Refolding of FhuA proteins through dialysis[1]

1. Preparation of inclusion bodies:

a. Harvest expressing bacteria after induction for about 3-6 hours.

b. Lyse bacteria by microfluidizer in the following buffer:[2, 3]

50 mM Tris-HCl, pH 8.0
100 mM NaCl
5 mM EDTA[4]
1 x protease inhibitors
1-5 mM DTT[5]

c. Centrifuge at 1000 xg to collect unbroken and cell debris.

d. Centrifuge the supernatant at ≥ 16000 xg to collect the inclusion bodies.

e. Wash the inclusion bodies with lysing buffer with Triton-X 100.

Inclusion bodies washing buffer[6]:

50 mM Tris-HCl, pH 8.0
100 mM NaCl
5 mM EDTA
1% Triton-X 100

g. Repeat the wash step at least 3 times.[7]

2. Dissolve the washed inclusion bodies:

1- Dissolve the inclusion bodies in 25 ml of the lysis buffer with 8 M urea (you may exclude protease inhibitors). Dissolve the pellet completely by the homogenizer.

2- Spin down at 150 000 xg.

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[1] This protocol has been done only on FhuA ΔC/Δ4L_$_{cys\ 373}$ mutant. But, I expect it to work on other FhuA similar proteins as well.
[2] I generally lyse cells in 50-75 ml per 4 liters of expressing cells. But, volume can be changes depending on the density of the cells. Stated volume is true when the final OD for expressing cells is 1.3-1.6 at 600 nm.
[3] I usually run the cells 5-10 times through the microfluidizer with no ice.
[4] This concentration must be dropped to .08 mM if the IMAC columns from BioRad are to be used for His^+ tagged proteins.
[5] Generally, the concentration of DTT and EDTA depends on the next step of purification. Some column beads can hand reducing or chelating agents. For BioRad IMAC columns, they can hand 5 mM and .1 mM DTT and EDTA, respectively. Also, I use 5 mM DTT if my protein has cys mutagenesis.
[6] I stick with 50 ml washing buffer per protein pellet from 4 liter expressing cells. I used the homogenizer for this step.
[7] I sometimes wash more than 3 times if the solution still yellowish.

3- Save the supernatant as it contains your denatured proteins.

2. Purification the denatured proteins:

1- Follow the protocol that uses IMAC column from BioRad. It is up to you what protocol to follow for purification. The next step requires purified proteins.

2- Measure the protein concentration.

3. **Refolding**

a- Preparing the protein for refolding:

1- Aim at having ~ 1.5 mg/ml in your final protein purified sample.[8]
2- Make 3 % DDM solution in lysis buffer (excluding the protease inhibitors).[9]
3- Add equal volume of 3 % DDM solution to the purified proteins sample. Final urea concentration is 4 M, and DDM is 1.5 %.

b- Refolding the protein through multi-step dialysis:

Dialysis buffer:

50 mM Tris-HCl, pH 8.0
5 mM EDTA
1 mM DTT.

With 3 M, 2 M, 1 M and 0 M Urea.[10]

Steps of dialysis[11]:

1- Dialyze against dialysis buffer with 3 M urea for 24hrs
2- Dialyze against dialysis buffer with 2 M urea for 24hrs
3- Dialyze against dialysis buffer with 1 M urea for 24hrs
4- Dialyze against dialysis buffer with 0 M urea for 24hrs[12]

[8] I have not tried more than this concentration. But, I can imagine, the more you start with the more aggregates you may have at the end.
[9] I used DDM because the cut off for our dialysis tubings ~ 10 kDa. One can easily use different detergents, for example, OG with smaller cut off tubings (if they exist!!!)
[10] I have not tried to start from 8 to 0 M urea. In other words, I never tried to add 1.5% DDM in the protein sample when it is in 8 M urea.
[11] If you notice any white precipitant I any of the steps, do not proceed further. The precipitants indicates a failure in refolding.
[12] This step can be longer than 24 hours, as it may give protein more time to finalize its refolding.

c- **Checking the refolding of proteins:**

1- I usually check the refolding by size exclusion column (SEC). In this way you can have a rough estimate how much of proteins have been refolded vs. aggregates. The refolded protein should elute ~ 110 kDa (included the DDM micelles, 50 kDa).
2- Ultimately, you check the channel insertion activity of the refolded proteins from the fractions that show the refolded protein from SEC.