

SOP: RP007msmeg

Production of Recombinant GlcB/Rv1837c from *M. smegmatis* 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

Protocol:

1. _____ Inoculate 300 ml LB Hyg¹⁰⁰ Kan²⁵ culture with Rv1837c in the pvv16 in the *M. smeg* MC²155 expression strain and grow for 2 days at 37°C with shaking.(note 5)
2. _____ Autoclave 2L of LB broth in a 4L Erlenmeyer Flask. Allow media to cool and add Hyg¹⁰⁰kan²⁵.
3. _____ Inoculate the 2L broth with the pellet from the 300ml culture. Grow at 37°C with shaking for 2 days.

4. _____ Inoculate 5x2L LB broth with Hyg¹⁰⁰kan²⁵ with the pellet from the 2L flask. Grow at 37°C with shaking for 3 days.(note 14)
4. _____ Harvest cells by centrifugation at 10,000 x g in 450 ml centrifuge bottles or by pipeting the pellet into a sterile conical.
5. _____ Resuspend the cell pellet to .5g/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 6). Freeze at -20°C or continue.
6. _____ Incubate at 30°C for 15 minutes (Or 2 hours if frozen).
7. _____ Break cells by French Pressure Cell or sonication. 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.(Note 13)
8. _____ 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 insoluble form. (Note 7)
9. _____ Decant lysate into graduated 50 mL conical tube to record volume. Freeze at -20°C or continue.
10. _____ Perform BCA to determine protein concentration.
11. _____ 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.
12. _____ Equilibrate the appropriate number of His-Bind columns (note 8).
13. _____ Apply the lysate to the His-Bind columns by pipetting slowly.
14. _____ Collect Flow-through in 15 ml conical tubes.
15. _____ Apply 10 column volumes of 1X Binding Buffer, collect fraction in 15 ml conical tube.
16. _____ Apply 6 CV of 1X Wash Buffer to the columns, collect fraction as before.
17. _____ Apply 10 CV of 10 mM Tris-HCl in ET free water to remove residual salts from the columns. Collect fraction as before.
18. _____ 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).
19. _____ Apply another 10 CV of the ET free 10 mM Tris-HCl to remove any excess detergent. Collect fraction as before.
20. _____ Apply 5 ml of the elution buffer, ET free 10 mM Tris-HCl, 1M Imidazole. Collect fraction. Freeze fraction at -20°C or continue with dialysis.
21. _____ 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.
22. _____ Prepare dialysis chamber with 4L of 10 mM Ammonium Bicarbonate.

23. _____ 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.
24. _____ Dialyze with slow stirring at 4°C for 8 hours, exchange buffer 3 more times to ensure complete removal of contaminants.
25. _____ Pat dry the dialysis membrane to remove any potential contaminating buffer containing endotoxin.
26. _____ Cut open the dialysis membrane at the top and carefully pour the sample into a clean, sterile 50 mL conical tube.
27. _____ 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.
28. _____ Perform BCA analysis to determine protein concentration.
29. _____ If concentration is lower than .45 mg/ml (for .5mg aliquots) or .9 mg/ml (for 1mg aliquots) lyophilize or concentrate on the savant.
30. _____ 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.
31. _____ If concentrated, repeat the BCA analysis to determine protein concentration. If concentration is adequate, proceed to the next step otherwise start at step 33.
32. _____ Perform SDS-PAGE analysis to determine protein purity.(note 10)
33. _____ Perform LAL testing to determine endotoxin contamination (note 11).
34. _____ Protein can be lyophilized in desired aliquots (note 12).

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 about .1 mg.
6. Add Complete EDTA-free protease inhibitor per instructions with Complete.
Add 30µl stock DNase to 50 ml
Add lysozyme to 200µg/ml
7. If denatured Rv1837c is acceptable for your applications you may start RP003b with the pellet from this step. The amount of GlcB in an insoluble form from M. smegmatis is unknown. Run a gel and perform a BCA prior to attempting purification.
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. Significant breakdown does occur with this protein so mass spec and western blots should be used to confirm bands are breakdown products and not a contaminating protein.
11. Optimal endotoxin level is less than 10ng endotoxin/mg protein.
12. The standard aliquot for recombinant proteins is 1 mg.
13. French Press will be much more effective as sonication is much less efficient for breaking cells. French press will require about 9-12 passes. If sonication is used increase sonication cycles. Check lysis by acid fast staining.
14. The volume of media may be adjusted as needed just maintain similar inoculation sizes to maintain the same growth time and conditions.