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We thank Mrs. Renata Knirshova for excellent technical assistance. This work was supported by Grant GA999135 from the Slovak Academy of Sciences. *Address correspondence to J. Kormanec, Institute of Molecular Biology, Slovak Academy of Sciences, Dubravská cesta 21, 842 51 Bratislava, Slovak Republic.*
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Received 25 April 1994; accepted 16 August 1994.

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A Rapid Protocol for DNA Extraction and Primer Annealing for PCR Sequencing

The sequencing of polymerase chain reaction (PCR) products is often achieved through lengthy and expensive procedures that produce single-stranded products. Such procedures include asymmetric PCR and the use of streptavidin-coated magnetic particles. Double-stranded sequencing is usually more rapid, but often requires the use of expensive systems for the extraction of DNA from an agarose gel (e.g., through the use of DNA-binding resin matrices). Such procedures are time-consuming and unsuitable for the extraction of very small DNA fragments.

We have used elements of existing protocols (1–3) to produce a modified double-stranded sequencing system that is extremely fast, reliable and cost-effective. In this procedure the denaturation and annealing of the sequencing primer is performed while extracting

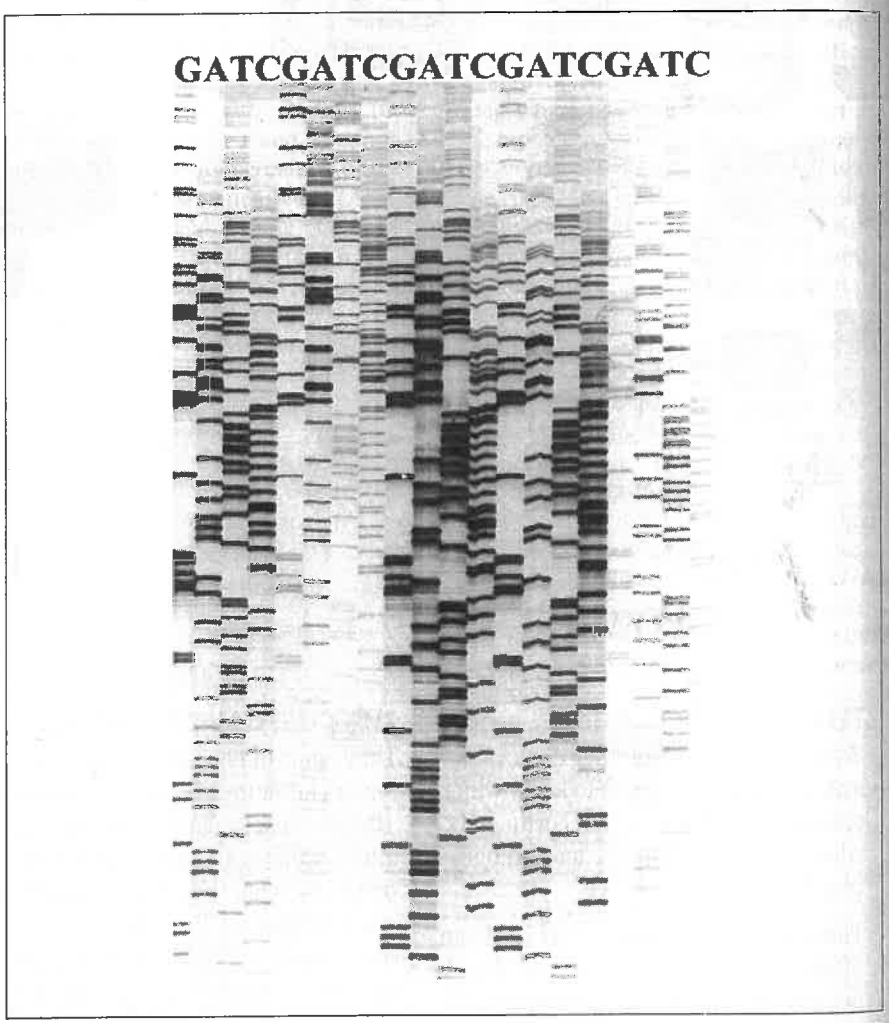

Figure 1. Sequencing gel from a 307-bp avian cytochrome b fragment. Shown is approximately 240 bp from 5 samples amplified from liver DNA and sequenced with the rapid protocol described (base ordering GATC). In this case, most samples were readable from primer to primer. Each sequence was generated from low-melt agarose gel cuts containing the products of a single 25-mL PCR amplification. DNA extraction and primer annealing were carried out as described in the text. Supernatant from the DNA extraction (7–12 mL) was added to 5.5 mL of a sequencing mixture containing 1.0 mL dithiothreitol (DTT), 0.4 mL 5× labeling mixture, 1.1 mL doubled-distilled H_2O, 1.0 mL Nonidet P-40, 0.25 mL ^{35}S-ATP, 0.5 mL Sequenase Mn buffer, 1.0 mL Sequenase dilution buffer and 0.25 mL Sequenase Version 2.0. It was then incubated at room temperature for 8 min. Termination reactions were carried out at 42°C for 8 min. The sequences shown were part of a 24-reaction sequencing run.

DNA from the agarose gel, thus speeding up the procedure.

The products of 25-µL or 50-µL PCRs are run on a 2% low melting point agarose gel in Tris-acetate (TA) buffer, the resulting bands excised and either stored at -20°C or used immediately. These agarose slices are placed in microcentrifuge tubes and 2 µL 5% Nonidet® P-40, 3 µL Sequenase® Version 2.0 buffer (United States Biochemical, Cleveland, OH, USA), 1 µL formamide and 1 µL 10 µM sequencing primer are added. The tubes are put in a boiling water bath for 5 min and then snap frozen in a -80°C absolute ethanol bath. The tubes are then centrifuged at 10 000 rpm for 10 min at a temperature of 5°C. Twelve microliters of the resulting supernatant are then used in a modified Sequenase chain termination reaction (Figure 1).

This procedure produces rapid results with various PCR products and has been invaluable for sequencing very small fragments generated during ancient DNA investigations. Continuous sequences of up to 300 bp are produced regularly using mtDNA primers in the cytochrome b and 12S RNA small subunit regions. Primers for a low copy number human repetitive element have also been used with success.

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Received 28 June 1994; accepted 3 August 1994.

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PCR-Based Detection of Mycobacteria in Sputum Samples Using a Simple and Reliable DNA Extraction Protocol

Detecting pathogenic mycobacteria in clinical samples by conventional methods, such as microscopy or culture, are either low in sensitivity and specifity or time-consuming. The polymerase chain reaction (PCR) has recently been investigated for detecting *Mycobacterium tuberculosis* and appears to have significant diagnostic potential (8). One of the remaining issues regarding the use of this technique is the extraction of DNA from clinical material prior to performing the PCR. Several procedures to release DNA from these acid-fast bacteria have been reported (2,3,5,6). Nevertheless, there is no extraction method available that is simple and reliable enough to allow its application in routine clinical practice (9).

Here we present a rapid freeze-thaw protocol for efficient extraction of mycobacterial nucleic acids as template molecules for a subsequent PCR. Sputum specimens were decontaminated by the standard *N*-acetyl-L-cysteine sodium hydroxide procedure, centrifuged at 10000× *g* for 5 min, and the pellet was subsequently suspended in 50 µL of extraction buffer (1% Triton® X-100, 0.5% Tween® 20, 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). In screw-capped microcentrifuge tubes, the turbid suspension was subjected to five cycles of 3-min freezing in liquid nitrogen and 1-min heating in a boiling water bath. After that treatment, the rigid and lipopolysaccharide-rich mycobacterial cell wall was disrupted and, following a short centrifugation step, the released genomic DNA from the supernatant could be reliably used for amplification. We found that freezing in liquid nitrogen is crucial for this procedure and this supports the hypothesis that lipid acids are cracked by a rapid temperature range of approximately 270°C.

The sensitivity of our procedure was determined by performing PCR on serial dilutions of *Mycobacterium avium* liquid culture with known concentration in mycobacteria-negative sputum. Using PCR primers according to Böddinghaus et al. (1) and following a standard 40-cycle PCR protocol, we were able to detect as few as ten organisms. In agreement with the results of Buck et al. (3), the use of acetone/dry-ice instead of liquid nitrogen throughout our DNA extraction protocol results in a remarkably lower efficiency since only 10^3 organisms were detectable this way.

Additionally, PCR was capable of

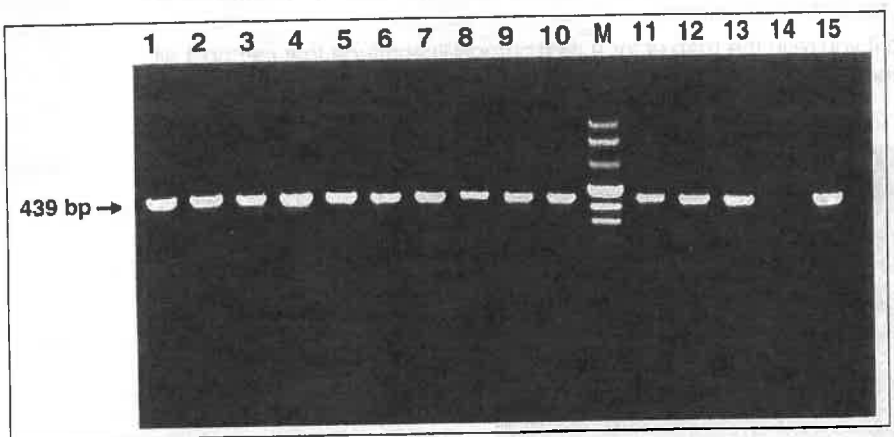

Figure 1. Analysis of PCR products after amplification of mycobacterial DNA prepared by the modified freeze-thaw protocol (1% agarose gel). Sputum specimens containing *M. tuberculosis* show the 439-bp band (lanes 1–13). Lane 14 represents a mycobacteria-negative sputum. Lane 15 represents a positive control, containing the amplified product from a pure culture of *M. tuberculosis*. Lane M contains a DNA marker (DNA molecular weight marker VIII, Boehringer Mannheim GmbH, Mannheim, FRG).