Protocol, Isolation of high molecular weight genomic DNA from Mycobacteria (CTAB procedure)

Materials

1. Solid medium: Löwenstein Jensen, or Löwenstein Jensen supplemented with pyruvate.
2. Liquid medium: Mgit or Mbact
3. 10X TE buffer: dissolve 100 mM Tris/HCl, pH 8.0 and 10 mM EDTA in distilled water. Autoclave. Store at room temperature (RT) for no longer than one year.
4. 1X TE buffer: add 1 volume of 10X TE buffer to 9 volumes of distilled water. Autoclave. Store at RT for no longer than one year.
5. 10 mg/mL lysozyme (Boehringer Mannheim, Mannheim, Germany) in distilled water. Store in small aliquots at -20°C for no longer than one year.
6. 10% SDS: 10 gram sodium dodecyl sulfate (SDS) (BDH Laboratory Supplies, Poole, England) / 100 mL distilled water (*see* **Note 1**). Dissolve by heating at 65 °C for 20 min. Do not autoclave. Store at RT for no longer than one month.
7. 10 mg/mL proteinase K (Boehringer Mannheim) in distilled water. Store in small aliquots at -20 °C for no longer than one year.
8. SDS/Proteinase K solution. Prepare a fresh mix for all samples. Mix 70 µl 10% SDS (see item 6) and 5 µl 10 mg/mL proteinase K (see item 7) for each sample. Vortex briefly.
9. 5 M NaCl: 29.2 gram NaCl (Merck, p.a., Darmstadt, Germany) / 100 mL distilled water. Autoclave. Store at RT for no longer than one year.
10. CTAB/NaCl solution. Dissolve 4.1 gram NaCl (Merck, p.a.) in 80 mL distilled water. While stirring, add 10 gram N-cetyl-N,N,N,-trimethyl ammonium bromide (CTAB) (Merck, p.a.). To dissolve heat the solution at 65 °C. Adjust the volume to 100 mL with distilled water. Store at RT for no longer than 6 months.
11. Chloroform/isoamyl alcohol (24:1) (*see* **Note 2**). Mix 24 volumes of chloroform (Merck, p.a.) with 1 volume of isoamyl alcohol (Merck, p.a.). Store at RT for no longer than one year.
12. Isopropanol (Merck, p.a.). Store at RT for no longer than one year.
13. 70% ethanol. Mix 7 volumes ethanol abs. (Merck, p.a.) with 3 volumes distilled water. Store at -20 °C.
14. Waterbath 37 °C, 65 °C, 80 °C.
15. Thermomixer (Thermomixer 5436, Eppendorf, Hamburg, Germany) (*see* **Note 3**).
16. Microcentrifuge.
17. Espirator (*see* **Note 4**).
18. Vortex.
19. -20 °C freezer.

Method

Caution: for mycobacteria pathogenic to humans appropriate containment facilities should be used while handling before heat-inactivation.

1. Transfer at least two loops of mycobacteria into a microcentrifuge tube containing 400 µL of 1X TE (see **Notes 5** and **6**).
2. Kill the cells by heating for 20 min at 80 °C, and cool at RT (*see* **Note 7**).
3. Put the CTAB/NaCl solution at 65 °C, for use in step 6.
4. Add 50 µL of 10 mg/mL lysozyme, vortex and incubate, while shaking, for at least one h at 37 °C (see **Notes 3** and **8**).
5. Add 75 µL of 10% SDS/proteinase K solution, vortex, and incubate for 10 min at 65 °C (*see* **Note 3**).
6. Add 100 µL of 5 M NaCl and 100 µL of prewarmed CTAB/NaCl solution, and vortex until the liquid content becomes white ("milky"). Incubate for 10 min at 65 °C (see **Notes 3** and **9**).
7. Add 750 µL of chloroform/isoamyl alcohol and vortex for at least 10 sec (see **Notes 2, 3** and **10**).
8. Centrifuge for 8 min at ± 11,000 x*g*.
9. Transfer the aqueous phase (top) to a fresh microcentrifuge tube, by pipetting small aliquots of e.g. 180 µL (see **Note 11**).
10. Carefully add 0.6 volume (450 µL) of isopropanol (see **Note 12**).
11. Manually move the tube slowly upside down to precipitate the nucleic acids and estimate the amount of 1X TE in which the DNA should be redissolved in step 20. Write the estimated volume e.g. on the tube (see **Note 13**).
12. Place at -20 °C for at least 30 min (see **Note 14**).
13. Centrifuge for 15 min at ±11,000 x*g*.
14. Discard most of the supernatant; leave about 20 µL (3 mm height) above the pellet (see **Note 4**).
15. Add 1 mL of cold 70% ethanol (from the -20 °C freezer) and turn the tube a few times upside down to wash the DNA precipitate.
16. Centrifuge for 5 min at ±11,000 x*g*.
17. Discard most of the supernatant; leave about 20 µL (3 mm height) above the pellet (see **Note 4**).
18. Centrifuge for 1 min at ±11,000 x*g*.
19. Remove the remaining supernatant from above the pellet by pipetting very carefully with a 20 µL pipette (see **Note 15**).
20. Permit the pellet to dry for ± 15 min at RT. Check whether all ethanol is evaporated. If not, then extend the drying time.
21. Dissolve the pellet in the amount of 1X TE estimated in step 10 (see **Note 16**).

Notes

1. Prevent inhalation of SDS when handling solid SDS. Use an exhaust hood and a mouth cap.
2. Be careful with chloroform. Its inhalation is unhealthy and it can damage certain plastics. Always use an exhaust hood. Do not spill chloroform on plastic racks.
3. For all incubations during the DNA isolation procedure a waterbath can be used. If shaking is required use 45 movements per min. Alternatively, a thermomixer can be used. This is an apparatus that heats and shakes the tubes simultaneously (see item 15). For shaking incubations use position 7, for vortexing use the highest shaking position. This apparatus can be used for the entire DNA isolation procedure, but it is important to check whether the vortexing went well after addition of CTAB/NaCl. We advise not to use this apparatus for heat-killing the bacteria and vortexing after the addition of chloroform/isoamylalcohol. These steps should preferably be done manually.
4. It is convenient to use an aspirator. Alternatively a pipette can be used.
5. If mycobacteria are isolated which grow well on solid media, such as e.g. *M. tuberculosis*, then use at least two loops (0.5 cm diameter) of bacteria. Alternatively, in case of e.g. *M. avium* complex strains, or other mycobacteria growing very smooth on solid media, take a well grown 50 mL liquid culture. Transfer the liquid culture to a suitable centrifuge tube and centrifuge for 15 min at 3000x*g* using aerosol-containment buckets. Discard the supernatant and add 200 µL of 1X TE buffer to the tube. Resuspend the pellet by vortexing. Transfer 200 µL resuspended pellet to a microcentrifuge tube and add 200 µL 1X TE.
6. Do not use a mycobacterial culture grown on 7H10 medium, because for unknown reasons DNA isolated from mycobacteria grown on this medium is not well digested by restriction enzymes.
7. Use microcentrifuge tubes with a safe-lock, or jam the microcentrifuge tubes in such a way that the lids cannot open spontaneously.
8. Incubation should preferably occur overnight, especially when DNA is isolated from *M. bovis* or *M. microti* strains.
9. Pre warming the CTAB/NaCl solution in a waterbath at 65°C will make the solution less viscous and therefore easier to pipette. The aim of CTAB treatment is to remove cell wall debris, denatured protein, and polysaccharides complexed to CTAB, while retaining the nucleic acids in solution. Adding salt is very important, since a CTAB-nucleic acid precipitate will form if the salt concentration drops below about 0.5 M at RT.
10. The chloroform/isoamyl alcohol extraction precipitates the CTAB-protein/polysaccharide complexes. A white interface should be visible after centrifugation.
11. Be careful not to transfer anything of the inter phase, this will result in impure DNA.
12. There is no need to add salt for precipitation of the DNA since the NaCl concentration is already sufficient.
13. While turning the tube upside down precipitated DNA may or may not become visible, depending on the amount of mycobacterial cells started with. Stop shaking when the precipitate is formed and the solution becomes clear. If there is no precipitate of nucleic acids visible, then dissolve the pellet in step 20 in 20 µL 1X TE. If there is a small precipitate visible, then dissolve the pellet in 35 µL. Medium and large precipitates require 50 and 80 µL, respectively.
14. This step is not necessary, but ensures that all DNA precipitates. The incubation time can be

extended as long as is convenient, since DNA can be kept in these conditions for even years.

15. Be sure that all traces of ethanol are removed, otherwise the pellet cannot dry and the precipitated DNA can even redissolve after a while.
16. Dissolving the DNA pellet at RT may take some time. To dissolve the DNA more quickly, incubate at 37°C for 1 h. Alternatively, the DNA can be incubated overnight at 4°C. Dissolved DNA can be stored, until use, at 4°C. For longer periods of time the DNA can be stored at -20°C (for years).