

SOP: RP019

Production of Recombinant BFRB SOP

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 Shaking Incubator
13. 37°C Plate Incubator
14. Heat block with 96-well plate incubator
15. Optical Plate Reader with 405 and 550 λ filters
16. Pyrogen-free tubes
17. Vortexer
18. LB Broth
19. 4L Erlenmeyer Flask
20. IPTG
21. Ampicillin
22. Chloramphenicol
23. High-speed Centrifuge
24. Centrifuge Bottles
25. Burdick and Jackson Water (Cat#365-4)
26. 1X Binding Buffer (20 mM Tris-HCl, 500 mM NaCl, 5 mM Imidazole, pH 7.9) (note 2)
27. 1X Charge Buffer (50 mM NiSO₄) (notes 1 and 2)
28. 1X Wash Buffer (20 mM Tris-HCl, 500 mM NaCl, 60 mM Imidazole, pH 7.9) (note 2)
29. 10 mM Tris-HCl, pH 8 made with Endotoxin free water (note 2)
30. 1X Elution Buffer (10 mM Tris-HCl, 1M Imidazole, pH 8 made with endotoxin free water) (note 2)
31. 0.5% ASB-14 (Calbiochem Cat# 182750) in 10 mM Tris-HCl (note 3)
32. Lysozyme (10 mg/ml in Milli-Q Water)
33. DNase (3mg/ml in Milli-Q Water)
34. Complete protease inhibitors (Roche Cat#1873580 or 1836170) (note 4)
35. Endotoxin free water
36. Dialysis Membrane 3500 MWCO (Spectra-Por cat# 132 724)
37. 1000 mL beaker
38. Dialysis chamber
39. magnetic stir-bar
40. stir plate
41. 4°C Cold Room
42. Ammonium Bicarbonate
43. 10 mM Ammonium Bicarbonate made with ET free water
44. Urea

Protocol:

1. _____ Inoculate 30 ml LB Amp¹⁰⁰ Cam³⁴ culture with Rv2626c in the *E. coli* BL21 DE3 pLys E expression strain and grow overnight at 37°C with shaking.(note 5)
2. _____ Autoclave 2L of LB broth in a 4L Erlenmeyer Flask. Allow media to cool and add Amp¹⁰⁰Cam³⁴.

3. ____ Centrifuge the overnight 30 ml culture to pellet the cells. Decant spent media and resuspend pellet in fresh LB Amp¹⁰⁰Cam³⁴.
4. ____ Inoculate the 2L broth with the resuspended pellet. Grow at 37°C with shaking to an OD₆₀₀ of 0.5.
5. ____ Once culture has reached the proper OD, remove from the 37°C incubator.
6. ____ Add sterile IPTG to a concentration of 0.5 mM.
7. ____ Place culture into a shaking 37°C incubator, allow to grow 4-6 hrs.
8. ____ Harvest cells by centrifugation at 10,000 x g in 450 ml centrifuge bottles.
9. ____ Resuspend the cell pellet in 15 mL 1X Binding Buffer + 6M Urea (all buffers should be made with Burdick and Jackson water or Milli-Q water to minimize endotoxin levels in the buffers). Add complete protease inhibitors, DNase, and Lysozyme (note 6). Freeze at -20°C or continue.
10. ____ Incubate at 30°C for 15 minutes (Or 2 hours if frozen).
11. ____ Break cells by sonication or French Pressure Cell. Sonicate on iced ethanol using probe sonicator with 60 second cycles followed by 90 second intervals to cool the lysate. Repeat until the viscosity of the lysate changes.
12. ____ Centrifuge the lysate at 16,000 x g for 90 minutes to remove cellular debris and to clarify. Cell pellet may contain protein in the form of Inclusion Bodies. (Note 7)
13. ____ Decant lysate into graduated 50 mL conical tube to record volume. Freeze at -20°C or continue.
14. ____ Perform BCA to determine protein concentration.
15. ____ 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 approximately 12 mg of recombinant His-tagged protein.
16. ____ Equilibrate the appropriate number of His-Bind columns (note 8).
17. ____ Apply the lysate to the His-Bind columns by pipetting slowly.
18. ____ Collect Flow-through in 15 ml conical tubes.
19. ____ Apply 10 column volumes of 1X Binding Buffer+ 6M Urea, collect fraction in 15 ml conical tube.
20. ____ Apply 6 CV of 1X Wash Buffer + 6M Urea to the columns, collect fraction as before.
21. ____ Apply 10 CV of 10 mM Tris-HCl in ET free water to remove residual salts from the columns. Collect fraction as before.
22. ____ 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 9).
23. ____ Apply another 10 CV of the ET free 10 mM Tris-HCl to remove any excess detergent. Collect fraction as before.

24. ____ Apply 5 ml of the elution buffer, ET free 10 mM Tris-HCl, 1M Imidazole, 6M Urea. Collect fraction. Freeze fraction at -20°C or continue with dialysis.
25. ____ 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.
26. ____ Prepare dialysis chamber with 1L of 10 mM Ammonium Bicarbonate, 4M Urea.
27. ____ 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.
28. ____ 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.
29. ____ Pat dry the dialysis membrane to remove any potential contaminating buffer containing endotoxin.
30. ____ Cut open the dialysis membrane at the top and carefully pour the sample into a clean, sterile 50 mL conical tube.
31. ____ Rinse the dialysis membrane carefully with a small amount of 10 mM Ammonium Bicarbonate made with ET free water. Decant into the conical tube. Freeze at -20°C or continue with QC.
32. ____ Perform BCA analysis to determine protein concentration.
33. ____ If concentration is lower than .45 mg/ml (for .5mg aliquots) or .9 mg/ml (for 1mg aliquots) lyophilize or concentrate on the savant.
34. ____ 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.
35. ____ If concentrated, repeat the BCA analysis to determine protein concentration. If concentration is adequate, proceed to the next step otherwise start at step 33.
36. ____ Perform SDS-PAGE analysis to determine protein purity.
37. ____ Perform LAL testing to determine endotoxin contamination (note 10).
38. ____ Protein can be lyophilized in desired aliquots (note 11).

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 .2µm 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. The general yield per liter of culture is unknown.
6. Add Complete EDTA-free protease inhibitor per instructions with Complete.
Add 30µl stock DNase to 50 ml
Add lysozyme to 200µg/ml
7. Some protein may be in inclusion bodies. Using the pellet from step 12 follow below procedure to extract inclusion bodies.
 1. Thaw the frozen pellet from step 12 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. Combine supernatant with previous supernatant and continue with step 14.
8. 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.
 6. Column is ready to add sample to.

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

9. After the detergent removal step, be very careful everything used from this point on is endotoxin free.
10. Optimal endotoxin level is less than 10ng endotoxin/mg protein. (If endotoxin levels exceed allowable levels the protein lyophilized and resuspended in Binding Buffer and put back on the column. See Step 16.)
11. The standard aliquot for recombinant proteins is 1 mg.