SOP: RP025

Production of KatG under Non-Denaturing Conditions 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^{o}C Shaking Incubator
13. 37^{o}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 $NiSO_4$) (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^{o}C Cold Room
42. Ammonium Bicarbonate
43. 10 mM Ammonium Bicarbonate made with ET free water
44. DEAE Buffer A(20mM Tris HCl, pH 8.4)(note 2)
45. DEAE Buffer B(20mM Tris HCl, 1M NaCl,pH 8.4)(note 2)
46. HiTrap DEAE FF(Amersham Biosciences Cat# 17-5055-01)
47. BioRad Econo Gradient Pump
48. 1.7ml microcentrifuge tubes

Protocol:

1. _____ Inoculate 10 ml LB Amp^{100} Cam^{34} culture with target protein in the *E. coli* BL21 DE3 pLys S Star and grow overnight at 37^{o}C with shaking.

2. _____ Autoclave 2L of LB broth in a 4L Erlenmeyer Flask. Allow media to cool and add $Amp^{100}Cam^{34}$.

3. _____ Centrifuge the overnight 10 ml culture to pellet the cells. Decant spent media and resuspend pellet in fresh LB $Amp^{100}Cam^{34}$.

4. _____ Inoculate the 2L broth with the resuspended pellet. Grow at 37°C with shaking to an OD_{600} 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 (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 5). 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. If the pellet appears light mocha or whitish in color, store pellet at -20°C and see RP003b.

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 6).

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, collect fraction in 15 ml conical tube.

20. _____ Apply 6 CV of 1X Wash Buffer 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 7).

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. Collect fraction. Freeze fraction at -20ºC 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 4L of 10 mM Ammonium Bicarbonate.

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^{o}C for 8 hours, exchange buffer 3 more times to ensure complete removal of contaminants.

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 -20ºC or continue with QC.

32. _____ Perform BCA analysis to determine protein concentration.

33. _____ Lyophilize and resuspend in DEAE Buffer A or transfer to DEAE Buffer A with an Amicon.

34. _____ Use the Econo Gradient Pump to run 10CV of sterile ET free water through the column.

35. _____ Use the Econo Gradient Pump to run 10CV of DEAE Buffer A through the column.

36. _____ Use the Econo Gradient Pump to run Kat G through the column collecting the flow through.

37. _____ Use the Econo Gradient Pump to run 30 CV of Buffer A through the column collecting the flow through.

38. _____ Use the Econo Gradient Pump to run a 25%-75% gradient of Buffer A to Buffer B at 1ml/min for 25 min. Collect 1ml fractions.

39. _____ Use the Econo Gradient Pump to run 15 CV of Buffer B through the column collecting the flow through.

40. _____ Run a SDS-PAGE gel on each fraction including initial flow through, buffer A flow through, fractions, buffer B flow through. Kat G should primarily be in the fractions.

41. _____ Combine the pure looking fractions into one.

42. _____ Perform Dialysis as in steps 25-31.

43. _____ Perform BCA analysis to determine protein concentration.

44. _____ If concentration is lower than .45 mg/ml (for .5mg aliquots) or .9 mg/ml (for 1mg aliquots) lyophilize or concentrate on the savant.

45. _____ 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.

46. _____ If concentrated, repeat the BCA analysis to determine protein concentration. If concentration is adequate, proceed to the next step otherwise start at step 33.

47. _____ Perform SDS-PAGE analysis to determine protein purity.

48. _____ Perform LAL testing to determine endotoxin contamination (note 8).

49. _____ Protein can be lyophilized in desired aliquots (note 9).

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. Add Complete EDTA-free protease inhibitor per instructions with Complete.

Add 30µl stock DNase to 50 ml

Add lysozyme to 200µg/ml

6. 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 4ºC with at least 1ml of binding buffer covering the top of the resin.

7. After the detergent removal step, be very careful everything used from this point on is endotoxin free.
8. Optimal endotoxin level is less than 10ng endotoxin/mg protein.
9. The standard aliquot for recombinant proteins is 0.5 mg.