methods

Rapid determination of apolipoprotein E genotype using a heteroduplex generator

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Abstract The apoE gene exhibits two common polymorphisms that have been associated with both coronary artery disease and Alzheimer's disease. The polymorphisms create the three allelic isoforms E2, E3, and E4 which are encoded by Cys–Cys, Cys–Arg, and Arg–Arg at amino acid positions 112 and 158, respectively. Numerous methods have been described to identify these three apoE alleles although there are disadvantages and ambiguities associated with all of them. Here we describe a method by which the two common apoE polymorphisms can be identified simultaneously. The method involves PCR of the region containing the two polymorphic sites, followed by hybridization of this PCR product to a synthetic molecule called a universal heteroduplex generator (UHG). The UHG is used to induce heteroduplex formation which is visualized on a non-denaturing mini-gel using ethidium bromide staining. This technique which can also identify other rare mutations in the amplified region of DNA under investigation, is an unequivocal method of genotyping and is simpler and faster than many methods, including using restriction enzyme digestion.— Bolla, M. K., N. Wood, and S. E. Humphries. **Rapid determination of apolipoprotein E genotype using a heteroduplex generator. *J. Lipid Res.* 1999.** 40: **2340–2345.**

Supplementary key words apoE • coronary artery disease • Alzheimer's disease • heteroduplex analysis • universal heteroduplex generator

The apolipoprotein, apoE, is now known to be involved in key processes in plasma lipid metabolism (1–11) and is a major risk factor in Alzheimer's disease (AD) (12–16). In the general population, apoE is present in three common isoforms, E2, E3, and E4 (6, 7). These are the result of the substitution Cys and Arg at amino acid residues 112 and 158 resulting from a single base substitution of a T to a C at each of these two corresponding sites in the gene (8). This genetic variation plays a key role in regulating apoE-mediated pathways. Both E2 and E4 are associated with differences in plasma lipid levels and hence play a role in the development of coronary artery disease (17, 18). The E4 allele has been implicated as a major risk factor for Alzheimer's disease (12–15), whereas the E2 appears to have a protective effect against this disease (16). Hence, accurate and rapid methods for apoE genotyping are in ever-increasing demand by research and diagnostic institutions (reviewed in ref. 19).

Numerous different methods have already been established for determining the three apoE isoforms. Originally this was carried out at the protein level by isoelectric focusing (IEF) (20), and more recently at the DNA level by methods such as amplification refractory mutation system (ARMS) (21), single strand conformation polymorphism (SSCP) (22), allele-specific oligonucleotide probes (ASO) (23), and restriction enzyme analysis (24). Numerous improvements to these methods have been described (25–30), but all of these have disadvantages. The method used here is based on the design of a synthetic DNA molecule called a "universal heteroduplex generator" (UHG), which is an amplifiable copy of the target sequence containing strategic sequence modifications in close proximity to the point of mutation (31–35). When a UHG is mixed, denatured and reannealed with the amplicon of interest, DNA heteroduplexes form which are subject to far greater electrophoretic retardation than homoduplexes and in-trans heteroduplexes. Heteroduplexes generated by this cross-hybridization give specific banding patterns on native polyacrylamide gels. Given these considerations this technique was assessed as an improved method of apoE genotyping.

MATERIALS AND METHODS

Preparation of human genomic DNA

Genomic DNA was isolated from 5 ml potassium EDTA anticoagulated whole blood using a salting out method (36) and dissolved in 1 ml of TE buffer (pH 7.6). An aliquot of the stock DNA

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TABLE 1. Sequence of the four oligonucleotides ligated together to form the UHG molecule

	Sequence (5′ to 3′)
Oligonucleotide 1	GAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGG GCGCGGACATGGAGGACGTGT
Oligonucleotide 2	CTCCTCGGTGCTCTGGCCGAGCATGGCCTGCACCTCGCCGCGGTACTGCACCAGGCGGCCGCTTTTAC ACGTCCTC CATGTCCGCGCC
Oligonucleotide 3	CACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGAC CTGCAGAAGC
Oligonucleotide 4	GGATGGCGCTGAGGCCGCGCTCGGCGCCCTCGCGGGCCCCGGCCTGGTACACTGCCAGGCTTTTGCTTCTGCAGGT CATC

The underlined bases indicate the eight inserted bases.

template was diluted 1 in 20 into a 96-deep-well array (1.2 ml deep-well plate, Advanced Biotechnologies, Surrey, UK). A working replica of this array was made, from which 2.5 μl (approx. 25 ng of DNA) was used to set up PCRs.

Synthesis of UHG

The UHG molecule was constructed by the synthesis, fusion, and subsequent amplification of four overlapping oligonucleotides (**Table 1**) using the cycling conditions: 94°C for 1 min, 57°C for 1 min, 72°C for 1 min (30 cycles). The amplification resulted in a 303 bp construct. The crude product was visualized on a 2% agarose gel. Pooled UHG amplicons from six reactions were electrophoresed on a preparative polyacrylamide mini gel (10% Protogel, National Diagnostics, Hull, UK). The UHG band was visualized by SYBR green (Molecular Probes, Leiden, Netherlands) staining and excised. The DNA was eluted from the gel fragments using a Bio-Rad Mini Protean II Model 422 electro-eluter (Bio-Rad, Hemel Hempstead, UK) and precipitated using a standard ethanol/ammonium acetate procedure. The pellets were washed in 70% ethanol, dried, and redissolved in 200 μl of sterile distilled water. The UHG was serially diluted (five dilutions: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}). The optimum concentration for amplification of generator for heteroduplex analysis was determined to be the 10^{-5} dilution.

PCR amplification of genomic DNA and the UHG

For amplication of the genomic DNA, 2.5 μl of each DNA template was pipetted from the deep well array into a sterile 96-well omniplate (Hybaid, Middlesex, UK) using a multi-channel pipette (Finnpipette; Life Sciences, Basingstoke, UK). The DNA template was dried on a PCR block for 10 min at 75°C. To each dried DNA template, a 20 μl PCR reaction mix was added. Each reaction contained: 2 μl 10 X polmix (500 mmol/L KCl, 100 mmol/L Tris, pH8.3, 0.1 g/L gelatin, 2 mmol/L of each dNTP), 0.6 μl 50 mm $MgCl_2$, 1 μl dimethyl sulfoxide (DMSO; BDH, Leicestershire, UK), 0.08 μl of each PCR primer FH49 and FH50 at 100 pmol/μl each (FH49: 5′-GAACAACTGACCCCGGTGGCGG; FH50: 5′-GGATGGCGCTGAGGCCGCGCTC; Gibco, Paisley, UK), 16.12 μl sterile distilled water and 0.12 μl Taq DNA polymerase (5 U/ml; Gibco, Paisley, UK). Each 20 μl PCR reaction mix was overlaid with 20 μl paraffin oil (BDH, Leicestershire, UK). The plate was sealed with a microplate sealer (cat. no. 676001, Greiner, Gloucestershire, UK) and centrifuged for 1 min at 1600 *g* (Sorvall T60000B; DuPont, Newtown, CT). Additional UHG was amplified using 1 μl of the 10^{-5} dilution of the UHG using the exact PCR reacton cocktail described above. The UHG and genomic DNA were amplified separately using the MJ Tetrad peltier thermal cycler (GRI, Essex, UK) using the following cycling conditions: 95°C for 10 min, 95°C for 1 min, 72°C for 3 min (5 cycles), 95°C for 1 min, 55°C for 1 min, 72°C for 1 min (40 cycles). Amplification of the genomic DNA results in a PCR product of size 295 bp, whilst amplification of the UHG results in a product of size 303 bp due to the insertion of the eight adenine bases.

Preparation of polyacrylamide mini gels

Two sets of glass plates (20 cm × 18.5 cm; Cambridge Electrophoresis, Cambridge, UK) were used to pour two polyacrylamide gels, each of 1.35 cm thickness. For each pair of gels, 100 ml of a 10% polyacrylamide was made using the following: 33.4 ml 30% acrylamide (0.8% w/v acrylamide; 37.5:1 acrylamide:N,N′-methylenebisacrylamide; Protogel, National Diagnostics, Hull, UK), 20 ml 10 × Tris-borate-EDTA buffer (TBE) pH8.3, and 46.6 ml distilled water. To polymerize each gel (50 ml), 100 μl TEMED (NNN′,N′-tetramethylethylenediamine, BDH, Leicestershire, UK) and 300 μl 10% ammonium persulphate (APS; BDH, Leicestershire, UK) were added and the mix was poured between a set of two plates. A comb with 24 teeth (each tooth 5 mm wide) was inserted along the top edge of the gel and the gel allowed to set for about 30 min. Once the gels had set, the combs were removed and both sets of plates were clamped onto each side of the vertical gel electrophoresis apparatus (Cambridge Electrophoresis, Cambridge, UK) ready for loading.

Generation of heteroduplexes and heteroduplex analysis

A total of 48 samples (half of a microtitre plate) were analyzed at any one time. For each sample, 4.0 μl loading dye (cat. no. G-7654 Sigma, Dorset, UK), 10 μl amplified UHG, and 15 μl amplified genomic DNA were mixed together in a clean microtitre plate, sealed with a microplate sealer (cat. no. 676001, Greiner, Gloucestershire, UK), and denatured on a PCR block at 94°C for 10 min. The samples were allowed to cool slowly in the block for a further 15 min to allow renaturation and formation of the heteroduplexes. For each denatured sample, all of the sample (about 25 μl) was loaded onto the mini gel. Once all 24 samples had been loaded onto each of the two mini gels, the gels were electrophoresed in 1 × TBE buffer (pH8.3) at 130 V for 20 h in an air-conditioned room at a temperature of 19°C. Alternatively, the gels were electrophoresed at 300 V for 3.5 h with the use of a cooling system (Multi-temp III thermostatic circulator, Pharmacia Biotech, Herts, UK). Once electrophoresis was complete, the gels were removed, post-stained for 20 min in 1 × TBE containing 0.1% ethidium bromide, and then visualized under the UV transilluminator. A digital image of the gels was obtained with a charged couple device (CCD) camera and frame grabber (UV products, Cambridge, UK).

ApoE genotyping by restriction fragment length polymorphism (RFLP)

Samples were amplified using primers and conditions as described previously (30). Digestion with HhaI (New England Biolabs, Herts, UK) was carried out using 4 units enzyme per sample and fragments were separated on 7.5% acrylamide microtitre array gel electrophoresis (MADGE) gels as described previously (29).

Sequencing

DNA from the rare variant was amplified for the apoE gene as described before and sequenced using the Applied Biosystems

PRISM BigDye Terminator Cycle Sequencing Ready Reaction kit. The sample was loaded onto a 5% denaturing gel on an ABI377 DNA Sequencer (Perkin Elmer, Cheshire, UK).

RESULTS

The UHG had a sequence identical to the genomic region covering the polymorphic area but with the insertion of four adenine bases adjacent and downstream of the polymorphic sites coding for amino acids 112 and 158 respectively (**Fig. 1**). Following hybridization of the UHG with the amplified apoE fragment from subjects of known genotype, the apoE-UHG was able to generate characteristic and discriminatory DNA heteroduplexes for all six apoE genotypes. **Figure 2A** shows the band patterns obtained from the analysis of apoE using the heteroduplex method. Each apoE genotype was readily recognizable and there is no ambiguity between them. Analysis of the apparent size of the heteroduplexes showed that they were running at around 1 kb (not shown), which is approximately 3–4 times the size of the homoduplexes.

The reliability of this method was examined and found to be better than that using restriction enzyme analysis. This was done by analyzing one 96-well microtitre plate using both the heteroduplex and the restriction enzyme method and 100% concordance was found between the two techniques. However, there were less unreadable and unambiguous results using the heteroduplex technique compared with the restriction enzyme method. Out of 96 samples analyzed by both methods, 6 were unreadable using the restriction enzyme method whilst there were no unreadable samples using the heteroduplex method. There was no need to re-check any samples typed using heteroduplex analysis whilst several of those samples typed using HhaI had to be re-analyzed mainly due to poor amplification resulting in faint bands.

Whilst carrying out apoE genotyping using heteroduplex analysis a variant (Fig. 2B, lane 16) was found. This individual has a E4/E4 genotype but the additional bands suggested a further genetic change. ABI sequencing revealed a point mutation (C to A) which converts glutamine (CAG) to lysine (AAG) at amino acid position 156.

DISCUSSION

Heteroduplex analysis has been shown to be a simple and rapid method for the identification of mutations (31–35, 37, 38), involving the amplification of the mutated and normal genomic DNA followed by non-denaturing polyacrylamide gel electrophoresis. Heteroduplexes, which are formed between the complementary DNA strands of two alleles, have a reduced mobility compared to DNA homoduplexes when subjected to gel electrophoresis and numerous methods have been described where the heteroduplexes naturally show different band patterns upon electrophoresis (37–39). The method used here is an adaptation of this approach.

The construction and design of the UHG is dependent on the sequence and mutation in the region of DNA being investigated. Generally, UHGs contain 'controlled' nucleotide substitutions, deletions or insertions at nucleotide positions adjacent to known mutations sites. The introduction of these 'identifiers', and the subsequent induction of heteroduplex formation with the PCR product containing the mutation, results in an increased degree of mismatch between the heteroduplex strands which is responsible for the greatly reduced mobility of heteroduplexes on a non-denaturing polyacrylamide gel (33, 35). These heteroduplexes tend to migrate at an apparent molecular weight 3- to 4-fold greater than the actual molecular weight of the amplicon. The UHG is most effective when the 3′ terminii of the primers are at least 30 bases away from the mutation and its identifier. From previous optimization studies (40) the most informative modifica-

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ApoE WT    GAACAACTGACCCCGGTGGCGGAGGAGACGCGGGCACGGCTGTCCAAGGAGCTGCAGGCGGCGCAGGCCCGGCTGG
UHG        ----------------------------------------------------------------------------
ApoE mut   ----------------------------------------------------------------------------

ApoE WT    GCGCGGACATGGAGGACGTGT    GCGGCCGCCTGGTGCAGTACCGCGGCGAGGTGCAGGCCATGCTCGGCCAGA
UHG        --------------------AAAA----------------------------------------------------
ApoE mut   --------------------C    ----------------------------------------------------

ApoE WT    GCACCGAGGAGCTGCGGGTGCGCCTCGCCTCCCACCTGCGCAAGCTGCGTAAGCGGCTCCTCCGCGATGCCGATGA
UHG        ----------------------------------------------------------------------------
ApoE mut   ----------------------------------------------------------------------------

ApoE WT    CCTGCAGAAGC    GCCTGGCAGTGTACCAGGCCGGGGCCCGCGAGGGCGCCGAGCGCGGCCTCAGCGCCATCC
UHG        -----------AAAA------------------------------------------------------------
ApoE mut   ----------T    ------------------------------------------------------------
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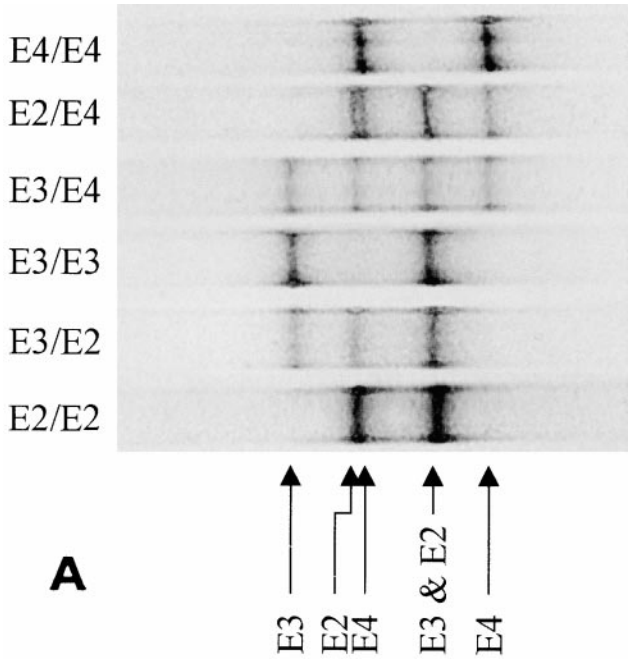
Fig. 1. Nucleotide sequence (5′ to3′) of part of the apoE gene including the 112 (T to C) and 158 (C to T) polymorphic sites and the corresponding sequence of the UHG. The UHG contains four base (adenine) insertions downstream of the 112 and 158 sites respectively. Dashes indicate nucleotide identity. The location of the primers (FH49 and FH50) used in the PCR are underlined.



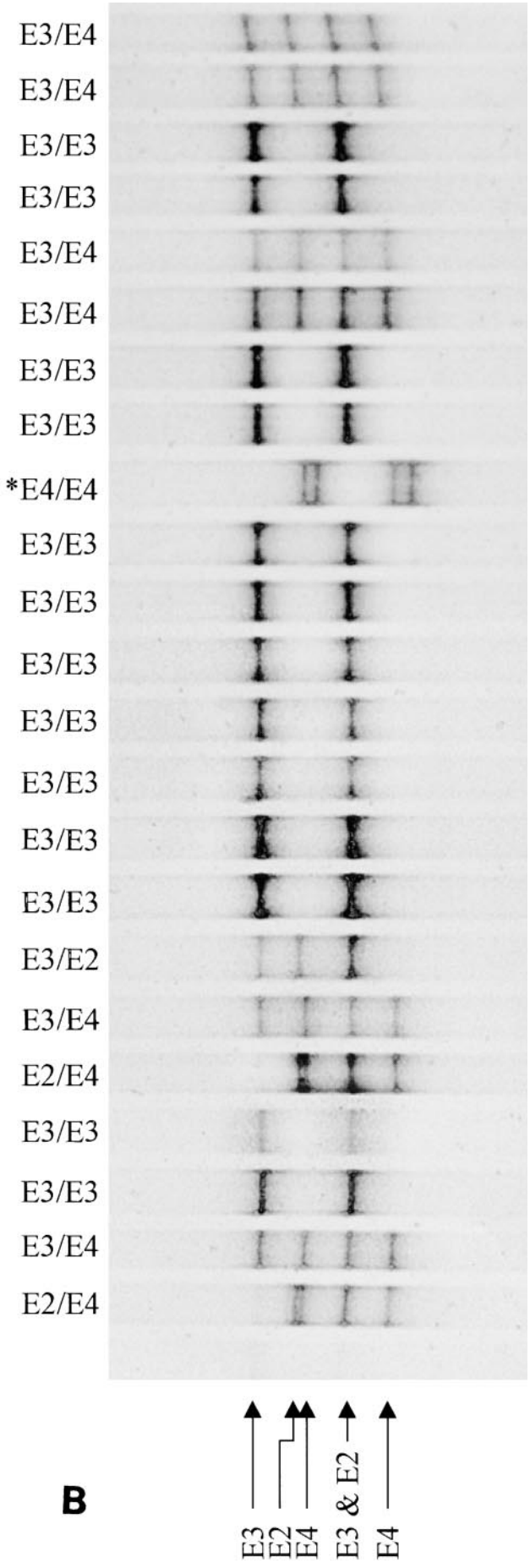


Fig. 2. ApoE genotyping by heteroduplex analysis using polyacrylamide minigel electrophoresis. Characteristic band patterns are observed for all six combinations of E2, E3, and E4 alleles. The heteroduplexes were found to run at apparent molecular weights of: E2 (1.3 and 1.5 kb), E3 (1.3 and 1.7 kb) and E4 (1.2 and 1.5 kb). (A) Gel showing 6 samples which had previously been apoE genotyped using the restriction enzyme digestion method. (B) Gel showing apoE genotyping of samples typed using the heteroduplex method only. * Lane 16 shows an E4/E4 genotype with an additional rare variant (Gln156Lys).

tion to the wild-type sequence is a four-base polyadenine insertion, downstream of the point of mutation. On the basis of these considerations, the UHG used here for the heteroduplex analysis of apoE had four adenine bases inserted downstream of the 112 site and four adenine bases dowstream of the 158 site.

Wood and Bidwell (40) have found certain conditions to enhance heteroduplex resolution and these were incorporated into the development of the running conditions used. The ionic concentration in the gel is critical, and the use of 2 × Tris-borate-EDTA (TBE) buffer in the gel with the usual 1 × TBE as the running buffer has been found to improve resolution of closely or co-migratory bands. The degree of cross linkage, ratio of acrylamide to bisacrylamide in the gel is critical. A cross-linking of 2.6% as in Protogel has been shown to give the optimum resolution between the heteroduplex bands (40). Total acrylamide concentration required is a function of the fragment length being resolved, 80 bp–120 bp: 15%–20%; 150 bp–200 bp: 12%–15%; greater than 200 bp: 10%–12%. Finally, minimization of the heating of the gel during electrophoresis is critical, as excess heating of the gel degrades the resolution of the heteroduplexes. Gel thickness is another factor critical to the resolution and visualization; thin gels (about 1 mm seems to be optimum), which dissipate heat more efficiently and give rise to less "fluorescence bleed," and sharper gel bands, which aid the interpretation of results.

All previously described methods of determining the three apoE isoforms have drawbacks. For isoelectric focusing (IEF), the requirement for plasma or sera limits the method and there is the possibility of artifacts caused by prolonged storage of sera (41). Misclassification of apoE phenotypes can be caused by post-translational modifications of the apoE protein (42–45) and by interference from serum amyloid A or apoAI (46). The major advantage of apoE genotyping over phenotyping is the fact that DNA is stable when stored at −80°C. Several methods for apoE genotyping have been described (21–24), but all these methods have limitations and disadvantages for large scale screening in population studies. For example, although ASO (23) is a sensitive method, it is time consuming and requires the labeling of the oligonucleotide probe with radioisotopes (47). SSCP involves the use of radioisotopes or cumbersome silver staining for the detection of band patterns (22), which are also very sensitive to the conditions under which SSCP has been carried out (48). The ARMS method (21) involves no labeling with radioactivity, and gels can easily be stained with ethidium bromide, but for each sample four PCR reactions have to be performed. The current method of choice for apoE genotyping has been by the digestion of a PCR product with the restriction enzyme HhaI (24). We previously described a high throughput method (29) based on this restriction enzyme analysis but occasionally experienced difficulty in the discrimination between the E2/E2 and E3/E2 genotype, as there is only a subtle difference between the banding pattern resulting from the two different genotypes. Typing was also found to be difficult with samples which have from amplified only weakly. In such cases, heterozygotes (such as an E3/E4 individual) can be difficult to identify because the distinguishing fragment is small, and in a "weak" PCR can be missed. Partial digestion also makes it difficult to distinguish between genotypes such as E2/E2 and E3/E2.

There are several advantages of the UHG method described here. The denaturing–annealing step is quicker (20 min) compared to the restriction enzyme digestion (several hours), and UHG avoids the problems associated with partial digestion. Both methods require the separation of fragments on a gel and have similar constraints involved with loading and visualization, but, in our experience UHG patterns are easier to interpretate. As the patterns seen are due to the presence of 2, 3, and 4 bands of equal intensity in each lane, there is no risk of misgenotyping due to one band being below the level of detection, as with PCR and HhaI digestion. Genotyping is therefore either unequivocal or "no result." Our results suggest heteroduplex analysis to be more robust and reliable than restriction enzyme analysis, especially for large scale screening in population-based studies. Both require PCR amplification, but once the UHG has been synthesized, there is an infinite supply of it. When more is required, all that is necessary is to re-amplify it in large volumes and then store this at −20°C. An extra advantage of this method is the possibility of identifying rare mutations during routine analysis. The variant detected here, Gln156Lys, has not been reported previously, and a more detailed description of the subject and the effect of the variant on plasma lipid levels will be presented elsewhere.

Thus, although apoE genotyping by HhaI restriction enzyme digestion is the current method of choice for high throughput screening, the technique described here using heteroduplex analysis is a simpler, more rapid, and more reliable method of apoE genotyping, particularly with sub-optimal samples.

We thank Dr. Robert Kong at the Middlesex Hospital, London, for the samples, and Renate Hamvas at the Institute of Child Health, London, for technical advice. This work was supported by BHF Grants RG95007 and SG98003. The UHG used for this work is patented to Bristol University, British Patent No. 2280266.

Manuscript received 28 April 1999 and in revised form 15 September 1999.

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