Direct single stranded sequencing from agarose of polymerase chain reaction products

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The polymerase chain reaction (PCR) has made possible the rapid isolation and amplification of specific DNA segments, which can then be used for a wide range of applications (1). Direct sequencing of PCR products has been described, using assymetric PCR, which requires purification of the PCR product, or inclusion of 10% DMSO in the sequencing reaction (2, 3, 4). However, these methods of sequencing have depended on the production of a pure uncontaminated DNA fragment. Direct sequencing from low melting point agarose has been successfully performed, but still requires a double stranded template (5). Single stranded DNA for sequencing has also been produced from biotinylated PCR products. One PCR primer has a biotin moiety attached at the 5′ end, and the resultant PCR product can be bound to solid phase using streptavidin-biotin bonding to hold DNA onto streptavidin coated magnetic beads (6). Schofield *et al.* modified this method, avoiding the need for prior disruption of the template cells, and incorporating radio-labelled deoxynucleotides, rather than end-labelling the sequencing primer (7). We have further developed this method to be applied to direct sequencing of multiple biotinylated PCR products, without the need for cloning, using agarose gel purification of the amplified DNA. Heating simultaneously melts the agarose gel slice and denatures the DNA and the single biotinylated strand of DNA is captured on solid phase for sequencing.

The template DNA was transferred from glycerol stocks into PCR reaction mix using a disposable toothpick. The reaction mix consisted of 50 μl, containing 1 μM forward and reverse universal M13 primers, 200 μM deoxynucleotides, 4 units of Taq polymerase (USB), 50 mM Tris-HCl pH 8.3, 0.01% gelatin, 1.5 mM KCl, and 1.5 mM $MgCl_2$. The forward M13 primer had been previously biotinylated. The reaction mix was overlaid with 50 μl of light mineral oil (Sigma). PCR reactions were performed in a programmable thermal cycler (Techne PHC-1). The reaction conditions consisted of initial denaturing at 95°C for 2 minutes, then 35 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute, with a final polymerisation step of 72°C for 5 minutes. The PCR reaction mix was loaded on a 1% low melting point agarose (BRL) gel, containing 0.1 μg/ml ethidium bromide, and run in TAE buffer at 40 volts for 2 hours (see Fig. 1). The band containing the insert was cut out, melted at 80°C for 5 minutes in a heating block (Grant BT1), in the presence of 3×10^6 streptavidin coated magnetic beads (Dynal), and treated according to the revised solid phase biotin method of Jones *et al.* (8). The reaction products were run on a 6% polyacrylamide/7 M urea gel, which was then fixed for 15 minutes in a 5% methanol/5% acetic acid solution, vacuum dried, and exposed directly to X-ray film for 16 hours at room temperature (see Fig. 2).

This method enables multiple PCR products from the same reaction to be individually sequenced, without the need for isolation, extraction and cloning of each band. The problem of optimal annealing temperature for the sequencing reaction in low melting point agarose, as raised by Casanova *et al.* (4), is

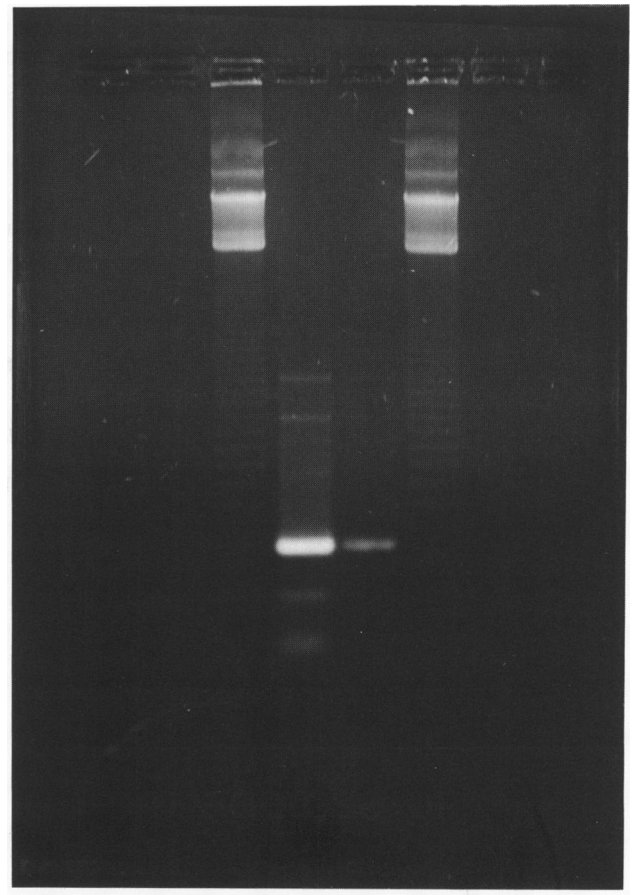

Figure 1. 1% agarose gel containing 1 μg/ml ethidium bromide, fluorescent under UV light, demonstrating multiple PCR products and 123 base pair DNA ladders in lanes 1 and 4. The template was a 254 base pair clone containing satellite repetitive DNA derived from a human genomic library constructed from HpaII digested placental genomic DNA.

Figure 2. Autoradiograph of the sequencing reaction of the extracted 254 base pair band. The gel was loaded in the order ACGT.

overcome by the production of a single-stranded sequencing template. This method should be of great value in the rapid sequence analysis of genomic PCR products and of multiple PCR products derived from degenerate primers.

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