rPrP Preparation Protocol

rPrP Prep Solutions:

1. **Sodium Phosphate Stocks**

0.2M Monobasic Stock
24g Anhydrous Sodium Phosphate Monobasic in 1L H2O

0.2M Dibasic Stock
28.4g Anhydrous Sodium Phosphate Dibasic in 1L H2O

2. **Denaturing Buffer pH = 8.0 (300mL)**

100mM Sodium Phosphate
142.1mL 0.2M Dibasic
7.96mL 0.2M Monobasic

10mM Tris
10mM x 300mL x 121.14g/mole = 0.3634g Tris

6M Guanidine
6M x 300mL x 95.5g/mole = 171.9g

*Bring to 295mL volume, check pH (8.0) and bring to 300mL

3. **Refolding Buffer pH = 8.0 (600mL)**

100mM Sodium Phosphate
284.1mL 0.2M Dibasic
15.9mL 0.2M Monobasic

10mM Tris
10mM x 600mL x 121.14g/mole = 0.7268g Tris

*Bring to 590mL volume, check pH (8.0) and bring to 600mL

4. **Elution Buffer pH = 5.8**

100mM Sodium Phosphate
20mL 0.2M Dibasic
230mL 0.2M Monobasic

10mM Tris
10mM x 500mL x 121.14g/mole = 0.6056g Tris

500mM Immidizole
500mM x 500mL x 68.08g/mole = 17.017g Immidizole

*A lot of acid (HCl) is needed to adjust the pH to 5.8
QS to 500mL

5. **Dialysis Buffer pH = 5.8**

10mM Sodium Phosphate
0.2M Dibasic = 14.3mL
0.2M Monobasic = 166mL

*Bring to 3.6L volume and check pH (5.8).

Procedure: Express and Grow bacterial stock

1. Kanamycin culture plates are streaked with glycerol bacterial stock of the rPrP construct of interest and put upside down in a 37°C incubator for overnight to grow colonies.
   Note: More recently we have been skipping this culture plate step and directly inoculating 2 x 4ml media cultures, as described in step 2 & 3, from the glycerol bacterial stock.
2. Make up media: 1L LB-M Broth, 1mL Kanamycin (50mg/mL), 1mL Chloramphenicol (34 mg/mL in 100% EtOH).
3. Inoculate 2 x 4ml mini-cultures of media either directly from the glycerol stock or from colonies on the culture plate.
4. Grow mini-cultures at 37°C with shaking for 5-6 hrs (until cultures are strongly turbid).
5. Add Overnight Express (Autoinduction System 1) to the remainder of the original 1L of media and then add all 8mls of the turbid mini-cultures.
6. Put in a 3-4L autoclaved flask and grow at ~200rpm and 37°C for ~20-24hrs. 600 nm OD should be at least 3.0 (using LB broth as a blank).
7. We split the resulting 1L culture into 4 x 250ml portions in 250ml conical centrifuge tubes and then spin at 3750 rpm for 20 min. Decant supernatant and freeze pellets for storage. (The resulting cell pellets have typically weighed 3 to 4grams per 250ml tube.)
   Note: Our previous protocols describe multiple freeze/thaw steps using liquid nitrogen. We no longer follow these steps as we have found that the use of the OMNI TH (Tissue Homogenizer) described below is sufficient to break up the cell pellets.

Procedure: INCLUSION BODY PREPARATION

Batch sizes: What we originally called a 1X batch consisted of the pellet of a single 250ml conical centrifuge tube, derived from 250mls of the original 1L Overnight Express culture. It later became typical for us to purify from a scaled up 3X batch consisting of 3 x 250ml tubes. A 1X batch typically yields 15-20mgs of rPrP and a 3X batch typically yields 50-70mgs. The Inclusion Body Preparation protocol below describes what is done for each individual 250ml conical centrifuge tube containing the pellet derived from a quarter of the original 1L culture.

1. Thaw cell pellet by placing 250ml tube at 37C for a few minutes.
2. Add 12ml of 1X Bug Buster Master Mix to each 250ml tube.
3. Using a disposable plastic spatula, I scoop the majority of the pellet out of the tube and transfer it to a blue cap BD 50ml tube. I then swirl the solution remaining in the 250ml tube to incorporate the remaining pellet and then pour this also into the same blue cap tube. Any remaining fluid can be transferred with a pipette.
   Note: We specifically use the blue cap BD 50 mL tubes for the following centrifugation steps due to their resistance to cracking at these high rpms)
4. We use a ***OMNI International TH*** (Tissue Homogenizer) with disposable tip to fully resuspend the pellet, taking care to minimized bubbles.
   Note: I highly recommend the use of this tool because it is very efficient at homogenization and the disposable tips mean that you don't have to thoroughly clean the device between preps. This works much much better than pipetting up and down. (NOTE: Each time you homogenize, first break up all the big chunks at low speed and then continue to blend at a little higher speed (around 50% power) until all the visible chunks are broken up. This takes only a minute or so per tube. Don't crank the homogenizer to full power as this will create bubbles.)
5. Incubate each of the 50ml tubes on rotating mixer for 20min at RT.
6. Centrifuge at 13,000 X g (9,000 rpm; JA12 rotator) for 20min at 4°C.
   Note: 16,000 X g is what the original protocol called for, but it was cracking the tubes, so I lowered the g force.
7. Discard the supernatant by pouring it off.
8. Resuspend pellet in 10ml 1X Bug Buster Master Mix, again using the same tip on the Tissue Homogenizer.
9. Again, incubate at room temp on the rotator for 15min.

10. Q.S. to 40mL with 1:10 (0.1X) Bug Buster reagent and mix by inversion.
11. Centrifuge at 7,900 X g for 15min at 4°C to collect inclusion bodies.
12. Pour off and discard supernatant and resuspend pellet in 20mL of the 0.1X Bug Buster reagent. using homogenizer with the same tip.
13. Q.S. to 40mL with 0.1X Bug Buster reagent and centrifuge at 13,000 X g (9,000 rpm; JA12 rotator) for 15min at 4°C.
14. Pour off and discard supernatant and freeze inclusion body pellet for later use.

Procedure: PROTEIN PURIFICATION

1. Make two (for regular batch) or three (for 3X scale up batch) batches of dialysis buffer (3.6L each) and put at 4°C.
2. Filter all buffers (other than the dialysis Bf) before use, and remember to check buffer levels periodically during the following FPLC purification to ensure lines do not go dry.
3. Dissolve each inclusion body pellet (in 50ml blue cap tube) into 14mL 8M Guanidine (38g Guanidine in 0.1M NaPO4 pH = 8.0 and Q.S. to 50mL; do not pH final solution) using the OMNI International Tissue Homogenizer and disposable tip and then put on rotator for ~50min.
   Note: That is 3 individual tubes of 14ml per tube for a 3X scale up batch. They will be pooled when you pour your column.
4. Rinse 18g of Ni-NTA superflow resin beads (18g in each of 3 fresh tubes for 3X scale up batch) with ~100mL water using the in house vacuum and a 100mL Qiagen 0.22 micron bottle top filter. (**NOTE: Make sure not to dry out the beads. Pull vacuum just until you start to see cracking/white color)
5. Weigh out 18g (3 X 18g for 3X scale up batch) and transfer to a fresh 50mL tube (into each of 3 tubes for 3X scale up batch). Q.S. each tube to 30mL with Denaturing buffer and equilibrate for ~50min on a rotator at room temperature. (**NOTE: This is for a ~4g cell paste, so if your cell pastes are smaller, combine them to get approximately 4g total)
   Note: These last two steps are how we do it, but basically you just need to get ~18g of beads into each fresh tube, one for each inclusion body pellet tube that you are using. You need to find a way to replace the bead storage Bf with the denaturation Bf for equilibration.
6. Spin solubilized inclusion body pellet at 13,000 X g for 5min to remove insoluble debris and add supernatant to equilibrated beads (one ~4g pellet equivalent to each tube containing 18g beads).
7. Put on rotator for 40min to allow protein to bind to beads.
8. Wash FPLC (we use an AKTA) with milliQ water to clean out both lines (A & B) and then run Denaturing buffer through both lines (A & B) to prime the system (the column is not yet attached).
9. Load resin onto column (Akta #XK16 for regular batch and Akta #XK26 for 3X scale up batch), keeping sure to minimize air bubbles.
10. Attach column to FPLC and run gradient refold at 0.75 mL/min for 240min (1X batch) or 2.25 mL/min for 240min (3X scale up batch) following the gradient refolding program: 100% A (Denaturing Buffer) and 0% B (Refolding Buffer) → 100% B and 0% A.
11. Following the gradient refolding program, let 100% Refolding Buffer continue to flow through column for an additional 30min at 0.75mL/min (regular batch) or at 2.25 mL/min (3X scale up batch).
12. Rinse line A initially with water, and then with Elution buffer (bypassing column) to clean out the Denaturing buffer.
13. To elute protein, run a gradient elution over 50min from 100%B (Refolding Buffer) to 100%A Elution Buffer (2mL/min for regular batch or 6mL/min for 3X batch). The primary protein peak should **start** to elute about 1/3 of the way through the gradient.
14. Watch for chromatography of UV 280 to increase, then collect the center of the large peak. Dilute this peak immediately with about 1/3 volume of Dialysis Buffer to help correct pH and stabilize the protein. (a 1X peak is usually 10-15mL and is brought to 20-25mL total volume with dialysis buffer) (for the 3X batches, I have been adding ~20mls of dialysis buffer to ~30mls of elution peak for a total volume of ~50ml) (note: Diluting the protein immediately in dialysis buffer helps

to reduce protein loss because it accelerates the incomplete pH change remaining from the gradient elution.)

15. Filter protein with 0.2 micron filter (**prewash filter with MilliQ Water to remove filter contaminants such as glycerol)
16. Gently put into Snakeskin dialysis tubing (**MW cut-off of 10,000 (Pierce)) and dialyze in 3.6L of pre-chilled dialysis buffer for >1hr and then transfer to a fresh 3.6L dialysis buffer for further dialysis ON. For the larger 3X batch volumes, we have also been transferring the prep to a third 3.6L dialysis bucket for several hours to get more complete buffer exchange.
17. Filter protein again following dialysis with a pre-washed 0.2 micron filter.
18. Spec protein at 280nm to determine protein concentration (**Use correct extinction coefficient)
19. Concentrate if necessary using Amicon Ultra 50mL tubes (Note: This is uncommon and only done when there is a poor yield, which usually only happens when something went wrong during the prep.)
    a. Typical yield from a 4g cell pellet is ~15-30mg
    b. Protein concentrations are typically around 0.4 to 0.6mg/ml on good batches following dialysis.
20. Check protein on gel and/or with mass spec for contaminants or fragmentation.
21. Immediately following filtration we make 1ml aliquots of the protein and immediately place the batches into the -80C freezer.
22. When the aliquots are thawed, we filter with a 100KDa filter, as described in the assay protocol, to remove oligomeric rPrP. This freeze/thaw step does cause some additional precipitation (maybe 10 or 20%). (This is not ideal protein handling, but it allows us to get consistent results from a batch over time.)

Note: All purification steps done at room temp unless otherwise stated. We do not do the prep work in a cold room environment.

Note: To further explain steps 14-17 above, I'll add the following information. It is important to move quickly after protein elution from the column and to keep the protein cold from this time forward. Collect the elution in a tube held in an ice bucket. The direct addition of cold dialysis buffer immediately after elution will reduce precipitation by accelerating the pH correction. Move directly from gently pooling the eluted fractions that you wish to keep to filtering and gently transferring to the dialysis tubing. Even with these efforts you will likely see visible precipitation of protein and/or Imidazole precipitation. Filtering again immediately after recovering the protein solution from the dialysis tubing the next day will again remove any aggregation seeds that will accelerate protein precipitation.