SOP: PP042.2
Modified: 12/16/2022 by RAS

Large scale purification of Non-Mtb *Mycobacterium spp* 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. MilliQ water
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 (-20ºC)
17. Agarose (BioRad 161-3102)
18. SYBRSafe agarose gel stain (Life technologies)
19. Beckman Coulter Avanti JXN-26 centrifuge
20. Beckman Coulter centrifuge rotor JA-25.50
21. 37ºC Water bath
22. 60ºC 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, 4ºC
30. Freezer, -20ºC
31. Pipet tips, 10 µl
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
39. 5M NaCl
40. 10% CTAB (in .7M NaCl)
41. Lowenstein-Jenson agar slant with Sodium pyruvate
42. 2:1 chloroform:methanol
43. Nitrogen bath

Protocol:

1._____ Thaw frozen pellet/cell suspension of bacterial cells (note 1)

2._____ Heat kill cells by entirely submerging in 80°C water bath for a full 30 minutes. (Freeze -80C if needed after)

3._____ Optional- Add an equal volume of 2:1 (chloroform:methanol) and invert several times. This step will delipidate the cells making them easier to lyse. At this point the cells are inactivated and the protocol can be carried out in the BSL-2. Decant the upper layer. Using a fume hood, allow the 2:1 suspension to dry completely, preferably in a N_2 bath over-night. Once dried, resuspend the cells in 5ml TE buffer.

4._____ Add 700 µl 10% SDS, 1500 µl Proteinase K, and add 10mg/mL stock lysozyme (70 µL) to a final concentration of 100 µg/mL. Invert several times and place in 60°C water bath for 1 hr (notes 2 and 3).

5._____ Add 1 ml of 5M NaCl and 1 ml of 10% CTAB. Invert several times and place in 60°C water bath for 20 min. (note 13)

6._____ Freeze for 15 minutes at -80°C. Remove and allow to thaw. Freeze again for 15 minutes and again allow to thaw.

7._____ Add a sterile mini stir bar, place on stir plate and incubate overnight at 37°C (note 4).

8._____ Remove tube from incubation and transfer cellular solution to a sterile 50 ml Teflon Oakridge tube. Add an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) and place on platform rocker for 30 minutes (note 5).

9._____ Centrifuge at 12,000 xg for 30 minutes at 4°C temp.

10._____ Transfer the upper, aqueous layer to a 50 ml sterile Teflon Oakridge tube. Discard of phenol layer as hazardous waste.

11._____ Add 200 µl of 1 mg/ml RNase. Invert several times and set in a 37°C water bath for 3 hrs.

12._____ Add an equal volume of chloroform-isoamyl alcohol (24:1) to the aqueous phase and place on platform rocker for at least 30 minutes.

13._____ Centrifuge at 12,000 xg for 30 minutes at 4°C temp.

14._____ Transfer upper, aqueous layer to a 50 ml sterile Teflon Oakridge tube. Discard 24:1 layer as hazardous waste (note 6).

15._____ To the final aqueous phase add 0.1 volumes of 3M sodium acetate, pH 5.2 and 1 volume of isopropanol (note 7).

16._____ Mix by inverting several times and place at 4°C for 1 to 16 hours (note 8).

17._____ Centrifuge at 12,000 xg for 30 minutes at 4°C temp and decant the supernatant.

18._____ 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.

19._____ Centrifuge at 12,000 xg for 30 minutes at 4°C temp and decant the supernatant.

20._____ Allow the precipitated and washed DNA pellet to air dry.

21._____ Suspend the pellet in 5 ml of sterile TE buffer and place at 4°C (note 9).

22._____ 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. If contamination is present, repeat extractions starting at either step 7 (for protein contamination) or step 11 (for phenol contamination) (note 10).

23._____ Make a 0.4% agarose gel; add 10 µl of SYBRSafe gel stain per 100ml of gel suspension before pouring in caster.

24._____ 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) (note 11).

25._____ Visualize gel by Bio-Rad Gel-Doc system containing a UV light and record a picture of gel (note 12). If RNA contamination is present, refer back to step 11 and repeat from the addition of 100 µl RNase.

26._____ Make 100 µg (or 2 mg aliquots for *M. leprae*) aliquots into sterile, DNase and RNase free 1.7 ml or 0.65 ml Eppendorf tubes, dry on savant, and store at 4°C.

Notes:

1. If isolating genomic DNA from *M. bovis*, 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 with Sodium Pyruvate (SPAS) medium for two to three weeks at 37°C on an orbital shaker platform. This will typically yield a bacterial pellet of 5 to 10 g (wet weight). If isolating *M. leprae* genomic DNA, obtain cells according to SOP: PP050 and start with ~30 mg. If isolating genomic DNA from *M. africanum*, use SOP: PP009.3.
2. Do not vortex the suspension after the addition of lysozyme or shearing of DNA will occur.
3. 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 60°C for an additional hour.
4. After this step, *M. bovis* bacilli have been inactivated and the remainder of the protocol may be done under BSL-2 conditions. Perform a quality assurance by inoculating an LJ slant containing Sodium pyruvate with an extract of the organic layer. Incubate the slants in warm room at 37°C for about 2 to 2.5 weeks. If no growth is observed, then the remainder of the protocol may be done under BSL-2 conditions. Alternatively, if BSL-3 equipment is available, the entire genomic extraction could take place within the BSL-3, thus inoculate an LJ slant with a small extract of the final genomic product before removing from the BSL-3.
5. This extraction will remove contaminating proteins.
6. This extraction will remove contaminating phenol. If the smell of phenol is present after transferring the aqueous layer to a new Oakridge tube (step 13), then repeat steps 10-13 once more. Make chloroform-isoamyl alcohol (24:1) using fresh solvents.
7. This will precipitate the DNA.
8. One hour is acceptable for precipitation, but overnight is preferable.
9. 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. Due to low yield, resuspending M. leprae in 2.5 ml may be preferred.
10. M. leprae yield may be low making dilution unnecessary. See SOP SP014. DNA concentration may be calculated by the following formula:

    (A_{260}) x (50 µg/ml) x (dilution factor) = µg/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, a low ratio indicates protein contamination. The absorbance at 230nm should also be taken, calculating the A_{260}/A_{230} ratio should be about 2.0-2.2, a low ratio indicates phenol contamination or guanidine contamination (if lysis buffer from extraction kits were used). Alternatively, an A_{330} or A_{320} can be used to indicate light scattering from particulate contamination. The A_{330} or A_{320} value should be equal to or close to 0 to indicate a pure sample.
11. Because recoveries of *M. leprae* DNA are generally very small, as little as 300 ng of DNA can be used and should be sufficient to visualize on a gel.
12. 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. Shearing will appear as a long “smudge” running down the length of the ladder in the sample lane. RNA contamination will appear as a “cloudy” band toward the bottom of gel in its lane.

13. To make 100 ml of 10% CTAB: Dissolve 4.1g of NaCl in 80 ml of MilliQ water. While stirring, add 10 g of CTAB. To dissolve CTAB, heat solution at 65 C until fully dissolved. Make volume to 100 ml using MilliQ water.

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.

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