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Convenient single-step, one tube purification of PCR products for direct sequencing

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Structural alterations of the apolipoprotein E (apo E) are known to influence the lipid metabolism. The three most common isoproteins (E2: Cys^{112}, Cys^{158}; E3: Cys^{112}, Arg^{158}; E4: Arg^{112}, Arg^{158}) are encoded by three co-dominant alleles ($\epsilon 2$, $\epsilon 3$, $\epsilon 4$). Phenotyping is cumbersome and, therefore, several methods for apo E genotyping have been developed. Of these, PCR-RFLP (restriction fragment length polymorphism) is currently most rapid and cost-effective (1). To detect mutations not affecting restriction sites, direct PCR sequencing of apo E fragments is the method of choice. This, however, requires purification of PCR products, which is routinely accomplished by techniques like gel electrophoresis or chromatography.

We have established a new purification method using exonuclease I (exo I) and shrimp alkaline phosphatase (sAP) to degrade excess primers and nucleotides, which are the main factors interfering with dideoxy PCR sequencing. This procedure simply requires the addition of the enzymes to the PCR product, avoiding frequent sample handling and DNA loss through technical manipulation.

Human DNA was extracted from 50 µl EDTA blood with Tris–EDTA lysis and proteinase K digestion. The PCR reaction mix (50 µl) was optimized to contain 10 µl of DNA extract, 30 pmol of the primers F4 and F6 (2), 5 µl of dimethyl sulfoxide (DMSO), 10 pmol of each dNTP, 2 U of *Taq* polymerase (AmpliTaq® , Perkin-Elmer, Norwalk, Connecticut, USA), and 5 µl of 10× PCR buffer (GeneAMP; Perkin-Elmer). After initial denaturation (95°C 5 min) 35 cycles (95°C 20 sec, 60°C 30 sec, 72°C 30 sec) of amplification were performed, followed by a final extension (72°C 10 min; DNA Thermal Cycler 480, Perkin-Elmer). 2 µl of PCR product (244 bp) were mixed with 1 U exo I and 1 U sAP (USB, Cleveland, USA) and incubated for 1 h at 37°C. The enzymes were inactivated for 15 min at 72°C. The purified PCR product was diluted to a final volume of 10 µl for cycle sequencing (3) with 0.5 pmol biotinylated primer F6 (Δ Taq™ Cycle Sequencing Kit; USB) under the same conditions as for PCR of genomic DNA. For chemiluminescence detection (4) we used the 'Sequenase® Images™ non-isotopic DNA sequence detection kit' (USB). Sequencing data obtained with sAP/exo I purified DNA are at least equal to those after column chromatography and superior to those using unpurified templates (Figure 1). Adjusting the purified DNA solutions for dilution we saw a slightly better efficiency of cycle sequencing in the enzymatically purified DNA.

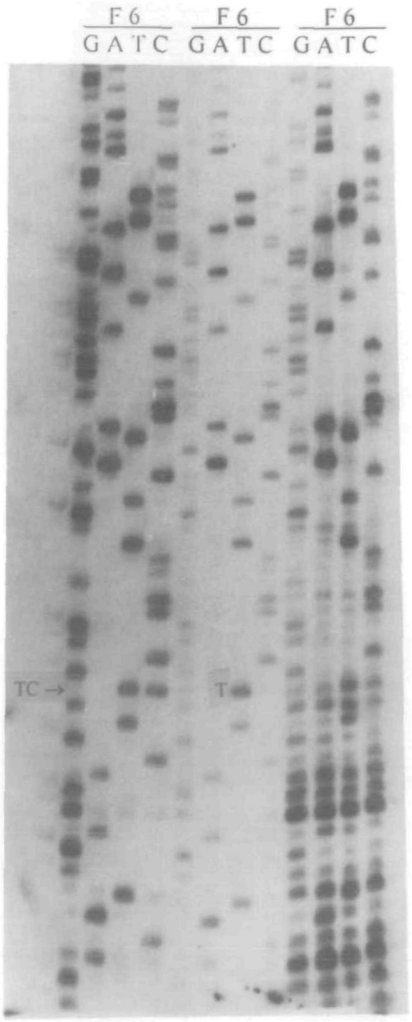

Figure 1. Sequence analysis of the receptor binding region of the apolipoprotein E gene using primer F6 (sense orientation). The effect of purification of 2 µl PCR reaction by sAP/exo I treatment (left 4 lanes) and of 50 µl of PCR reaction by column chromatography (QIAquick-spin PCR purification kit; Qiagen, Chatsworth, CA; middle 4 lanes) as compared to no purification (right 4 lanes) is shown. An equivalent of 2 µl of the PCR reaction was used as sequencing template in all cases. Mutation sites are indicated.

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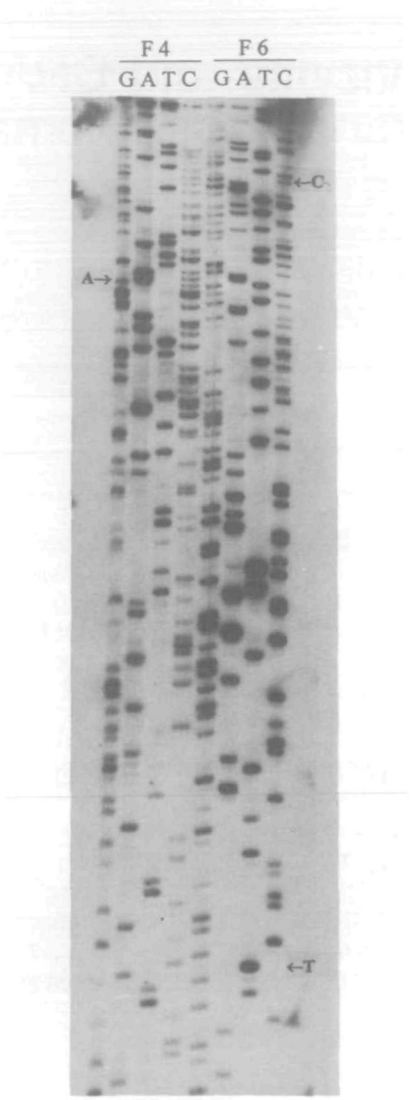

Figure 2. Sequence analysis of the 244 bp PCR product in the receptor binding region of the apolipoprotein E gene (ε3/ε3 genotype; GC content 70%). Primers F6 (sense orientation) and F4 (anti-sense orientation) were used and sAP/exo I purification was performed prior to non-isotopic cycle sequencing. Mutation sites are indicated.

Using our protocol, the purification of PCR products for direct non-isotopic sequencing can be reproducibly achieved with simple pipetting steps (Figure 2). This minimizes sample handling, risk of cross-contamination and amount of DNA template required. Our technique has turned out to be reliable, convenient and cost-effective. It has a broad applicability in the growing field of mutational analysis by PCR sequencing and should furthermore serve to facilitate its automation.

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