refold buffer:

    200mM Tris-HCl pH8
    10mM EDTA
    1M L-arginine
    0.1 mM PMSF
    6.5 mM cysteamine
    3.7 mM cystamine

    For 200 ml volume:
    40 ml 1 M Tris-HCl pH8.0
    4 ml 0.5 M EDTA pH8.0
    42.14g L-arginine (MW 210.7)
    200 µl 100 mM PMSF
    0.1476g cysteamine
    0.1666g cystamine

Adding the L-arg is endothermic which helps cool down the buffer considerably. The arginine is there to reduce aggregation.

2. Put the PMSF and redox couple in just before it is about to be used
3. Keep at 4°C with a magnetic flea spinning reasonably fast but ensuring there aren't too many bubbles.
4. Make up enough refold buffer such that when all the protein has been added, the final concentration is <0.1 mg/ml e.g. add ~20mg of protein in 1.5 ml GnHCl to 200 ml refold buffer in a 500ml conical flask.
5. Add the protein/GnHCl solution dropwise (with a few seconds between drops) using a P200 pipette over a period of about 30 minutes to the refold buffer with the flea going quite fast.

Once it's all added you can turn down the speed of the flea a bit and leave the whole thing for 24/48 hours at 4°C.
NB. It is possible that adding a second 'pulse' of GnHCl denatured protein after 24 hours may increase the yield although this is not strictly necessary.

6. Concentrate down to a suitable volume for gel filtration, or whichever method is to be used for purification, using an Amicon Stirred Cell or a Vivascience Vivacell 250, both of which operate under nitrogen gas pressure.

This can take a long time. There will be some precipitated protein - there always is - but spin that down at the earliest opportunity and discard it (15000g, 20 minutes). There should be some nice soluble protein in solution (along with some soluble aggregate probably).
One method is to simply do one gel filtration run - aggregate comes off first, followed by a nice homogenous peak of active protein. A His-tag can't be used in this refolding

buffer because of the arginine and the EDTA - some do several dialysis steps after the refold to remove the arginine and EDTA but this can use a lot of buffer if dialysing a 200ml sample.

Things to optimise:

1. For the refolding, the pH and the amount of arginine can be altered (1M is quite a high concentration but it's best to start with a large amount).
2. The arginine or the redox couple can be replaced - for potential alternatives check the contents of the Novagen refolding kit or the Hampton research products 'FoldIt' kit (instructions online at http://www.hamptonresearch.com).
3. Different ratios of the redox pair, or a different redox couple altogether could be used (often reduced and oxidised glutathione is used). Also, the refold could be done at lower protein concentrations of < 0.05 mg ml. Novagen and Hampton Research have more available literature on their web sites.
4. Some people also refold at higher protein concentrations, often in dialysis tubing - i.e. the refold is done by dialysis rather than rapid dilution. Basically the denatured protein solution is put in tubing (check the exclusion size of the tubing) and dialysed for about 12-24 hours against a large volume of refold buffer, then the refold buffer is changed several times. This uses more buffer and it may be preferable to concentrate the 200-400ml of dilute refolded protein solution in an Amicon Stirred Cell overnight.

(If your protein is His-tagged, there are also methods for refolding whilst the protein is attached to a Nickel column in the Novagen literature.)