High-Throughput Method for Determination of Apolipoprotein E Genotypes with Use of Restriction Digestion Analysis by Microplate Array Diagonal Gel Electrophoresis

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Molecular epidemiological research has identified the association of a common apolipoprotein E (apo E) isoform (E4 as opposed to E3), with risk both of coronary artery disease and of Alzheimer dementia. In addition, the role of apo E genotype (usually E2/E2) in Type III hyperlipidemia is well known. However, both for diagnostic and research purposes, apo E genotyping is cumbersome. The preferred approach is electrophoretic sizing of restriction digestion fragments, enabling simultaneous analysis of the two codons (112 and 158) that represent the six common genotypes (E2/E2; E2/E3; E2/E4; E3/E3; E3/E4; E4/E4). However, the consequent demands of high-yield PCR, high-resolution, high-throughput electrophoresis, and sufficient detection sensitivity have left shortfalls in published protocols. In conjunction with a high-throughput electrophoresis system we described recently, microplate array diagonal gel electrophoresis (MADGE), we have constructed extensively optimized, simplified protocols for DNA isolation from mouthwash samples for PCR setup and high-yield PCR, for restriction digestion, and for subsequent MADGE gel image analysis. The integral system enables one worker to readily undertake apo E genotyping of as many as hundreds of DNA samples per day, without special equipment.

Indexing Terms: polymerase chain reaction/coronary artery disease/Alzheimer dementia/gel image analysis

Apolipoprotein E (apo E) is an important component in lipoprotein metabolism (reviewed in 1), acting in intermediate-density lipoprotein as a ligand for the low-density lipoprotein (LDL) receptor.⁴ It is also expressed in the nervous system (reviewed in 2) in astrocytes and is induced 250–350-fold at sites of nerve injury (3). In the general population, polymorphism of the protein is expressed with three common alleles, E2, E3, and E4 (4, 5). At amino acids 112 and 158, the alleles encode Cys-Cys (6), Cys-Arg, and Arg-Arg, respectively. The frequency of the E2/E2 genotype is ~1% and, in conjunction with secondary triggers, a proportion (2–5%) of E2/E2 individuals develop Type III hyperlipidemia with the accompanying risk of peripheral and coronary arterial disease (1, 7). Strong evidence exists that the presence of Cys rather than Arg impairs binding to the LDL receptor, leading to slow clearance of intermediate-density lipoprotein from plasma (8). The frequency of E4 alleles is 10–15% but, compared with E3 alleles, E4 is overrepresented in frequency in groups with coronary artery disease (9) and in those with Alzheimer dementia (10). In neither instance is the molecular basis of the genetic epidemiological observation fully understood, but differential effects on cholesterol concentrations (11) and differential interaction with β-amyloid (12) are known to occur. At present, the identification of E2/E2 genotype is of diagnostic value in the context of classification of Type III hyperlipidemia (7), whereas identification of E3 and E4 alleles does not currently alter therapeutic choices. However, the study of associations of E3 and E4 alleles with many different diseases and traits can be expected to be undertaken in the near future (13–15), research into the functional differences between the protein isoforms continues, and there will be a search for ways to modulate the effects of apo E4 (16). Thus, a demand exists for apo E genotype or phenotype analysis in large numbers of individuals for either research or diagnostic purposes.

Apo E phenotyping can be undertaken by isoelectric focusing (4). Determination of phenotype has been compared with determination of genotype; i.e., a few phenotyping discrepancies may occur (17, 18). Apo E genotyping can be undertaken by combining the polymerase chain reaction (PCR) with a subsequent analytical procedure, e.g., oligonucleotide binding (19), amplification refractory mutation detection system PCR and electrophoresis (20), or restriction digestion and electrophoresis (21). A range of other rarer genotypes can also be identified by restriction digestion (22). The preferred approach is electrophoretic sizing of restriction digestion fragments, which allows simultaneous analysis of the two codons (112 and 158) representing the six common genotypes (E2/E2; E2/E3; E2/E4; E3/E3; E3/E4; E4/E4), and numerous improvements to this approach have been presented (23, 24). However, the consequent demands of high-yield PCR, high-resolution high-throughput electrophoresis, and sufficient detection sensitivity have not been adequately met in published protocols and, in terms of speed and overall cost, phenotyping is still more efficient (25). Apo E gene PCR is particularly prone to low yield and misproducts, possibly because of high % (G+C) nucleotide content (26), and because most pro-

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Nonstandard abbreviations: LDL, low-density lipoprotein; apo E, apolipoprotein E; MADGE, microplate array diagonal gel electrophoresis; TBE, Tris-borate-EDTA; BSA, bovine serum albumin; CCD, charge-coupled device; and DMSO, dimethyl sulfoxide.

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tocals have used highly purified DNA from blood (see, e.g., 27). The procedure for purification from blood is itself relatively laborious and precludes large-scale implementation in the clinical or research laboratory.

Electrophoresis of DNA has been performed traditionally either in agarose or acrylamide gel matrix (28). Much effort has been directed to improved-quality agaroses capable of high resolution (29), but for small fragments, such as those from PCR and post-PCR digests, polyacrylamide still offers the highest resolution. Although agarose gels can easily be prepared in an open-faced format to gain the conveniences of horizontal electrophoresis, including the use of arrays of wells, acrylamide does not polymerize in the presence of air, and the usual configurations for gel preparation lead to electrophoresis in the vertical dimension. We recently described (30–33) a very simple device and method for preparing and manipulating horizontal polyacrylamide gels. To accommodate the practice that many procedures are undertaken in standard 96-well microplates, we have also designed a device that preserves the exact configuration of the 8 × 12 well array and enables electrophoresis in tracks along a 71.6° diagonal between wells (microplate array diagonal gel electrophoresis; MADGE) with the use of either polyacrylamide or agarose. This procedure eliminates almost all of the staff time taken in setup, loading, and record-keeping, and it offers high resolution for pattern recognition in genotyping. The nature and size of the gels allow direct stacking of gels in one tank, so that a tank used typically to analyze 30–60 samples can readily be used to analyze 1000–2000 samples.

Here we have addressed each of the problematic aspects of apo E genotyping in conjunction with MADGE, and we have constructed an integral system for large-scale apo E genotyping, which we estimate is one to two orders of magnitude cheaper and faster than previous approaches. This system is currently in use in our laboratory for genetic epidemiological research, but it would also be applicable in the clinical laboratory for identifying apo E2/E2 genotype.

Materials and Methods

General

In our experience, many apparently minor variables (which do not affect many other PCRs) affect the success of apo E gene PCR. Reoptimization may be necessary if the reaction is to be carried out in a different type of PCR equipment or if different sources of reagents or template DNA prepared by a different method is to be used, even within the same laboratory. For the current work, we used the equipment and reagents listed below.

Equipment. Deep 96-well plates (Beckman, High Wycombe, UK), 96-well Omniplates (Hybaid, Teddington, UK), and loose-fitting lids (Falcon, Becton Dickinson, Oxford, UK); 0.5–10-μL eight-channel multipipette (Finnpipette; Life Sciences, Basingstoke, UK); 100-μL repeat pipette (Biohit; Alpha Labs., Eastleigh, UK); centrifuge (Sorvall T60000B; duPont, Newtown, CT); Omnimune PCR machine (Hybaid); polystyrene block (10 × 7.5 cm); Incubator (Sanyo-Gallenkamp, Leicester, UK); horizontal gel electrophoresis tank (11.5 × 21 cm); 75 g/L MADGE gels (genetiX, Wimborne Minster, UK); ultraviolet transilluminator, charge-coupled device (CCD) camera, Imagestore 5000 frame grabber for digital images, and video copy processor (UV Products, Cambridge, UK).

Reagents. 10× “polmix” [500 mmol/L KCl, 100 mmol/L Tris (pH 8.3), 0.1 g/L gelatin, 2 mmol/L of each dNTP]; 15 mmol/L MgCl2; dimethyl sulfoxide (DMSO); BDH, Lutterworth, Leicestershire, UK); PCR primers (Genosys, Cambridge, UK), 100 μmol/L each:

“FH49,” 5’-GAACAACTGACCCGTGGCGG

“FH50,” 5’-GAATGCCGCTGAGGCGCGCTC

Taq polymerase (5 kU/L, cat. no. 18038-026; Gibco-BRL, Renfrew, UK); paraffin oil; HhaI (20 000 kU/L; New England Biolabs, Hitchin, UK); NE buffer 4 (10×; 500 mmol/L potassium acetate, 200 mmol/L Tris-acetate, 100 mmol/L magnesium acetate, 10 mmol/L diethylthreitol, pH 7.9 at 25 °C; New England Biolabs); bovine serum albumin (BSA, 10 g/L; New England Biolabs); 10× Tris–borate–EDTA (TBE), pH 8.3; formamide loading buffer [980 mL/L ionized formamide, 10 mmol/L EDTA (pH 8.0), 0.25 mL/L xylene cyanole FF, and 0.25 mL/L bromophenol blue]; 1-kb molecular-mass marker ladder (Gibco-BRL); and ethidium bromide (10 g/L in water).

DNA Templates and Pre-PCR Set-Up

Blood DNA. We isolated genomic DNA from potassium EDTA-anticoagulated whole blood (either fresh or frozen at −20 °C until use), using the following serial steps, as previously described (27): cellular lysis with a sucrose buffer, nuclear lysis including sodium dodecyl sulfate, overnight protein digestion by proteinase K, salt precipitation of residual debris, and ethanol precipitation of DNA.

We redissolved template DNA stock to ~0.16 g/L in Tris-EDTA, and placed the aliquots in Beckman plates with 96 deep wells. We set up dilutions in water to ~25.6 mg/mL in a replica array, from which we drew 2.5-μL aliquots (containing 40 ng of DNA) to set up either 10- or 20-μL PCRs. DNA samples for this work were anonymous donations for research studies approved by local ethical committees.

Mouthwash DNA. We gave patients a 20-mL universal tube containing 10 mL of isotonic saline (NaCl 9 g/L) and asked them to draw the liquid into their mouths, swirl it around for ~30 s, and spit it back into the tube. Sample transit of 1–3 days at room temperature and (or) storage at ~20 °C until use gave acceptable results for PCR template DNA. Briefly, we vortex-mixed mouthwashes before removal of 1 mL into an Eppendorf tube, centrifugation at ~10 000g for 2 min, and removal of the supernate. The pellet was then resuspended in 400 μL of NaCl-EDTA (10 mmol/L
each), vortex-mixed, and centrifuged for 2 min more. The supernate was again removed, and the resulting pellet was fully resuspended in 100 μL of 20 mmol/L NaOH and heated to 95 °C for 15–20 min. The preparation was pulse-centrifuged to pellet any cell debris before removal of a 2.5-μL aliquot for use either neat or diluted with water as template for PCR. A 1:10 dilution was made of each DNA sample and pipetted into a deep-well Beckman plate, leaving a few of the 96 wells empty (for positive/negative controls). This array was used to transfer 2.5 μL of each DNA into its corresponding coordinate of a 96-well Omniplate, to the very base of the well. Into the empty wells on the plate we pipetted 2.5 μL of DNA from reference genotypes or 2.5 μL of water for negative controls. The DNAs (~40 ng of DNA in each well) were then allowed to dry, and the dried DNA array was stored at room temperature (for as long as 3 months) until required for PCR.

PCR Conditions

A total volume of 1000 μL of PCR mix, sufficient for one 96-well Omniplate (96 × 10 μL reactions), contained: 100 μL of 10× polmix; 100 μL of 15 mmol/L MgCl₂; 50 μL of DMSO; 40 μL of oligonucleotide pairs (FH49/50 at 20 μmol/L each); 704 μL of sterile distilled water; and 6 μL (30 U) of Taq polymerase stock. We pipetted 10 μL of this PCR mix into each of the 96 wells and overlaid this with an equal volume of paraffin oil. We covered the plate with a lid and centrifuged for 1 min at 1600g in preparation for thermal cycling.

The following cycling conditions were used: 95 °C for 10 min; 95 °C for 1 min, 72 °C for 3 min (5 cycles); and 95 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min (30 cycles).

Restriction Digests

We then digested the amplified DNA with 4 U of HhaI according to the procedure illustrated below. For one 96-well plate, we prepared 260 μL of digest mix as follows: sterile distilled water, 91 μL; 10× NE buffer 4, 130 μL; 100× BSA (10 g/L), 13 μL; HhaI (20 MU/L), 26 μL. We then placed aliquots of the digest mix into a column of eight wells on a clean 96-well plate and, using an eight-channel multipipette, pipetted 2.0 μL of the mix into each well of a clean plate. To each well we transferred 80 μL of PCR product from its identical coordinate on the PCR plate. The plate was centrifuged at 1600g for 1 min and the contents of each well were digested in an incubator at 37 °C for at least 1 h.

MADGE Imaging and Analysis

After digestion, 5.0 μL of each sample was added to 2.0 μL of formamide dye mix (formamide, 0.5 mol/L EDTA, and 0.25 g/L each of bromphenol blue and xylene cyanole), and 5.0 μL of each mix was loaded into the wells of a 7.5 g/L MADGE gel (prestained in a solution of 10 μL ethidium bromide in 100 mL of 1× TBE for 10 min) in an electrophoresis tank containing 1× TBE. MADGE gels were prepared as described previously or were the gift of genetiX. Briefly, the gels are supported on glass and contain 96 wells (2 mm³) for sample loading in an 8 × 12 array, with the long axis of the array at an angle of 71.6° relative to the electrodes and with the edges of the wells parallel and perpendicular to the line of electrophoresis. A 1-kb ladder was also loaded into one well of the gel. Electrophoresis was at 10 V/cm at room temperature for 45 min, after which time we observed the gel with the use of an ultraviolet transilluminator and acquired a digital image of the gel with a CCD camera and frame grabber.

Results

Sample Preparation

PCR. We tried a wide variety of conditions. To ensure a high yield of PCR product, the precise conditions were critical. In particular, the inclusion of DMSO was essential, as was the relatively high concentration of Taq polymerase used. In the interest of economy, minimizing the PCR volume (10 μL) and increasing the Taq polymerase concentration was much more effective than obtaining a larger volume of lower-concentration PCR product that would be amenable to concentration but would accordingly introduce an additional laborious step.

Figure 1 shows apo E genotyping MADGE of HhaI digests of apo E gene PCR products; a map of the PCR product and HhaI sites shows the sizes of the expected fragments for different alleles. All six apo E genotypes are recognizable from the observed band pattern. Each apo E genotype pattern can readily be recognized by eye (see schematic and closeup gel image, Fig. 1) and the format of the 96-well array is preserved for convenience of procedures. Read by eye, the presence of a closely spaced doublet of 91 and 83 bp indicates E3/E2 or E2/E2. The most common genotype, E3/E3, displays only the upper band of this doublet, although the presence of an E4 allele must be excluded for confirmation (see below). If the lower band is of an intensity roughly equal that of the upper, then E2/E2 is expected and is confirmed by the absence of bands of 48 and 35 bp. In the E3/E2 heterozygote, only ~25% of duplexes cleave to form the lower band of the doublet, because in the last plateau cycle(s) of the PCR, about half of the E2 strands would have annealed with E3 complementary strands to form noncleaving heteroduplexes; this reduced intensity, in conjunction with the presence of bands of 48 and 35 bp, confirms the E3/E2 genotype. Neither band of the upper doublet is present in E4/E4 homozygotes; instead, a 72-bp band characteristic of E4 is seen. In E4 heterozygotes, this band is also present, but because of heteroduplexes' resistance to cleavage, its intensity is typically 25% of that found in homozygotes; the identification of the other allele as E3 or E2 is made as above. A constant 63-bp band from the digest aids orientation, acting essentially as an internal size standard. The patterns are recognizable even without detailed mobility measurements.
Discussion

Apo E genotyping by HhaI restriction digestion simultaneously determines the genotypic status of two independent positions separated by 138 bases, with both positions representing HhaI polymorphisms. Many groups prefer this approach over the use of four separate oligonucleotides for determination of the status of each site by allele-specific oligonucleotide binding. The simplicity of the “one PCR—one digest—one electrophoresis track” method with HhaI has made this method both robust and popular for apo E genotyping. As shown in Fig. 1 (lower panels), MADGE is compatible with the industry standard 8 × 12 well, 9-mm pitch microtiter plate format available for sample storage and liquid-phase procedures such as PCR, and it benefits from the many related productivity enhancements such as simplification of information handling (sample identity, etc.) and industry standard plates and multichannel pipettes, which are available for the microplate format. The entire procedure from PCR to MADGE gel preserves with 8-, 12-, or 96-channel pipettes the 96-well coordinate position of each analysis relative to the position of the pre-PCR DNA template master array. The constant size fragment of 63 bp (see top diagram in Fig. 1) is appreciably noticeable in every track and effectively acts as an internal size standard. The presence of an 83-bp fragment uniquely identifies an E2 allele, and a 73-bp fragment uniquely identifies an E4 allele. The presence of one or two copies of the most common allele, E3, is established by the presence of 91-, 48-, and 35-bp fragments in the absence of either an 83-bp band (E2/E3 genotype) or a 73-bp band (E3/E4 genotype) or in the absence of 73- and 83-bp fragments (E3/E3 genotype).

If HhaI did not also cleave at further positions (constant for all alleles) located between the variable sites in the apo E gene PCR product, then unique fragment sizes would be observed, conditional on the state of both sites in the same molecule. In the more general situation, for contiguous restriction fragments ordered as a, b, c, with variable cutting sites between them, and all possibilities occurring, then the four haplotypes would be recognizable from unique fragment sizes b, a + b, b + c, or a + b + c. An

Fig. 1. Apo E genotyping by using HhaI digests in combination with MADGE.

(Top) Schematic showing fragment sizes (in base pairs) of HhaI digest products from PCR products from apo E alleles E2, E3, and E4. Arrows mark HhaI sites.

(Bottom left) View of full 96-well MADGE gel of a set of HhaI digests from a 96-well microtiter plate PCR. The 96 wells where samples were loaded are shown in an 8 × 12 array maintaining the 9-mm pitch of microtiter plates. The direction of electrophoresis is at 71.6° to the eight-well columns of the array (i.e., downward and to the right), and the 2-mm³ loading wells have their axes parallel and perpendicular to the direction of electrophoresis. Bands have a 26.5-mm track for migration before they would meet another well on a diagonal of 2 × 4 wells. (Bottom middle) Close-up of a part of the MADGE gel shown at left. (Bottom right) Schematic identifying six tracks, each representing one of the six possible genotypes. The direction of electrophoresis is from top to bottom; the identifying curves are placed above the slowest migrating band in each track—either a 91-bp band in tracks containing PCR product from an E3 or E2 allele, or a 72-bp band in tracks containing only E4 PCR product. Traces of nondigesting higher-molecular-mass PCR misproducts are common from the PCR and are evident just anodal to the loading wells, but we have omitted them from the schematic for clarity.
Electrophoretic approach based on sizing can thus be particularly convenient for direct determination of haplotypes (as well as phase-indeterminate genotypes) that include altered restriction sites for one or more enzymes. As long as PCR (34) becomes more routine, the direct characterization by PCR of haplotypes contingent on variations spaced hundreds or thousands of bases apart (35) will become feasible. Direct haplotype studies avoid the need for family analysis to determine haplotype and are important both in research (36, 37) and diagnostics (38, 39). MADGE, whether polyacrylamide or agarose, should contribute to the general efficiency of such analyses for the same reasons that it has enhanced our productivity for apo E genotyping.

In essence, MADGE is the electrophoretic equivalent of the microtiter plate. Here we have combined the MADGE system with careful optimization and simplification of sample preparation, PCR, and restriction digestion to achieve a cost-effective approach to apo E genotype determination in large numbers of individuals. Use of the highly simplified protocols of the MADGE system generally reduces the heavy expenditure in staff time inherent in previous approaches to sample acquisition, preparation, apo E gene PCR, HhaI digestion, and electrophoresis and requires no specialized equipment not already available in the molecular genetics laboratory.

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