This method, which uses two control amplicons in combination with standardized laser-induced capillary electrophoresis, allows fast, reliable, and sensitive quantification of gene dosages without the use of synthetic competitors, even in the presence of highly degrade DNA.

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Rapid Identification of *Apolipoprotein E* Genotypes by Multiplex Amplification Refractory Mutation System PCR and Capillary Gel Electrophoresis, *Gerard G. Donohoe,[1,3*] Anne Salomäki,[2] Terho Lehtimäki,[2] Kari Pulkki,[1] and Veli Kairisto[1]* ([1] University of Turku and Turku University Hospital, Department of Clinical Chemistry, FIN-20520 Turku, Finland; [2] Tampere University Hospital, Department of Clinical Chemistry, FIN-33520 Tampere, Finland; [3] Dublin Institute of Technology, Department of Biological Sciences, Dublin 8, Ireland; * author for correspondence: fax 358-2-2613920, e-mail gerdon@utu.fi)

Apolipoprotein E (*Apo E*) genotyping on its own is neither sufficiently sensitive nor specific enough for use as a predictive diagnostic test for Alzheimer disease (AD) *(1)*. Nevertheless, it is still of diagnostic value in the classification of type III hyperlipidemia *(2)*. Furthermore, there exists a great research need and interest for *Apo E* genotyping in conjunction with other data relating to genetic polymorphisms. Several independent studies have now established that the inheritance of one or more *Apo* ϵ4 alleles increases an individual's risk of developing atherosclerosis and AD *(3, 4)*. Conversely, the inheritance of one or more ϵ2 alleles confers protection against AD *(5)* and is associated with lower lipid concentrations *(6)*. Thus, a rapid and simple genotyping test is needed for any laboratory that takes part in clinical research related to Apo E.

Apo E is a polymorphic protein consisting of a single polypeptide chain, 299 amino acids long. In plasma, it exists mainly in a nonglycosylated form. The three major isoforms of the protein are Apo E2, Apo E3, and Apo E4. These differ from each other by cysteine-arginine interchanges at amino acid residues 112 and 158. The biosynthesis of each protein isoform is under the control of three independent codominant alleles, ϵ2, ϵ3, and ϵ4, located at a single *Apo E* gene locus on chromosome 19q13. Depending on the inheritance of any two alleles, six common *Apo E* genotypes are possible, three homozygotes (ϵ2ϵ2, ϵ3ϵ3, and ϵ4ϵ4) and three heterozygotes (ϵ2ϵ3, ϵ3ϵ4, and ϵ2ϵ4) *(7)*.

Currently, several different *Apo E* genotyping techniques have been described; these include minisequencing *(8)*, single-strand conformation polymorphism *(9)*, allele-specific oligonucleotide probes *(10)*, oligonucleotide ligation assays *(11)*, restriction isotyping with *Hha*I or *Afl*III/*Hae*II *(12, 13)*, and the amplification refractory mutation system (ARMS) *(14)*. The ARMS technique has many advantages when compared with these and other genotyping methods: it is fast, easy to perform, and nonisotopic. This is in contrast to single-strand conformation polymorphism, which is difficult to perform and may require radioactive probes or the use of labor-intensive silver staining. Moreover, ARMS requires less sample manipulation than restriction endonuclease analysis, and unlike allele-specific oligonucleotides, oligonucleotide ligation assays, or minisequencing, it does not require labeling.

ARMS *Apo E* genotyping as described by Wenham et al. *(14)* requires four separate PCR reactions. We wanted to reduce the workload by combining the different Wenham primers into two PCR reactions. However, when the combined primers were used, the target DNA failed to coamplify. We subsequently designed a new set of ARMS primers according to established rules for good primer design (matched T_m values and low self-complementary, matched GC content). The software program CloneWorks© for Windows 95, Ver. 1.99 (Anteater Software Corp.) was used for primer design. The following primer sets were constructed:

Cys158/Arg158 (5′-ATGCCGATGACCTGCAGAAT<u>T</u>-3′)/(5′ATGCCGATGACCTGCAGAAT<u>C</u>-3′)

Cys112/Arg112 (5′-CGCGGACATGGAGGACGT<u>T</u>T-3′)/(5′-CGCGGACATGGAGGACGT<u>T</u>C-3′).

The primers were designed so that the 3′-most nucleotide of the respective primer recognized either A or G corresponding to Arg or Cys at positions 112 or 158, respectively. The 3′ penultimate nucleotide (underlined above) was mismatched to enhance specificity of the primers *(14)*. In addition, a common primer was designed (5′-GTTCAGTGATTGTCGCTGGGCA-3′) that paired with Arg/Cys 158 or Arg/Cys 112 and produced an amplicon of 588 and 451 base pairs (bp), respectively. A 360-bp fragment of the α_1-antitrypsin gene was coamplified to function as an internal positive control *(14, 15)*.

Genomic DNA was extracted from 3 mL of fresh or frozen whole blood by either the standard proteinase K-phenol-chloroform method or by a rapid DNA extraction kit, Nucleon (Amersham). The isolated DNA samples were measured by ultraviolet spectrophotometry at 260 nm and diluted with distilled water to a final concentration of 20 mg/L (20 ng/μL). The Nucleon kit performed just as well as the standard DNA extraction method with either fresh or frozen whole blood samples and was subsequently used for additional DNA extractions.

Each PCR reaction mixture contained the following in a final volume of 25 μL: 50 ng of genomic DNA; 200 μmol/L of dATP, dCTP, dGTP, and dTTP (Pharmacia Biotech); PCR reaction buffer (10 mmol/L Tris-HCL, pH 8.8, 1.5 mmol/L $MgCl_2$, 50 mmol/L KCl, and 1 mL/L Triton X-100; Finnzymes); 80 g/L dimethyl sulfoxide (DMSO; Sigma); 1 U of DyNAzyme II DNA Polymerase (Finnzymes); 8 nmol/L of α_1-antitrypsin primers, and 0.8 μmol/L ARMS common primer. Reaction mixture A contained, in addition to the above, 0.8 μmol/L Cys158 and 0.4 μmol/L Cys112 primers. Similarly, reaction mixture B contained 0.8 μmol/L Arg158 and 0.4 μmol/L Arg112 primers. Amplification was performed with a Perkin-Elmer/Cetus DNA thermocycler (N801-0177). Each PCR run had blank controls with no DNA to exclude contamination. The PCR amplification profile was as follows: initial denaturation at 95 °C for 4 min, 35 cycles with denaturation at 96 °C for 45 s, annealing at 66 °C for 45 s, and extension at 72 °C for 45 s, followed by a final cycle of extension at 72 °C for 5 min.

Amplified multiplex product (9 μL) was mixed with 3 μL of 6× gel loading dye type I (Sigma) and separated in a 1.6% agarose gel (Sigma) that contained 0.1 mg/L ethidium bromide (Bio-Rad). The samples were electrophoresed for 50 min at 12 V/cm in a minigel (Pharmacia Biotech), using 0.5× Tris-borate-EDTA running buffer (1× Tris-borate-EDTA: 90 mmol/L Tris borate, 2 mmol/L EDTA, pH 8.0, and 0.08 mg/L ethidium bromide). The amplicons were sized using a 50-bp molecular mass marker (Boehringer Mannheim).

We found as others have reported that DMSO is essential for the successful amplification of the *Apo E* gene *(16)*. We investigated this further by testing various DMSO concentrations (60–160 g/L) and found 80 g/L to be the optimal concentration. DMSO concentrations below this produced no visible bands on the gel, and concentrations above this produced weak or absent PCR bands.

We obtained *Apo E* reference samples with known *Apo E* genotypes, analyzed by either minisequencing or restriction digestion with *Afl*III and *Hae*II. These samples were tested with the newly developed ARMS technique (Fig. 1A). The results obtained were in complete agreement with all 10 reference samples. Furthermore, all three bands in the ARMS multiplex gave relatively equal band intensities, even with the relatively low concentration of ethidium bromide used. However, an extra band between the 451 and 588 bands was also produced. This intermediate band was seen only when both the 451- and 588-bp fragments were coamplified and not when they were amplified individually (Fig. 1A). When the PCR products in single-stranded form were separated using a denaturing polyacrylamide gel, this intermediate band was no longer visible. Therefore, this band was produced when longer 588-bp band and shorter 451-bp band annealed to each other, because of their complementary sequence, and its presence did not interfere with the interpretation of the *Apo E* genotype.

To test the robustness of the technique, we analyzed >100 population samples (informed consent was obtained). In addition, the reproducibility, sensitivity, and reliability of the technique were evaluated by analyzing 20 samples four different times. All samples tested gave an unambiguous *Apo E* genotype. The distribution of the genotypes in our study group were in accordance with previous Finnish population studies, which have shown a higher prevalence of the ϵ4 allele than in other populations *(17)*.

We also tested the amplified product of the 10 reference samples by running them on capillary electrophoresis (Fig. 1B). Analysis was performed with the HP3D Capillary Electrophoresis System (Hewlett-Packard). CEP-coated fused-silica capillaries provided with the pGEM Double Stranded DNA Analysis Kit (Hewlett-Packard) with a 40-cm effective length and 48.5-cm total length were used. The separation/flush buffer consisted of 89 mmol/L Tris, 332 mmol/L boric acid, and 2 mmol/L EDTA, pH 7.4, containing 7.5 g/L hydroxymethyl cellulose (Hewlett-Packard) and 1 mg/L ethidium bromide.

The buffer was filtered and degassed by sonication. Samples were introduced into the capillary by electrokinetic (−10 kV for 15 s) injection, with a separation voltage of −16.5 kV (340 V/cm). The temperature of the capillary was 20 °C, and the ultraviolet absorbance was monitored at 258 nm, using 380 nm as the reference wavelength. Before each injection, the capillary was flushed for 10 min with buffer to ensure the reproducibility of migration times. A typical assay took ~13 min. The pGEM DNA size marker was analyzed similarly but with the injection

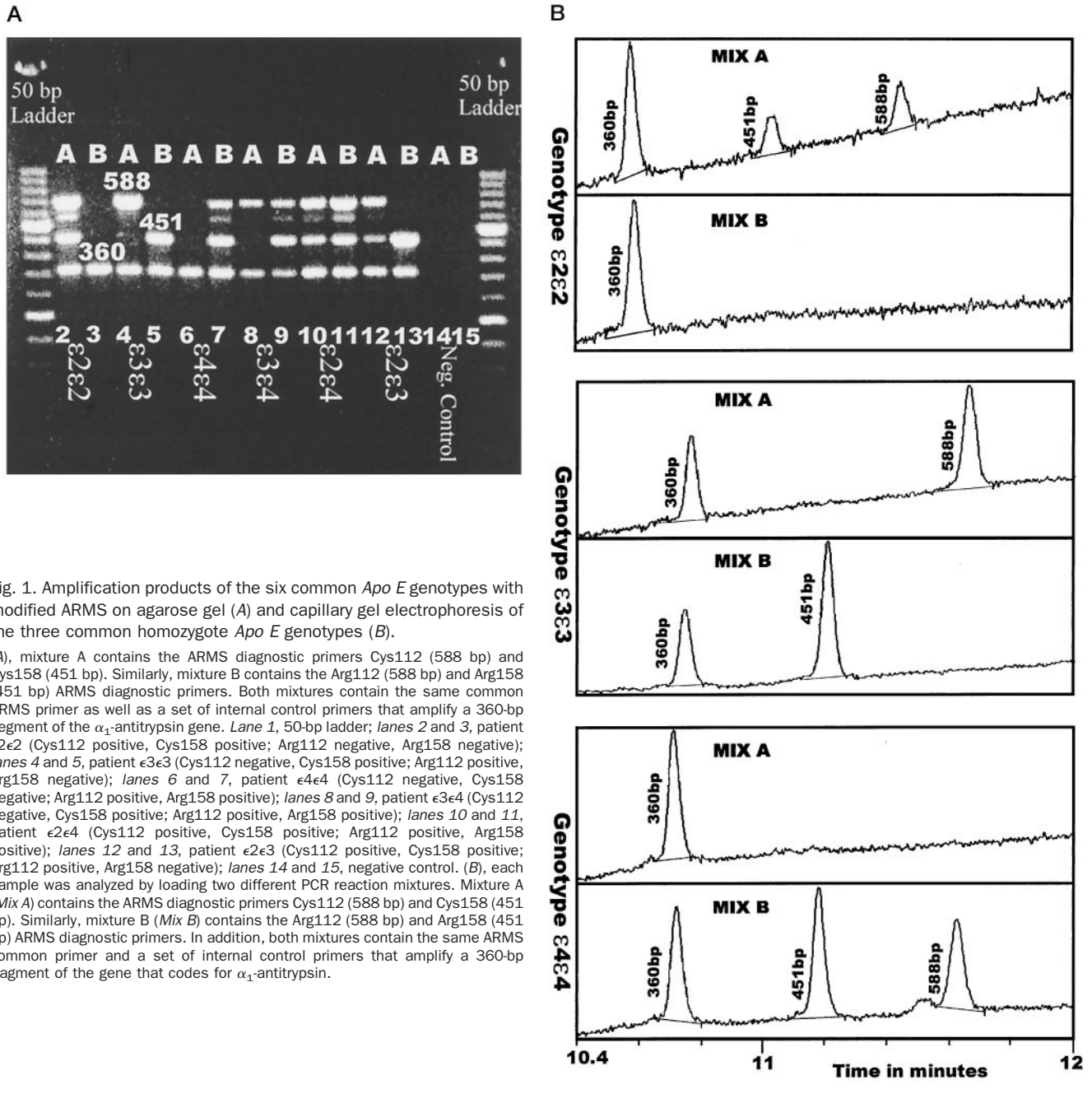

Fig. 1. Amplification products of the six common *Apo E* genotypes with modified ARMS on agarose gel (*A*) and capillary gel electrophoresis of the three common homozygote *Apo E* genotypes (*B*).

(*A*), mixture A contains the ARMS diagnostic primers Cys112 (588 bp) and Cys158 (451 bp). Similarly, mixture B contains the Arg112 (588 bp) and Arg158 (451 bp) ARMS diagnostic primers. Both mixtures contain the same common ARMS primer as well as a set of internal control primers that amplify a 360-bp segment of the α_1-antitrypsin gene. *Lane 1*, 50-bp ladder; *lanes 2* and *3*, patient $\epsilon 2\epsilon 2$ (Cys112 positive, Cys158 positive; Arg112 negative, Arg158 negative); *lanes 4* and *5*, patient $\epsilon 3\epsilon 3$ (Cys112 negative, Cys158 positive; Arg112 positive, Arg158 negative); *lanes 6* and *7*, patient $\epsilon 4\epsilon 4$ (Cys112 negative, Cys158 negative; Arg112 positive, Arg158 positive); *lanes 8* and *9*, patient $\epsilon 3\epsilon 4$ (Cys112 negative, Cys158 positive; Arg112 positive, Arg158 positive); *lanes 10* and *11*, patient $\epsilon 2\epsilon 4$ (Cys112 positive, Cys158 positive; Arg112 positive, Arg158 positive); *lanes 12* and *13*, patient $\epsilon 2\epsilon 3$ (Cys112 positive, Cys158 positive; Arg112 positive, Arg158 negative); *lanes 14* and *15*, negative control. (*B*), each sample was analyzed by loading two different PCR reaction mixtures. Mixture A (*Mix A*) contains the ARMS diagnostic primers Cys112 (588 bp) and Cys158 (451 bp). Similarly, mixture B (*Mix B*) contains the Arg112 (588 bp) and Arg158 (451 bp) ARMS diagnostic primers. In addition, both mixtures contain the same ARMS common primer and a set of internal control primers that amplify a 360-bp fragment of the gene that codes for α_1-antitrypsin.

made at −5 kV for 5 s. The identification of the various DNA fragments by size was obtained by plotting 1og (bp) vs 1/migration time for the electropherogram of the pGEM size marker. We found all the reference *Apo E* samples to be in complete agreement with the expected genotypes.

In conclusion, we have successfully designed a new ARMS method that requires only two PCR reactions instead of four for each *Apo E* genotyping. This method can easily be implemented into a minimally equipped laboratory, and the detection of the PCR products can be automated by the use of capillary gel electrophoresis. However, a technical failure in the PCR could lead to misclassification of a patient's *Apo E* genotype. Therefore, those wishing to use our method should standardize the assay first by testing it with all six *Apo E* genotypes. Because our method does not rely on restriction digestion, it has distinct advantages over other *Apo E* capillary gel electrophoresis methods *(18–20)*. For example, it does not require the use of expensive laser-induced fluorescent detection equipment, which has been used to distinguish the small fragment sizes produce by the restriction digestion method *(19, 20)*. In comparison, our method, which produces fragments of a much larger size (360–588 bp),

can use simple ultraviolet detection. Moreover, because our PCR products can be loaded directly onto the capillary, no preparative steps such as overnight digestion, precipitation, desalting, or extensive washing of the samples are required. This greatly speeds up the analysis and reduces both the labor and cost. Thus, our technique is ideally suited to semi-automation, and it can readily be applied to high-volume genotyping or screening programs.

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Observations on the Zinc Protoporphyrin/Heme Ratio in Whole Blood, *Robert F. Labbé,[1*] Anjana Dewanji,[2] Kathleen McLaughlin[1]* ([1] Department of Laboratory Medicine, Box 359743, Harborview Medical Center, Seattle WA 98104-2499, and [2] Biological Sciences Division, Indian Statistical Institute, Calcutta, 700 035 India; * author for correspondence: fax 206-731-3930, e-mail boblabbe@ix.netcom.com)

Zinc protoporphyrin (ZPP) is a normal metabolite that accumulates at trace amounts in erythrocytes during hemoglobin synthesis. In states of inadequate iron delivery to developing red cells, excess ZPP forms as a by-product of the heme biosynthetic pathway. This ZPP response occurs because iron and zinc interact as ferrochelatase substrates, with zinc utilization increasing when the iron supply is diminished *(1)*. The resulting high zinc protoporphyrin/heme ratio (ZPP/H) in circulating erythrocytes reflects a state of relative iron-deficient erythropoiesis *(2)*. Either the whole blood or erythrocyte ZPP/H ratio in μmol/mol can be determined conveniently using hematofluorometry *(3)*, a technique for front-surface fluorescence/absorption measurement *(4)*.

Despite its ease of determination and its clinical utility *(5)*, ZPP/H has found only limited application in patient care. Criticism of ZPP/H determination has been based largely on interference from plasma components, primarily bilirubin *(6–8)*. Other less common analytical pitfalls that have been cited include incomplete hemoglobin oxygenation *(9)* and the degree of hemolysis *(10)*. Accordingly, we undertook an evaluation of the analytical procedure to try to understand and alleviate these factors associated with hematofluorometry. When the ZPP/H ratio is determined as prescribed, we believe that clinically acceptable analytical quality can be maintained. Furthermore, the highly sensitive, cost-effective ZPP/H ratio has merit as a primary test for assessing iron status and for monitoring iron therapy.

For this evaluation, we used anticoagulated whole blood specimens obtained from our clinical hematology laboratories after all ordered tests had been completed. The blood had been collected in Vacutainer 3.0-mL Hemogard Tubes containing 3.0 mg EDTA (K3). Approval was obtained from the Human Subjects Division, Office of Research for the use of these leftover specimens without patient identification. Blood specimens were used either as collected or were processed as described for particular experiments.