SOP: PP009.3
Updated: 05/07/2015 PK

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 (-20ºC)
17. Agarose (BioRad 161-3102)
18. SYBRSafe agarose gel stain (Life technologies)
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, 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. Lowenstein-Jenson medium slants

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 N_2 air bath (with heat) for 10 to 15 minutes, or until the odor of organic solvents can no longer be detected (note 3). Cell Pellet may be left on N_2 air bath overnight.

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. Place in 55°C water bath for 30 min, then vortex again to break up clumps.

9._____ Add lysozyme stock solution to a final concentration of 300 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 100 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, inverting every half hour (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. If contamination is present, refer back to step 13 (note 12).

27._____ Make a 0.4% agarose gel; add 30 μl of SYBRSafe gel stain per 100 ml of gel suspension 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). If RNA contamination is present, refer back to step 10 and repeat from the addition of 100 μl RNase.

30._____ If DNA passes QC, make 100 μg aliquots (default quantity is 100 μg) into sterile 1.7 ml or 0.65 ml eppendorf tubes, and store at 4°C.

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. Perform a quality assurance by inoculating an LJ slant 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. 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. Use caution while working with phenol. Always handle phenol in a chemical fume hood, wearing proper PPE, and only use glass pipets. Phenol is not compatible with all centrifuge tubes (the recommendation here is Teflon). Check chemical compatibilities of all materials before use.
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}) 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, and the calculated A_{230}/A_{260} 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.

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. 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.

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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