

SOP: RP003b**Production of Recombinant Proteins Under Denaturing Conditions****Materials and Reagents:**

1. His-Bind Resin (Novagen Cat# 69670)
2. Poly-Prep Chromatography Columns (BioRad Cat# 731-1550)
3. 15 ml Falcon Conical Tubes (Fisher Cat# 14-959-70C)
4. 10 ml pipets (VWR Cat# 12777-014)
5. Auto-Pipettor
6. 200 µl pipet
7. 200 µl sterile, pyrogen-free pipet tips
8. 96 well sterile plates
9. BCA Kit (Pierce Cat# 23225)
10. SDS-PAGE Gel supplies
11. LAL Kit (Bio-Whittaker Cat# 50-648U)
12. 37°C Plate Incubator
13. Heat block with 96-well plate incubator
14. Optical Plate Reader with 405 and 550 λ filters
15. Pyrogen-free tubes
16. Vorter
17. High-speed Centrifuge
18. Centrifuge Bottles
19. Burdick and Jackson Water (Cat#365-4)
20. 1X Binding Buffer + Urea (20 mM Tris-HCl, 500 mM NaCl, 5 mM Imidazole, 6M Urea, pH 7.9) (note 2)
21. 1X Charge Buffer (50 mM NiSO_{4,s}) (notes 1 and 2)
22. 1X Wash Buffer + Urea (20 mM Tris-HCl, 500 mM NaCl, 60 mM Imidazole, 6M Urea, pH 7.9) (note 2)
23. 1X Elute Buffer + Urea (20 mM Tris-HCl, 1M Imidazole, 6M Urea, pH 7.9) (note 2)
24. 10 mM Tris-HCl, pH 8 made with Endotoxin free water (note 2)
25. 0.5% ASB-14 (Calbiochem Cat# 182750) in 10 mM Tris-HCl, (note 3)
26. Complete protease inhibitors (Roche Cat# 1873580 or 1836170) (note 4)
27. Lysozyme (10 mg/ml in Milli-Q Water)
28. Urea
29. Endotoxin free water
30. Dialysis Membrane 3500 MWCO (Spectra-Por cat# 132 724)
31. 1000 mL beaker
32. Dialysis chamber
33. Magnetic stir-bar
34. Stir plate
35. 4°C Cold Room
36. Ammonium Bicarbonate
37. 10 mM Ammonium Bicarbonate made with ET free water

Protocol:

1. ____ Thaw the frozen pellet from step 12 in RP003a in ice and resuspend in 10 ml of 1X Binding buffer containing complete protease inhibitors and 6M Urea.
2. ____ Add lysozyme to a concentration of 100 µg/ml. Incubate on ice for 1 hour. Vortex after 1 hour.
3. ____ Centrifuge the inclusion body lysate at 16,000 x g for 30 minutes to pellet the cell debris.
4. ____ If the lysate still appears cloudy repeat step 3 again increasing the time to 1 hour as necessary.
5. ____ If the pelleted material still appears to contain inclusion bodies, repeat steps 1-4 as necessary.

6. ____ Decant lysate into graduated 50 mL conical tube to record volume.
7. ____ Perform BCA to determine protein concentration.
8. ____ Perform SDS-PAGE analysis to estimate protein amount in total lysate. This will allow the number of His-Bind columns needed for purification to be calculated. Each 1.5 ml column is capable of binding 12 mg of recombinant His-tagged protein.
9. ____ Equilibrate the appropriate number of columns (note 5).
10. ____ Apply the lysate to the appropriate number of His-Bind columns by pipetting slowly.
11. ____ Collect Flow-through in 15 ml conical tubes.
12. ____ Apply 10 column volumes of 1X Binding Buffer with 6M Urea, collect fraction in 15 ml conical tube.
13. ____ Apply 6 CV of 1X Wash Buffer with 6M Urea to the columns, collect fraction as before.
14. ____ Apply 10 CV of 10 mM Tris-HCl in ET free water to remove residual salts from the columns. Collect fraction as before.
15. ____ Apply 10 CV of 0.5% ASB-14 in ET free 10 mM Tris-HCl. This is the endotoxin removal step. Collect fraction as before (note 6).
16. ____ Apply another 10 CV of the ET free 10 mM Tris-HCl to remove any excess detergent. Collect fraction as before.
17. ____ Apply 5 ml of the elution buffer, ET free 10 mM Tris-HCl, 1M Imidazole, 6M Urea. Collect fraction.
18. ____ Prepare 3500 MWCO dialysis membrane by sealing both ends with clips to prevent ET from reaching the inside of the membrane. Boil in 1000 mL beaker with Burdick and Jackson water.
19. ____ Prepare dialysis chamber with 1L of 10 mM Ammonium Bicarbonate, 4 M Urea.
20. ____ Cut ends off of dialysis membrane, tie off the bottom end and re-clamp. Add protein sample to the dialysis membrane. Tie off top end and clamp.
21. ____ Dialyze with slow stirring at 4°C for 8 hours, exchange buffer 4 times, but the exchanges should be from 4M Urea, to 2M Urea to no Urea. The final exchange should be a second one with no Urea to ensure its complete removal. Increase exchange volumes to 4L on the third exchange.
22. ____ Pat dry the dialysis membrane to remove any potential contaminating buffer containing endotoxin.
23. ____ Cut open the dialysis membrane at the top and carefully pour the sample into a clean, sterile 50 mL conical tube.
24. ____ Rinse the dialysis membrane carefully with a small amount of 10 mM Ammonium Bicarbonate made with ET free water. Decant into the conical tube.
25. ____ Perform BCA analysis to determine protein concentration.
26. ____ If concentration is lower than .45 mg/ml (for .5mg aliquots) or .9 mg/ml (for 1mg aliquots) lyophilize or concentrate on the savant.

27. ____ If concentrated, make sure protein is fully suspended and uniform. Sonicate if necessary until suspended. If completely dried use ET free water to resuspend or 10mM ammonium bicarbonate.
28. ____ If concentrated, repeat the BCA analysis to determine protein concentration. If concentration is adequate, proceed to the next step otherwise start at step 26.
29. ____ Perform SDS-PAGE analysis to determine protein purity.
30. ____ Perform LAL testing to determine endotoxin contamination (note 7).
31. ____ Protein can be lyophilized in desired aliquots (note 8).

Notes:

1. Don't adjust the pH of charge buffer. It will precipitate if you adjust it.
2. After mixing up solutions and adjusting the pH filter sterilize the buffers with a .2 μ m filter.
3. Add appropriate amount of ASB-14 to your already prepared 10mM Tris-HCl just before use.
4. Make sure to use EDTA-free complete, as EDTA will interfere with protein binding to the nickel charged resin.
5. Equilibration:
 1. Add 3ml of resuspended resin (resin is in ethanol) to a poly-prep column (settled volume of 1.5ml)
 2. Allow it to flow through.
 3. Add 4ml of water to the column. Allow to flow through.
 4. Add 7ml of charge buffer to the column. Allow to flow through.
 5. Add 4ml of binding buffer to the column. Allow to flow through (unless storing see below).
 6. Column is ready to add sample to.

Columns can be stored for a day or two at 4C with at least 1ml of binding buffer covering the top of the resin.

6. After the detergent removal step, be very careful everything used from this point on is endotoxin free.
7. Optimal endotoxin level is less than 10ng endotoxin/mg protein.
8. The standard aliquot for recombinant proteins is 1 mg.