Preface

The aim of this book is to provide detailed protocols for studying the molecular biology of the pathogen *Mycobacterium tuberculosis*, and its interactions with host cells. As established mycobacterial laboratories move towards exploiting the genome, and laboratories with expertise in other fields apply them to mycobacteria, both traditional and novel methodologies need to be reviewed. Thus the chapters in *Mycobacterium tuberculosis Protocols* range from perspectives on storage of strains and safety issues to the application of the latest functional genomics technologies.

The last few years have been remarkable ones for research into *M. tuberculosis*. The most important landmark by far has been the completion of the genome sequence of the widely studied H37Rv strain (1). We can now predict every protein and RNA molecule made by the pathogen. This information is or will soon be enriched by the addition of genome sequences of other strains from the *M. tuberculosis* complex: a second strain of *M. tuberculosis*, *Mycobacterium bovis*, and the vaccine strain, *M. bovis* BCG. Valuable comparative data will also be provided by the genome sequences of *Mycobacterium leprae*, *Mycobacterium avium*, and *Streptomyces coelicolor*. Another recent milestone for *M. tuberculosis* has been the development of efficient mutagenesis methodologies, the lack of which has been a major handicap in functional studies. The new challenges to researchers are first to use this information and these techniques in combination with the battery of methodologies being developed around the world to exploit all genome data, so-called functional genomics research, which includes transcriptomics and proteomics. The second challenge is to integrate them with other disciplines of active research such as immunology, cell biology, and biochemistry. *Mycobacterium tuberculosis Protocols* incorporates both of these aspects in the methods described.

This book is aimed at people who are actively working on *M. tuberculosis.* However, there is much that will be relevant to work on other mycobacteria and on such phylogenetically related organisms as corynebacteria and streptomycetes. It is intended both for people with experience in handling *M. tuberculosis* and those who are new to the field.

The topics covered by the 24 chapters included are quite diverse. A major focus is the production of mutants, which plays a central role in functional studies. The recent successes in mutagenesis are reflected in the inclusion of four chapters (4–7) describing different strategies for both transposon mutagenesis and targeted allelic replacement.

Two of the most exciting recent technological developments make use of the genome sequence to allow us to look at the RNA and protein complements of the cell at a global level. Proteome analysis is described in Chapter 21, and the production and analysis of whole genome microarrays is described in Chapter 22. RNA is discussed in more detail elsewhere, with chapters on purification (Chap. 3), transcriptional start site analysis (Chap. 8), and quantitation using real-time PCR (Chap. 19).

Fractionation of the bacteria to look at protein carbohydrate and lipid components is described. The contributions focus on analysis of culture filtrates (Chap. 13), the capsule (Chap. 14), and lipids (Chap. 15). Cytological analysis of the bacteria allows the analysis of cellular properties in individual bacteria, and many of the major technological advances that have helped eukaryotic cell biology are beginning to be applied to bacteria (Chap. 9).

There is understandably enormous interest in how *M. tuberculosis* interacts with the host, and chapters discuss infection of macrophages both as a virulence assay (Chap. 17), and in order to understand the cell biology (Chap. 18). In addition, perhaps the most difficult topic to study, the persistence of bacteria, is addressed in Chap. 16.

The excitement of the post-genome era must not distract us from the fact that tuberculosis is a dreadful disease of which millions die each year, and control still suffers from difficulties in diagnosis. A relatively new method for detecting bacteria and identifying drug resistance using a cheap and sensitive phage-based method is described (Chap. 10). This method is phenotypic, in that the basis of the drug resistance need not be known. Genotypic methods are described that identify specific mutations by dot-blotting (Chap. 11) or real-time PCR (Chap. 19). Our understanding of the spread of bacteria has been revolutionized by DNA typing techniques, and the most up-to-date methodology for carrying out RFLP typing is in Chap. 12. DNA preparation from bacteria cultures and clinical isolates is discussed in Chap. 2.

Though most of *Mycobacterium tuberculosis Protocols* concentrates on laboratory methods, the genome sequence is a central resource for laboratory and bioinformatics research. A description of the main *M. tuberculosis* genome resources on the internet is therefore provided in Chap. 20.

Finally there are chapters providing basic but essential methods for work with *M. tuberculosis.* These are an up-to-date account of available cloning vectors (Chap. 1), how to store strains (Chap. 23). and last but by no means least, a discussion of some of the safety issues (Chap. 24).

This book aims to complement and update the earlier volume in this series, *Mycobacteria Protocols* (2). Some methods are deliberately complementary—for example, the computer analysis of IS*6110* fingerprints was described in the earlier book, whereas the production of the fingerprints is described here. Several diagnostic methods included in the earlier volume complement those presented here. Other topics in the earlier book that are relevant to tuberculosis research are those on pulsed field gel electrophoresis, preparation of cell-free extracts and cell wall fractions, the use of mycobacteriophages, and the analysis of gene expression using reporter genes and RT- and RAP-PCR.

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2

Isolation of DNA from *Mycobacterium tuberculosis*

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1. Introduction

Research into and identification of *Mycobacterium tuberculosis* can take on a number of facets, many of which involve the use of DNA at one stage or another. The quality and quantity of DNA required will depend on the end-use requirement. For example, good yields of pure, high-molecular-weight DNA uncontaminated by DNA from other sources (i.e., homogeneous) are optimal for the generation of cosmid libraries and sequencing ***(1)***, Southern hybridization ***(2–6)***, or microarray analysis ***(7)*** for genome studies, whereas relatively crude DNA (fragmented DNA or DNA from multiple sources [i.e., heterogeneous]) may be adequate for PCR-based diagnosis ***(8–12)*** or amplification of regions of the genome for other purposes, e.g., identification of mutations conferring drug resistance ***(13,14)***.

The source of material and the method selected for DNA preparation will define the purity and yield. It is important to bear in mind the aim of the project when deciding on the process to be followed. For example, strain genotyping using Southern hybridization ***(2–6)*** would generally require highly purified high-molecular-weight DNA from live cultures, whereas PCR-based examination of small areas of the genome can be done from archival material, such as formalin-fixed granulomas or Ziehl-Neelsen (ZN) slides ***(8–12)***. If fresh or frozen material is available, it may be preferable to establish a culture of *Mycobacterium tuberculosis* in liquid or on solid medium prior to attempting DNA recovery. This will facilitate recovery of high yields of clean, homogeneous high-molecular-weight DNA.

The tough cell wall of *Mycobacterium tuberculosis* can be both an advantage and a hindrance. The tough and complex cell wall ***(15)*** complicates purification

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in comparison to that of bacteria with relatively fragile walls. The mycobacteria have cell walls with copious amounts of polysaccharides, which can adversely influence subsequent manipulation of DNA. This cell wall can, however, allow the recovery of intact bacteria from a variety of sources and therefore help to remove much contaminating material, but the subsequent lysis of the bacterium and removal of the wall components to recover purified DNA is not trivial.

The extent to which purification needs to be done will also be a function of the starting material, since many biological samples contain unknown but potent inhibitors of enzymes ***(16)***, which may hamper further manipulation of the DNA.

Here we describe protocols for the preparation of DNA from *M. tuberculosis*. We have focused on the recovery of DNA from clinically derived samples, which can be used for diverse purposes, such as DNA fingerprinting, cloning, or PCR diagnostic work. Note that many of the appropriate methods for DNA purification from clinical material may result in a heterogeneous DNA preparation, which contains both mycobacterial and human genomic DNA ***(8–12,17)***. The sample preparation method always represents a trade-off between the requirements for the optimal methods, the source material and the aim of the procedure. In this regard, the number of organisms per volume of sample is critical. Factors to consider during sample preparation include the efficiency of target recovery, maintenance of intact DNA, the removal of known and unknown inhibitors, and safety factors for the worker. If DNA purification for diagnostic purposes is envisaged, care must be taken to avoid cross-contamination during all preparative stages ***(19)***. These are not the only methods that can be used or have been described; however, in our laboratory these methods are used on a regular basis and yield good quality material for further manipulation. For example, methods involving mechanical lysis ***(20)*** or use of guanidinium salts ***(21)*** have been described, but these harsher methods can yield sheared DNA, which may not be useful for purposes such as Southern blotting or cloning of larger fragments ***(22)***. The recovery of sheared or heterogeneous DNA does not necessarily exclude obtaining typing data, however, since PCR-based methods may still be useful in these cases ***(23)***.

Good quality and high yields of DNA can be obtained when *M. tuberculosis* is cultured in BACTEC vials, on Lowenstein-Jensen (LJ) slants, on 7H11 agar, or in 7H9 broth. The description of the culturing of the organism is not the objective of this chapter and the reader should refer to the relevant literature for further information ***(20)***. Clinical isolates of *M. tuberculosis* for DNA purification will frequently be established as cultures on LJ slants or in BACTEC vials. If the latter, a small aliquot can be streaked and grown on LJ or in liquid medium for further DNA isolation. DNA may also be recovered from other clinical sources, such as microscopy slides (ZN-stained), biopsy material, biological fluids (e.g., sputum, blood, urine, cerebrospinal fluid [CSF], or in vitro cul-

tures [e.g., macrophages]). The preparation and recovery of these is described, since the diagnosis and rapid characterization of clinical strains may play an important role in future strategies to curb the spread of the disease.

2. Materials

2.1. Recovery of Bacteria from Various Sources

Many sources of mycobacteria can be used for DNA preparation including: ZN- or auramine-stained microscopy slides, cultures on solid or in liquid medium, and biological specimens, e.g., sputum, biopsies, urine, CSF, in vitro cultures (*see* **Note 1**).

2.1.1. From Liquid Medium

1. Phosphate buffered saline (PBS): 8 m*M* NaCl, 2.6 m*M* KCl, 1.4 m*M* K_2HPO_4, 8 m*M* Na_2HPO_4, pH 7.4.
2. Oven or water bath set at 80°C.
3. Extraction buffer: 50 m*M* Tris-HCl, 25 m*M* EDTA, 5% mono-sodium glutamate, pH 7.4.
4. 50 mL Polypropylene tubes containing ±30 glass balls (5 mm).

2.1.2. From Solid Medium

1. Oven or water bath set at 80°C.
2. Extraction buffer: (*see* **Subheading 2.1.1.**, **item 3**).
3. Disposable inoculation loops.
4. 50 mL polypropylene tubes containing ±30 glass balls (5 mm).

2.1.3. Formalin-Fixed Tissue

1. Xylene.
2. Absolute ethanol.
3. Speedy-vac (Savant, New York).
4. Sterile distilled water.

2.1.4. Frozen or Fresh Tissue Sample

1. Mortar and pestle.
2. Dounce glass homogenizer with loose pestle.
3. Cell lysis buffer: 32 m*M* sucrose, 1% Triton X-100, 5 m*M* $MgCl_2$, 10 m*M* Tris-HCl, pH 7.6.

2.1.5. Microscopy Slides

1. PBS (*see* **Subheading 2.1.1.**, **item 1**).
2. Scalpel and blades.
3. 10% Saponin.
4. Sterile distilled water.
5. 20% Chelex 100 (Sigma, Poole, Dorset, UK).

2.1.6. Sputum

1. Decontamination solution: 4% (w/v) NaOH, 1% (w/v) *N*-acetyl-L-cysteine.
2. Extraction buffer (*see* **Subheading 2.1.1.**, **item 3**).
3. Lysozyme stock: 50 mg/mL, make immediately prior to use.
4. 10X proteinase K buffer: 100 m*M* Tris-HCl, 50 m*M* EDTA, 5% SDS, pH 7.8.
5. Proteinase K stock solution: 10 mg/mL. Store in aliquots at –20°C.
6. Buffer-saturated phenol ***(22)***: gently warm phenol to 68°C (place the bottle in extra container to contain spillages). Add hydroxyquinoline to 0.1%. Add an equal volume of 0.5 *M* Tris-HCl, pH 8.0. Stir for at least 10 min, then allow phase separation. Aspirate upper aqueous phase. Add an equal volume of 0.1 *M* Tris-HCl (pH 8.0). Repeat mixing and aspiration until pH of aqueous phase is >7.8. Add 0.1 vol of 0.1 *M* Tris-HCl, pH 8.0, containing 0.2% mercaptoethanol. Keep the solution in the dark at 4°C for up to 4–5 wk (*see* **Note 2**).
7. Phenol/chloroform/isoamylalcohol (25:24:1). Prepare with the buffer-saturated phenol (*see* **Note 2**).

2.1.7. Bone Marrow or Whole Blood

1. Histopaque 1077 (Sigma).
2. PBS (*see* **Subheading 2.1.1.**, **item 1**).
3. Lysis buffer: 10 m*M* Tris-HCl, pH 8.2, 400 m*M* NaCl, 2 m*M* EDTA. Add 50 μL of 10% SDS and 50 μL of 10 mg/mL proteinase K per mL of buffer before use.
4. 6 *M* NaCl.
5. Sterile distilled water.

2.1.8. Infected Macrophages

1. 10 m*M* EDTA, pH 7.5.
2. PBS (*see* **Subheading 2.1.1.**, **item 1**).
3. 10% SDS.

2.2. Enzymatic Lysis of Cells and Isolation of Genomic DNA

1. DNAse free RNAse: 10 mg/mL stock (*see* **Note 3**).
2. 50 mg/mL lysozyme stock: make immediately prior to use.

2.3. Removal of Protein and Cellular Contaminants

1. 10X proteinase K buffer.
2. Proteinase K stock solution: 10 mg/mL. Store in aliquots at –20°C.
3. Phenol/chloroform/isoamylalcohol (25:24:1) (*see* **Subheading 2.1.6.**, **item 7**).
4. Chloroform/isoamylalcohol (24:1)

2.4. DNA Precipitation and Recovery

1. 3 *M* sodium acetate, adjust to pH 5.5 with acetic acid.
2. Ice-cold isopropanol.
3. 70% ethanol.
4. 1X TE-buffer: 10 m*M* Tris-HCl, 1 m*M* EDTA, pH 8.0.

2.5. Quantitation and Purity of Genomic DNA

1. TE (*see* **Subheading 2.4.**, **item 4**).
2. Restriction enzyme and buffer.
3. Agarose gel electrophoresis equipment.
4. 3 *M* Na acetate, pH 5.5.
5. Absolute ethanol (at –20°C).

3. Methods

3.1. Recovery of Bacteria from Various Sources

Note that DNA recovered from pure cultures will be homogeneous, whereas clinical samples may yield heterogeneous DNA (*see* **Note 1**).

3.1.1. From Liquid Medium

1. Cells from liquid samples, including cultures ***(24)***, urine, and CSF (*see* **Note 4**) may be recovered by centrifugation in a category 3 biosafety laboratory. Centrifuge samples in sealed tubes (*see* **Note 5**) for 5 min in a microfuge or at 3000*g* in a bench centrifuge for 15 min.
2. Wash cells once with 1 mL of PBS without resuspending cells.
3. Heat-kill cells by placing in oven or water-bath at 80°C for 1 h (*see* **Note 6**).
4. Resuspend cells in extraction buffer and proceed with DNA extraction as described below (**Subheading 3.1.2.**, **step 3**). Use approx 6 mL of buffer for every 100 µL of cell pellet.

3.1.2. From Solid Medium

The example given is for isolation of DNA from *M. tuberculosis* grown on solid medium ***(20)***, for example, use an LJ slant culture of *M. tuberculosis* which has clearly visible colonies. Pure cultures will yield high molecular weight homogeneous DNA.

1. Heat sealed tube at 80°C for 1 h to kill bacteria (*see* **Note 6**). Subsequent steps can be carried out in a class 2 or 3 laminar flow cabinet.
2. Add 3 mL of extraction buffer and carefully scrape the colonies off the slant, using a disposable loop (estimated colony volume up to 100 µL).
3. Pour the buffer and bacteria into a 50 mL polypropylene tube containing approx 30 glass balls (5 mm diameter).
4. Add 3 mL of extraction buffer to the slant and remove remaining colonies — pool both extracts into polypropylene tube.
5. Vortex the suspension in a tightly sealed polypropylene tube at full speed for 2–3 min to disrupt the bacterial colonies. All clumps should be broken up. Proceed with DNA extraction by lysis of cells (*see* **Subheading 3.2.**).

3.1.3. Tissue (Formalin Fixed)

Heterogeneous DNA will be obtained from this source.

1. Collect one 10 μm tissue section from each paraffin block in a sterile 1.5 mL tube and deparaffinize as follows ***(25)*** (*see* **Note 7**).
2. Add 0.5 mL of xylene to the tube and vortex.
3. Incubate at room temperature for 5 min.
4. Centrifuge at 11,000*g* for 5 min.
5. Remove the supernatant and wash the pellet twice with ethanol.
6. Dry on a Speedy-vac sample concentrator.
7. Resuspend the pellet in 20 μL of sterile distilled water and boil for 10 min.
8. Proceed as in **Subheading 3.2.** (*see* **Note 8**).

3.1.4. Frozen or Fresh Tissue Samples

If it is not necessary to establish a culture prior to DNA extraction, the following procedure may be followed. The following steps should be done in a laminar flow cabinet in a class 3 biosafety facility.

1. Homogenize (grind) sample under liquid nitrogen in a mortar and pestle.
2. Add 6 vol of lysis buffer and homogenize with three strokes of a loose pestle in a Dounce glass homogenizer.
3. Treat as for sputum (*see* **Subheading 3.1.6.**).

3.1.5. Microscopy Stained Slide

1. Pipet 100 μL of PBS onto stained area of slide (*see* **Note 9**).
2. Scrape the material off with a scalpel and transfer to a 1.5 mL tube containing 900 μL of PBS and 50 μL of 10% Saponin.
3. Invert several times and incubate overnight at 4°C.
4. Centrifuge at 11,000*g* for 5 min, remove the supernatant and resuspend the pellet in 1 mL of PBS.
5. Incubate at 4°C for 2 h.
6. Centrifuge at 11,000*g* and discard the supernatant.
7. Add 50 μL of distilled water and 50 μL of 20% Chelex.
8. Incubate for 10 min at 95°C. Vortex every 2 min.
9. Spin for 5 min at 11,000*g* and recover the supernatant. Proceed as in **Subheading 3.2.** (*see* **Note 8**).

3.1.6. Sputum (see ***Note 8***)

1. Liquefy sputum by adding an equal volume of decontamination solution to a sputum sample in a sealable centrifuge tube (*see* **Note 10**).
2. Centrifuge at 11,000*g* for 10 min.
3. Aspirate supernatant (at this stage the pellet may be stored at –20°C for future use).
4. Heat for 10 min at 95°C (or 80°C for 1 h).
5. Resuspend the pellet in 100 μL of extraction buffer (*see* **Subheading 2.2.7.).**
6. Add 10 μL of 10 mg/mL lysozyme (*see* **Note 11**).
7. Incubate at 37°C for 2 h, with occasional mixing.

8. Add 10 μL of proteinase K buffer and 10 μL of proteinase K stock solution.
9. Incubate overnight (16 h) at 45°C.
10. Add an equal volume (150 μL) of phenol/chloroform/isoamylalcohol (25:24:1) and mix gently by inverting tube 6 times, ensuring that liquid remaining in the bottom of the tube is also mixed ("flick" with finger).
11. Centrifuge in a microfuge at 10,000*g* for 5 min.
12. Remove upper aqueous phase (approx 100 μL). Proceed to **Subheading 3.4.**

3.1.7. Bone Marrow or Whole Blood

1. Layer 1 mL of bone marrow aspirate or blood onto 3 mL of Histopaque 1077 in a conical tube and centrifuge at 400*g* for 30 min at room temperature.
2. Aspirate the upper layer to within 0.5 cm of the opaque interface containing mononuclear cells, and discard.
3. Pipet off the opaque interface into a conical centrifuge tube containing 5 mL PBS, taking care not to transfer any Histopaque. Centrifuge at 250*g* for 10 min.
4. Discard the supernatant and wash the pellet again with PBS.
5. Resuspend the pellet in 1 mL of lysis buffer.
6. Incubate at 65°C for 1 h.
7. Add 0.2 mL of 6 *M* NaC1 and mix.
8. Centrifuge at 2000*g* for 15 min.
9. Recover the supernatant and add 2 vol of cold ethanol. Incubate at –70°C for 30 min. Recover the DNA by "fishing out" or by centrifugation at 12,000*g* for 10 min.
10. Dry the DNA pellet on a Speedy-vac sample concentrator.
11. Redissolve the DNA in 30 μL of water or TE (*see* **Note 12**).

3.1.8. Infected Macrophages (in vitro)

1. Aspirate the culture medium from adherent cells in dish.
2. Add sufficient cold 10 m*M* EDTA pH 7.5 per well (for a 1 cm diameter well, use 110 μL) to just cover cells and incubate for 10 min.
3. Add 2 vol of PBS and 0.2 vol of 10% SDS and incubate at 4°C for 10 min.
4. Remove the suspension and proceed as in **Subheading 3.1.6.**, **step 4**, or **Subheading 3.1.1.**

3.2. Lysis of Bacterial Cells

This step is used primarily for cultures of *M. tuberculosis* (*see* **Subheading 3.1.2.**) and in this case yields high-quality homogeneous DNA. DNA from other starting material is also obtainable using this method, but may be contaminated with other DNA (e.g., from the host source) (*see* **Note 13**).

1. Add 400 μL of 50 mg/mL lysozyme stock and 10 μL of 10 mg/mL RNAase (*see* **Note 11**).
2. Incubate at 37°C for 2 h. Mix occasionally by gentle agitation.
3. Proceed to **Subheading 3.3.**

3.3. Removal of Protein and Cellular Contaminants

Cell lysates obtained by enzymatic, mechanical or other means of lysis or a crude preparation can be processed using this method. The volumes given are for 6 mL of cell suspension (*see* **Note 14**).

1. Add 0.1 vol (600 µL) of 10X proteinase K Buffer.
2. Add 150 µL of 10 mg/mL proteinase K.
3. Mix gently and incubate at 45°C for 16 h.
4. Add 5 mL of phenol/chloroform/isoamylalcohol and mix gently by inverting tube five times. Repeat the inversion steps four times over 30 min (*see* **Note 15**).
5. Centrifuge at 3000*g* at room temperature for 20 min.
6. Remove upper aqueous phase. If viscous, use a plastic pipet tip with narrow end cut off to enlarge aperture.
7. Add 5 mL of chloroform/isoamylalcohol, mix gently and centrifuge at 3000*g* at room temperature for 20 min.
8. Recover the upper aqueous phase and proceed to **Subheading 3.4.**

3.4. DNA Precipitation and Recovery

1. Add 0.1 vol (700 µL) of 3 *M* Na acetate, pH 5.5, to the supernatant (from **Subheading 3.3.**).
2. Add an equal volume of isopropanol.
3. Gently mix by inverting tube 2–4 times.
4. "Fish" the DNA out with a sealed tip Pasteur pipet (*see* **Note 16**).
5. Alternatively, incubate at –20°C for 30 min and centrifuge at 3000*g* for 30 min (*see* **Notes 17** and **18**).
6. Wash the pellet in 5 mL of 70% ethanol.
7. Air dry at 55°C until dry.
8. Dissolve the DNA pellet in 500 µL of TE. Incubate at 65°C until dissolved and store at –20°C (*see* **Note 12**).

3.5. Quantitation and Purity of Genomic DNA

3.5.1. Spectrophotometric Quantitation of DNA

1. Make a 1/50 dilution of an aliquot of the DNA stock in TE.
2. Read the absorbance at A_{260} and A_{280}.
3. Calculate the concentration of DNA using the formula (A_{260}) × (50 µg/mL) × (dilution fold) = concentration (µg/µL) of undiluted genomic DNA (*see* **Note 19**).
4. The A_{260}/A_{280} ratio should be 1.8 (*see* **Note 20**).

3.5.2. Electrophoretic Estimation of Quantity and Purity

1. Pipet approx 6 µg (estimated by spectrophotometry or other means) of DNA into a small tube.
2. Add 10 µL of 10X concentrate buffer appropriate for the restriction enzyme to be used, 3 µL of a 10 U/µL restriction enzyme stock and water to a final volume of 100 µL.

3. Incubate the mixture at the appropriate temperature (usually 37°C) for 3–16 h.
4. Electrophorese 1/10 of the sample (10 µL), as well as 100 ng of uncut DNA on a 1% agarose gel, using high molecular weight standards.
5. Stain the gel with ethidium bromide and visualize by UV transillumination (*see* **Note 21**).
6. Precipitate the cut DNA solution by adding 0.1 vol of 3 *M* sodium acetate, pH 5.5, and 3 vol of 100% ethanol at –20°C. Keep at –20°C overnight.
7. Spin at 10,000*g* and aspirate. Wash DNA with 70% ethanol. Dry and redissolve the DNA such that the final concentrations of cut DNA are all equal (*see* **Note 21**).

4. Notes

1. *M. tuberculosis* is a human pathogen, which requires extensive therapy to cure. Drug-resistant strains may be common and may be difficult, if not impossible to deal with once disease has progressed in an individual. Therefore all samples should be regarded as potentially hazardous and not be removed from a category 3 biosafety facility until inactivated. Work with live (or potentially live) organisms should be done only by properly trained persons, following appropriate safety guidelines.
2. Phenol and chloroform are corrosive, toxic and phenol may cause burns. Work should be done in an adequately ventilated environment (preferably fume hood) and appropriate safety measures taken (goggles, gloves, laboratory coat). The best quality phenol should always be used.
3. DNAse-free RNAse is best purchased commercially. Alternatively, it may be prepared by heating the enzyme to 100°C for 15 min ***(22)***.
4. Centrifugation of biological samples (e.g., CSF) will also pellet eukaryotic cells and result in a DNA preparation of mixed sources. This can be avoided by centrifugation over sucrose ***(26)***.
5. Centrifugation of samples containing live *M. tuberculosis* should be carried out in holders which seal with O-rings to minimize danger of creating aerosol suspensions.
6. Heat treatment at 80°C for 1 h will effectively kill the pathogen ***(18)***. Nevertheless, for added safety, subsequent steps (up to the phenol extraction step) should be carried out in a laminar flow cabinet. Extending the period of heating may lead to DNA degradation.
7. Decontamination of the microtome knife can be done with 70% ethanol after sectioning each block.
8. This preparation is not necessarily pure or homogeneous DNA, but is useful for direct PCR-based amplification.
9. The stained material (in the case of a ZN-slide) or microtome section cannot be scraped off while dry, as the material crumbles and shatters and is easily lost. The addition of PBS allows the scraped material to be easily recovered from the liquid.
10. A 10 µL aliquot of the liquefied sputum sample can be used directly for PCR analysis. The following method provides good reproducibility: overlay 0.5 mL of

the liquefied sputum sample onto 1 mL of sterile 50% (w/v) sucrose. Centrifuge at 12,000*g* for 5 min and wash the pellet in 100 μL of sterile saline, finally resuspend in 50 μL of saline. Use 10 μL of this suspension (with or without lysis of cells) directly for PCR amplification. This method may also be used for other samples, e.g., CSF, urine. The partial purification of the bacteria often improves results, as inhibitors of PCR are present in many fluids, whether of plant or animal origin. Prior to PCR-based analysis, lyse the bacteria by boiling and use the solution directly as the PCR substrate.

11. Lysozyme and RNAse A digestions must be done prior to addition of SDS and proteinase K.
12. If the DNA pellet fails to dissolve easily in TE or water, add a further volume and repeat 65°C heating step until dissolved.
13. Mechanical lysis results in sheared DNA which is not as useful as DNA prepared in this way.
14. Use tubes which seal to avoid spillage or generation of aerosols. Tubes should be polypropylene, which is resistant to the organic solvents used.
15. Hexadecyltrimethylammonium bromide (CTAB) treatment prior to the first phenol/chloroform extraction step can be carried out. This helps to remove polysaccharides which can copurify with DNA. Add 5 *M* NaCl or 3 *M* Na acetate, followed by addition of CTAB ***(20)*** to a final concentration of 10–12%. Mix and incubate for 10 min at 65°C. Proceed with extraction.
16. It is desirable to be able to recover a stringy precipitate with a rod or pipet tip, since this represents high molecular weight, good-quality DNA.
17. Isopropanol precipitation of DNA usually yields a large fluffy DNA conglomerate. If the cell number is low or for other reasons no conglomerate is seen, DNA may still be recovered by centrifugation and washing in 70% ethanol. If an oily residue is seen at these stages, discard prep and begin again. Usually, if the fluffy precipitate is not seen it is indicative of smaller fragments of DNA, which will give background problems if restriction fragment length polymorphism (RFLP) analysis or cloning of large fragments is planned.
18. DNA quantity is also dependent on the confluency of culture. Inadequate starting material will not yield sufficient DNA for "spooling" from alcohol precipitation.
19. This calculation is based on the approximation that 20 A_{260} U of DNA represents a concentration of 1 mg/mL. The dilution factor is 50, if 20 μL is pipeted into 1000 mL.
20. Pure double-stranded DNA has an A_{260}/A_{280} ratio of 1.8 ***(20,22)***. Ratios of 1.7 to 2.0 are acceptable, but indicate some degree of contamination, which may lead to inhibition of subsequent procedures. Further purification can be done by reextracting with phenol/chloroform/isoamylalcohol as described in **Subheading 3.3**.
21. Uncut DNA should show a band larger than 50kb. Diffuse material below this indicates sheared DNA. The digested DNA should be seen as a series of fragments over a broad size-range. There should be no DNA remaining in the intact genomic DNA zone. Quanititation is estimated on the basis of comparable fluo-

rescence intensity, between samples or with an accurately measured standard. For RFLP or dot-blot analysis, the concentration of DNA is important and should be determined as described.

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