Detection of Human Apolipoprotein E3, E2, and E4 Genotypes by an Allele-Specific Oligonucleotide-Primed Polymerase Chain Reaction Assay: Development and Validation

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A polymerase chain reaction (PCR) assay has been developed and validated by using allele-specific oligonucleotide (ASO) primers to specifically amplify E3, E2, and E4 polymorphic sequences of the human apolipoprotein E (apo E) genes. Degenerate ASOs containing one or two additional 3′ mismatches provided greater specificity than did ASOs containing a single mid-sequence or 3′ allele-specific mismatch with plasmid pEB4 or genomic DNA as template. Optimal specificity and efficiency of amplification did not correlate with primer annealing conditions, whether determined theoretically or via oligo-melting experiments. Pre-cycling denaturation times and high cycling denaturation temperatures were also required for optimal amplification, presumably because of the high G:C content (75–85%) of apo E gene sequences. Conditions permissive for amplification and discrimination with plasmid DNA did not transpose favorably to amplification from human genomic DNA from peripheral blood leukocytes; the latter required nested primer reactions. These data may be valuable in predicting PCR assay conditions for other G:C-rich sequences containing polymorphic sequence differences. The assay described is both more accurate and rapid (24 h) than previously described methods for phenotyping or genotyping human apo E from blood specimens.

Additional Keyphrases: PCR efficiency of plasmid DNA and human leukocyte DNA compared • phenotyping

The human apolipoprotein (apo) E gene located on chromosome 19 is highly polymorphic, with three alleles (designated e2, e3, and e4) encoding the main isoforms apo E2, E3, and E4 (I, 2).6 These isoforms represent amino acid substitutions at residues 112 (E3 Arg → E4 Cys) and 158 (E3 Cys → E2 Arg), corresponding to single-base changes of T to C or vice versa at the relevant position in the gene coding sequence (3). Several other isoforms have also been reported (4–8), principally E2x (E3 Cys → E2x Arg at residue 145) and E2

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6 Nonstandard abbreviations: apo, apolipoprotein; LDL, low-density lipoprotein; ASO, allele-specific oligonucleotide; PCR, polymerase chain reaction; and Tm, melting temperature.
146 (E3 Lys → E2 Gln at residue 146), although these are considered to be expressed at low frequencies. These sequence changes affect cholesterol concentrations in plasma, such that the apo E polymorphism is one of the most important genetic determinants of low-density lipoprotein (LDL) cholesterol concentrations (9–14). Homozygosity for the e2 allele has also been demonstrated to predispose individuals to familial type III hyperlipidemia (15) resulting from defective binding of apo E2 to the LDL receptor. Most studies that indicate such a role for apo E isoforms are based on phenotypic assignments (9–16).

Methods for apo E phenotyping from plasma by isoelectric focusing and immunoprophing with anti-apo E antisera (17) may be difficult to interpret and time consuming. Furthermore, phenotypes do not necessarily correlate with genotypes because changes in pi are similar for many genotypic variants (reviewed in ref. 11) and post-translational modifications of apo E alter the pi, leading to misassignment of the phenotype (2). Genotypes have been directly determined by using Southern blot (oligo-melting) assays with allele-specific oligonucleotides (ASOs) to probe human genomic DNA, with or without prior amplification by the PCR (18–22). More recently, Kontula et al. (23) demonstrated that accurate genotyping can be achieved by examination of Hha I restriction products generated from PCR-amplified DNA. However, all these assays require analysis times longer than three days and (or) the use of radiolabeled probes. Thus, although they are capable of accurate genotype determination, they are less useful for epidemiologic and routine clinical laboratory application.

To facilitate the investigation of the role of apo E isoforms in lipid regulation, we have investigated PCR as a means of rapidly detecting apo E alleles by using ASOs as one of the PCR primers (24–26). This has revealed important factors governing the efficiency and specificity of the PCR, especially given the high G:C content of the target sequences. We describe the development of the assay and its validation for apo E genotyping of human genomic DNA isolated from peripheral blood leukocytes.

Materials and Methods

Oligonucleotides. Oligonucleotides were synthesized by Dr. J. E. Fox (Alta Bioscience, Department of Biochemistry, University of Birmingham), using standard phosphoramidate chemistries and a BT8503 synthesizer (Biotech Instruments Ltd., Luton, U.K.). Sequences of oligonucleotides used as PCR primers are given in Table 1.

Table 1. Oligonucleotide Primer Sequences Used for PCR Amplification of Human Apo E Gene

<table>
<thead>
<tr>
<th>Common primers 5′ → 3′</th>
<th>PCR1</th>
<th>AAGGAGTTGAGGCGCTACAAT</th>
<th>PCR2</th>
<th>TCGCGGCCCCCTGCGTACA</th>
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<tr>
<td>R90</td>
<td>GAGGAACTACGTCCTCACGTCGAGG</td>
<td>R91</td>
<td>GCTGCCCTCTCCCTAATCCGGCGCGCAC</td>
<td></td>
</tr>
<tr>
<td>R82</td>
<td>GCGGAGTGACAGGCGATCTGCGCAC</td>
<td>GAGGAG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>R192</td>
<td>CACTGCCAGGCT(T/C)CCTCGCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ASO primers (mid-sequence mismatches)</th>
<th>3 2 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>112E3</td>
<td>TACTGACCGACGGCTACGCA</td>
</tr>
<tr>
<td>112E4</td>
<td>TACTGACCGACGGCTACGCA</td>
</tr>
<tr>
<td>145E3</td>
<td>TCGCGGAGGAGCCGATACG</td>
</tr>
<tr>
<td>145E2x</td>
<td>TCGCGGAGGAGCCGATACG</td>
</tr>
<tr>
<td>158E3</td>
<td>GCTGAGTACATCGGATACG</td>
</tr>
<tr>
<td>158E2</td>
<td>GCTGAGTACATCGGATACG</td>
</tr>
</tbody>
</table>

* ASOs contain one, two, or three 3′ mismatches as indicated by arabic numerals.

Plasmid and human genomic DNA. Plasmid pEB4 containing the human apo E3 gene sequence (27) was a kind gift from Dr. S. E. Humphries (Charling Cross Sunley Research Centre, London, U.K.). pEB4 DNA was prepared by standard procedures according to Mantias et al. (28).

Samples of human genomic DNA for which the apo E genotype had been determined by direct nucleotide sequencing (29) or Southern hybridization (18–22) were kind gifts from Drs. S. E. Humphries, R. R. Frants (Leiden, The Netherlands), and A.-C. Svanén (Orion Corp., Helsinki, Finland). Further samples of human genomic DNA were extracted by standard methods (28) from peripheral blood obtained from patients attending the Lipid Clinic at East Birmingham Hospital. Essentially, we mixed 0.75 mL of EDTA-treated blood with an equal volume of sterile phosphate-buffered saline, pH 7.4 (per liter, 120 mmol of NaCl, 2.7 mmol of KCl, and 10 mmol of sodium phosphate), and centrifuged this in an Eppendorf tube for 15 min at 4000 × g. The pellet was resuspended in 0.4 mL of lysis buffer (per liter, 10 mmol of Tris - HCl, pH 8.1, 1 g of sodium dodecyl sulfate, and 0.1 mmol of EDTA) containing 100 μg of protease K (Anglian Biotech) and incubated at 55 °C for 2 h. We then centrifuged the samples at 16 000 × g for 20 min and extracted the DNA from the supernates with phenol/chloroform (1/1 by vol).

Polymerase chain reaction. All amplifications were performed with a PHCII thermal cycler (Techne, Princeton, NJ), essentially according to Saiki et al. (30). Precise reaction conditions are described in the Figure legends and Results. However, the following conditions were typical: we denatured plasmid pEB4 DNA (0.575 ng) or human genomic DNA (1–10 μg) in 50 μL of reaction buffer [per liter, 100 mmol of Tris - HCl, pH 8.3, 500 μmol of KCl, 15 mmol of MgCl2, 0.1 g of gelatin, and 200 mmol each of dATP, dCTP, dGTP, dTTP (Pharma-cia/LKB, Bromma, Sweden) and 1 μmol of forward and reverse oligonucleotide primers]. After this denaturation, we added 2.5 units of Taq polymerase (AmpliTaq;
Perkin-Elmer Cetus, Norwalk, CT), overlaid the sample with 40 μL of light mineral oil (Sigma Chemical Co., Poole, U.K.), and subjected it to as many as 40 heating cycles, each comprising a denaturation (96 °C), annealing (variable), and extension step (72 °C).

Gel electrophoresis. DNA fragments and PCR reaction products were separated by electrophoresis in 1% agarose or 10% polyacrylamide gels by standard methods (22). Bands were made visible by ultraviolet illumination of ethidium bromide-stained gels.

Restriction digestions. We incubated 5 μL of PCR reaction products with restriction endonucleases (Northumbrian Biologicals Ltd., Northumbria, U.K.) *Pst* I, 10 units, or *Sac* II, 16 units, at 37 °C for 1 h and analyzed the fragments by polyacrylamide gel electrophoresis against a 123-bp ladder marker (Bio-Rad, Richmond, CA).

Results

PCR with Plasmid DNA Templates

We investigated several primer combinations for their ability to amplify the polymorphic region of the human apo E3 gene contained in plasmid pEB4 (see Figure 1 and Table 1). Optimal amplification required a pre-cycling denaturation step of 98 °C for 20–30 min before the addition of 2.5 units of Taq polymerase, and 25–40 cycles of 96 °C for 2 min, 65 °C for 1 min, and 72 °C for 2 min. It was also possible to amplify by using two temperature-cycling conditions (96 °C for 1 min and 65 °C for 2 min) (data not shown). Digestion of the correct-sized PCR products generated from pEB4 DNA with the restriction endonucleases *Pst* I and *Sac* II confirmed the gene specificity of amplified products (see Figure 2).

Allele-specific amplification could not be achieved under any conditions (e.g., various combinations of reactant concentrations, cycling parameters) when we used reverse primer in combination with ASOs containing an internal (mid-sequence) mismatch. Gene-specific, but not allele-specific, amplification of pEB4 DNA could also be achieved by using reverse primer R90 with forward ASO primers having the allele-specific mismatch at the 3' terminus of the oligonucleotide (Figure 3, Tracks 1–6). However, introduction of degeneracy by inclusion of a second or third non-ASO-specific mismatch into the 3' portion of the ASOs allowed discrimination and detection after 30 temperature cycles (see Figure 3, Tracks 7–12). The efficiency and specificity of the assay depended on the annealing temperature and the primer combinations used, such that a range of annealing temperatures and mismatches gave specific amplification. We decided to develop an assay requiring a single annealing temperature for all ASOs, judging this to be most suitable for routine clinical application, especially given that the majority of thermal cyclers contain a single temperature-controlled block. We then applied this strategy to the analysis of human genomic DNA samples.

PCR with Human Genomic DNA Templates

Initially, when we used human peripheral leukocyte-derived genomic DNA, none of the primer combinations and cycling conditions generated easily visible products after agarose gel electrophoresis of 10–30 μL of the reaction products and ethidium bromide staining/ultraviolet illumination (data not shown). This did not reflect contamination of the DNA with PCR inhibitors [e.g., porphyrins (31)] or inaccuracies in the amounts of DNA.

![Fig. 1. Schematic representation of positions of PCR primers for amplification of plasmid pEB4 and human genomic DNA. Restriction sites and expected product sizes for enzymes *Pst* I and *Sac* II digestion of PCR products are also indicated.](image)

![Fig. 2. Polyacrylamide gel electrophoresis separation of PCR products generated by using plasmid pEB4 DNA and primer R90 with each single mismatch ASO (Tracks 1, 3, 5, 7, 9, 11) and after digestion with *Pst* I (Tracks 2 and 4) or *Sac* II (Tracks 6, 8, 10, 12). Tracks 1–4 represent results with ASOs for 112 E3/E4, Tracks 5–8, ASOs 145 E3/E2c, and Tracks 9–12, ASOs 158 E3/E2c.](image)

![Fig. 3. Agarose gel electrophoresis separation of PCR products generated by amplification of pEB4 DNA after 30 cycles with primer R90 and ASOs containing one (Tracks 1–6) or two (Tracks 7–12) 3'-end mismatches.](image)
used because further purification of the DNA by using Sephadex G-50 (32) or by re-extracting with phenol/chloroform, or use of 60 ng–3 µg of target DNA in the reactions had no significant impact on efficiency.

We therefore used a nested primer approach (29) (Figure 1b), making a first-round amplification with flanking primers PCRE1 and PCRE2 (23, 29). Second-round amplification reactions with the same primers did not generate substantial amounts of specific products. First-round products, 5 µL diluted 100- to 10 000-fold, gave positive results after application of the ASO forward primers [mid and 3’ mismatch(es)] with use of reverse primer R90 in the second-round amplifications. Optimal specific amplification could be achieved by using the primers and conditions listed in Table 2. Selection of mixed ASOs containing either one, two, or three 3’ mismatches to give a single matched melting temperature (T_m) did not improve the specificity or efficiency of the amplifications.

Validation of the Assay

To fully validate the specificity of the assay, we analyzed human genomic DNA samples (representing E3, E2, and E4 homo- and heterozygotes), genotyped by direct sequencing (29) or Southern hybridization (8, 19) after PCR amplification. As demonstrated in Figure 4, in all circumstances, the genotypes determined by all three methods correlated exactly. An E2 sample containing a mutation at residue 146 (Lys → Gln), was identified as E3 on the basis of this assay (data not shown). The E2x assay has not been adequately validated because no such independently genotyped DNA was available; however, the assay was able to detect an E2x sequence in some of the human peripheral blood DNA samples (data not shown).

Discussion

Assay Development

Amplification of DNA (or mRNA) sequences by the PCR has been used as the basis of many strategies for direct sequencing, cloning, and discrimination between gene sequences containing point, insertional, or deletional mutations (4, 6, 30, 33). The potential of PCR in clinical laboratories has been hampered in part by difficulties in predicting the primers and conditions required to specifically amplify a given gene sequence. Several reports have suggested general rules dictating the efficiency and specificity of various PCR-based assays (34–37); however, the limited range of analyte sequences studied cannot guarantee which factors, if any, can be applied generally. The development of a diagnostically applicable PCR-based assay for detection of point-mutational differences therefore remains largely a matter of trial and error for any given target sequence.

Given its central role in lipid metabolism, the role of apo E isoforms in the pathophysiology of dyslipidemias has drawn investigators’ attention. However, most current information is based on phenotypic analyses, which lack discriminative power and reliability. Because phenotype and genotype do not necessarily correlate (owing to multiple mutations that yield a single pl value or to biochemical modifications of LDL before or during assay), data based on apo E phenotype may mask important factors determining apo E pathophysiology. The ability to accurately determine human apo E genotypes has been demonstrated by use of the PCR and (or) oligo-melting (18), Southern hybridization (19–22), Hha I restriction digestion patterns (23), and polynucleotide sequencing (29). Although the accuracy and reliability of these techniques cannot be questioned, they are technically complex, time-consuming procedures and generally involve the use of radioactive labels.

In developing a PCR-based method that utilizes ASO primers for the specific amplification and detection of three common allelic isoforms of human apo E, namely, apo E3 (wildtype), E4, and E2, we have gained several insights into the predictability of the conditions required for efficient and specific detection of allelic point mutations.

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Table 2. Conditions for ASO-PCR Assay to Detect Apo E3, E4, E2, and E2x Alleles in Human Genomic DNA

<table>
<thead>
<tr>
<th>Condition</th>
<th>1 µg of genomic DNA</th>
<th>5 µL of 1000-fold dilution of</th>
<th>Pre-cycling denaturation</th>
<th>Cycling conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary amplification</strong></td>
<td>PCRE1 &amp; PCRE2</td>
<td>R90 &amp; ASOs (two 3’ mismatches)</td>
<td>98 °C, 30 min</td>
<td>30 cycles of:</td>
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<td></td>
<td></td>
<td>99 °C, 2 min;</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>62 °C, 90 s;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>72 °C, 2 min</td>
</tr>
<tr>
<td><strong>Secondary amplification</strong></td>
<td></td>
<td></td>
<td>98 °C, 20 min</td>
<td>25 cycles of:</td>
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<td></td>
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<td>99 °C, 2 min;</td>
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<td></td>
<td></td>
<td>65 °C, 1 min;</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>72 °C, 2 min</td>
</tr>
</tbody>
</table>

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Fig. 4. Agarose gel electrophoresis separation of PCR products generated by using previously genotyped human genomic DNA samples, under the conditions given in Table 2
Initial experiments with plasmid pEB4 DNA containing a human apo E3 sequence insert (27) demonstrated that long pre-cycling denaturation times and high temperatures (96°C for >15 min) were required for optimal amplification. This agrees with previous reports that demonstrated decreases in both efficiency and specificity at denaturation temperatures <96°C (19, 20). However, when human genomic DNA was used as template, optimal amplification required a pre-cycling step of 98°C for 20-30 min. Even under these conditions, and using a range of in-house-designed or previously reported oligonucleotide primer pairs (see Table 1), we were unable to generate a visibly detectable product after electrophoresis of the reaction mixture. To generate products in sufficient quantity for detection in ethidium bromide-stained agarose gel electrophoresis requires a two-step amplification procedure with nested primers, in accord with Svävän et al. (29) and Kontula et al. (23). This finding was unaffected by changes in any other factor (e.g., target DNA quantity or purity, primer concentration, amount of Taq polymerase added, or buffer composition), despite suggestions in previous reports (35-37), probably because of the overall G:C-rich nature of the chromosomal region containing the apo E alleles, which adopts tertiary-folding patterns (e.g., loops and hairpins) and high-energy inter-chain bonding. Including deaza-guanine, as much as 100 µmol/L, in the reaction mixtures (38) also had a small effect but was insufficient to warrant the increased cost per tube. Thus we conclude that establishing an assay with plasmid DNA templates will not necessarily be applicable to detection of the genomic sequence, especially within G:C-rich regions.

Establishing the system for amplification of this region with the ability to discriminate between allelic point mutations requires a consideration of the variables controlling the PCR kinetics. Several methods have been described involving plasmid or genomic models, based on the ability to specifically hybridize ASOs (33, 37, 39, 40). However, the position, number, and type of mismatches required to give optimal discrimination also vary. Few general rules may apply, and there is no certain predictive model. Thus, for optimal discrimination, degenerate ASOs containing one or two additional 3’ mismatches were far superior to midsequence mismatches. The nature of the most effective mismatches could not have been predicted for apo E. For annealing temperatures, the general rule is that matched \( T_m \) values but unequal mismatches would be optimal; however, this approach to apo E produced the most inconsistent assay (data not shown). Rychlik et al. (35) suggested that optimal amplification and discrimination should be achieved by using annealing temperatures matching that of the lowest-\( T_m \) primer, or by using incremental increases in annealing temperature during cycling. Their suggestions do not apply to apo E. We found that the optimal annealing temperature was up to 5°C more than that of the greatest primer \( T_m \).

Varying the amount of template DNA, Taq polymerase (from 1 to 5 units per reaction), primer, Mg\(^{2+}\), or dNTP in the reaction buffer had no significant effects on the efficiency of amplifications. Increasing the original target template DNA (genomic) to >10 µg did, however, produce an inhibitory effect, with some genomic DNA samples probably reflecting slight contamination of DNA with porphyrins and so forth (31, 32).

Thus, the most important variables in determining optimal apo E allele amplification conditions were temperature and duration of the denaturation step before the addition of Taq, position of the mismatch(es) within the ASO primers, and optimal annealing temperatures (determined by the \( T_m \) of the ASOs and the end-point format of the assay).

Assay Validation

Application of this assay to human genomic DNA from phenotyped individuals revealed that the phenotype did not necessarily correlate with genotype results (manuscript in preparation). The perfect correlation between ASO-PCR genotypic assignments and assignments based on either sequencing or Southern hybridization validates the specificity of the present assay. However, these conditions may require further alteration for direct application in other laboratories. Several reports suggest that several factors, e.g., variable heating efficiencies and characteristics of different thermal cyclers, can influence PCR assay performances. Although a sample containing a mutation at residue 146 (Lys → Gln) was not detected by the assay, it should be relatively simple to design further primers for detection of this and other allelic forms (4-8). Application of such systems will allow determination of the pathophysiological importance of allelic variants of human apo E and their association with other disease states, e.g., myotonic dystrophy (41).

The need for two rounds of PCR amplification is undesirable but single-amplification strategies have been unsuccessful. However, a two-round amplification format does facilitate repeated analyses should there be such a need, e.g., for retrospective analyses and epidemiological studies involving multiple factor analysis.

In conclusion, we have described an assay for genotyping human DNA for apo E that has significant advantages over current methods. It is more rapid, taking 24 h instead of 72+ h for direct sequencing and Southern hybridization, and it does not require the use of radiolabeled markers. In its present form, the assay can be used to re-evaluate the data on gene frequencies of apo E3, E2, and E4 alleles and on the contribution of genotypes and phenotypic modifications to the pathophysiology of dyslipidemias. The rapidity and relative simplicity of the assay may also be improved in several ways (34, 39-43) that should also facilitate epidemiologic studies.

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References