

SOP: PP009.1

Large scale purification of mycobacterial genomic DNA

Materials and Reagents:

1. Mycobacterial cells, frozen (note 1)
2. Tris-EDTA (TE) buffer, 1 M, pH 8.0, sterile (VWR PI17890)
3. Oakridge centrifuge tubes, 50 ml Teflon, sterile
4. DNase/RNase, Pyrogen – free centrifuge tubes, 50 ml, sterile (VWR 21008-178)
5. Chloroform (VWR BJ049-1)
6. Methanol (VWR BJ023-1)
7. UltraPURE DNase/RNase-free Water (Gibco 10977-015)
8. Tris-HCl, 1M, pH 9.0, sterile
9. SDS, 10% solution, sterile (Gibco 15553-035)
10. Lysozyme (Roche 10 837 059 001), 10 mg/ml stock solution
11. Proteinase K (Roche 03 115 879 001), 10 mg/ml stock solution
12. RNase, DNase-free (Roche catalog 11 119 915 001)
13. Isoamyl Alcohol (VWR MK299204)
14. Sodium acetate, 3M, pH 5.2, sterile
15. Isopropanol (VWR BJ323-1)
16. Ethanol, 70%, cold (-20°C)
17. Agarose (BioRad 161-3102)
18. Ethidium bromide solution (10 mg/ml)
19. Sorvall centrifuge
20. Sorvall centrifuge rotor SS-34
21. 37°C Water bath
22. 55°C Water bath
23. Vortex
24. Glass pipets, 10 ml
25. Rubber pipet bulb
26. Phenol-chloroform-isoamyl alcohol (25:24:1) (Roche 101003)
27. Platform rocker
28. Transfer pipets
29. Refrigerator, 4°C
30. Freezer, -20°C
31. Pipet tips, 10µl
32. Pipet tips, 1000 µl
33. Pipetman, 10 µl
34. Pipetman, 1000 µl
35. Spectrophotometer, UV capable
36. Agarose gel electrophoresis unit
37. Power supply for gel electrophoresis unit
38. Gel-Doc system with UV light

Protocol:

1. ____ Thaw frozen pellet of bacteria (note 1)
2. ____ Suspend cells (5 to 10 g wet weight per tube) in 10 ml of sterile TE buffer and transfer to 50 ml sterile Teflon Oakridge tubes.
3. ____ Add 10 ml of chloroform-methanol (2:1) and mix by inverting several times; then incubate sample with rocking for 30 min @ room temperature (note 2).
4. ____ Centrifuge the cell suspension at 2500 x g, 4°C for 20 minutes. This will generate aqueous and organic phase separated by the bacterial pellet.

5. ____ Decant both the aqueous and organic layers, leaving the bacterial pellet in the centrifuge tube. Discard both layers appropriately.
6. ____ Place the tube containing the cell pellet on the N₂ air bath (with heat) for 10 to 15 minutes, or until the odor of organic solvents can no longer be detected (note 3).
7. ____ Add 5 ml of sterile TE buffer and re-suspend the cells by vortexing vigorously (note 4).
8. ____ Add 0.1 volumes of sterile 1M Tris-HCl, pH 9.0, to increase the pH of the cell suspension.
9. ____ Add lysozyme stock solution to a final concentration of 100 µg/ml and incubate by placing tubes in a 37°C water bath for 12 to 16 hours (note 5).
10. ____ Add 0.1 volumes of 10% SDS solution, 0.01 volumes of Proteinase K stock solution, and 10 µl of RNase, DNase-free stock solution to the cell lysate.
11. ____ Mix by inverting several times.
12. ____ Incubate in a 55°C water bath for 3 hours (note 6).
13. ____ Add an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and place on platform rocker for 30 minutes (note 7).
14. ____ Centrifuge at 12,000 x g for 30 minutes.
15. ____ Transfer the upper, aqueous layer to a 50 ml sterile teflon Oakridge tube. Discard of phenol layer as hazardous waste.
16. ____ Add an equal volume of chloroform-isoamyl alcohol (24:1) to the aqueous phase and place on platform rocker for 10 minutes (note 8).
17. ____ Centrifuge at 12,000 x g for 30 minutes.
18. ____ Transfer upper, aqueous layer to a 50 ml sterile teflon Oakridge tube. Discard 24:1 layer as hazardous waste.
19. ____ To the final aqueous phase add 0.1 volumes of 3M sodium acetate, pH 5.2 and 1 volume of isopropanol (note 9).
20. ____ Mix by inverting several times and place at 4°C for 1 to 16 hours (note 10).
21. ____ Centrifuge at 12,000 x g for 30 minutes and decant the supernatant.
22. ____ Add 30 ml of cold (-20°C) 70% ethanol to the DNA pellet, making sure the pellet is dislodged from the bottom of the Oakridge tube.
23. ____ Centrifuge at 12,000 x g for 30 minutes and decant the supernatant.
24. ____ Allow the precipitated and washed DNA pellet to air dry.
25. ____ Suspend the pellet in 5 ml of sterile TE buffer and place at 4°C (note 11).
26. ____ Make 1:10, 1:20 and 1:50 dilutions of DNA for spectrophotometric analysis. Measure absorbance at 260 nm and 280 nm to determine the purity and DNA concentration (note 12).

27. _____ Make a 0.4% agarose gel; add 10 µl of ethidium bromide stock solution to gel before pouring in caster.
28. _____ Load 2 µg, 4 µg and 8 µg of DNA, along with a high molecular weight DNA ladder, into the gel and electrophoresis (see SOP SP018).
29. _____ Visualize gel by BioRad Gel-Doc system containing a UV light and record a picture of gel (note 13).
30. _____ If DNA passes QC, make 100 µg, 250 µg, and 500 µg aliquots (default quantity is 100 µg) into sterile 1.7 ml or 0.65 ml eppendorf tubes, dry on savant, and store at 4°C.

QC procedures:

Notes:

1. If isolating genomic DNA from *M. tuberculosis*, then this must be done inside a BSL-3 facility. For this protocol, the bacteria should be grown in a 2.8 L fernbach flask containing 1L of GAS medium for two weeks at 37°C on an orbital shaker platform. This will typically yield a bacterial pellet of 5 to 10 g (wet weight).
2. After this step, *M. tuberculosis* bacilli have been inactivated and the remainder of the protocol may be done under BSL-2 conditions. Make chloroform-methanol (2:1) using fresh solvents.
3. Residual organic solvents will interfere with lysozyme activity and decrease the yield of genomic DNA.
4. Organic extraction of cells causes them to clump tightly, thus making them difficult to re-suspend in an aqueous solution.
5. Do not vortex the suspension after the addition of lysozyme or shearing of DNA will occur.
6. The suspension should be extremely viscous at this point. If it is not, then add another 0.1 volumes of 10% SDS solution and 0.01 volumes of Proteinase K stock solution and incubate at 55°C for an additional hour.
7. This extraction will remove contaminating proteins.
8. This extraction will remove contaminating phenol. If the smell of phenol is present after transferring the aqueous layer to a new Oakridge tube (step 18), then repeat this extraction once more. Make chloroform-isoamyl alcohol (24:1) using fresh solvents.
9. This will precipitate the DNA.
10. One hour is acceptable for precipitation, but overnight is preferable.
11. Sometimes it is difficult to completely re-suspend the DNA pellet in TE buffer. Usually allowing the DNA and TE buffer to sit at 4°C for 12 to 16 hours is sufficient. However, if the DNA has not completely re-suspended, more TE buffer may be added. Additionally the DNA may be placed at 37°C or, if necessary, at 55°C until in solution.
12. See SOP SP014. DNA concentration may be calculated by the following formula:

$$(A_{260}) \times (50 \mu\text{g/ml}) \times (\text{dilution factor}) = \mu\text{g/ml DNA}$$

DNA purity is calculated by the A_{260}/A_{280} ratio. Pure double stranded DNA is 1.8, but 1.7 to 2.0 is acceptable.

13. Genomic DNA will not run as a sharp band in a 0.4% agarose gel, but any RNA contamination and/or shearing will be readily apparent on the gel.

Reference:

Belisle, J. T. and M. G. Sonnenberg (1998) Isolation of Genomic DNA from Mycobacteria. *Methods in Molecular Biology, Vol 101: Mycobacteria Protocols.* (Parish T. and Stoker, N. G. ed), Humana Press, Inc., Towata, NJ., pp 31-44.