Table 1. Allele frequencies of *MEFV* mutations detected in our laboratory among FMF patients who fulfilled the diagnosis criteria according to Livneh et al. *(12)*.[a]

Sequence variation[b]	No. of alleles	Percentage of mutated alleles	Cumulative frequencies
M694V	568	62.2	62.2
V726A	133	14.7	76.9
M680I (G>C)	75	8.2	85.1
E148Q	46	5.0	90.1
M694I	34	3.7	93.8
F479L	21	2.3	96.1
R761H	20	2.2	98.3
K695R	4	0.4	98.7
A744S	2	0.2	98.9
P369S	2	0.2	99.1
M680I (G>A)	1	0.1	99.2
I692del	1	0.1	99.3
Other	6	0.7	100
No identified mutation	883		
Total	1796		

[a] The study group included Armenians (38.9%), Sephardim (22.8%), Arabs (11.5%), Turks (7.5%), Ashkenazim (0.5%), individuals of heterogeneous at-risk origin (2.4%), and individuals from classically non-at-risk origin (15.7%).

[b] Mutations detected by the FMF StripA^{ssay} are in bold.

We wish to thank Laurent Skopinski and Danielle Huot for valuable technical assistance, Dr. Catherine Dodé and Christophe Pêcheux (Service de Biochemiè-Génétique, Hôpital Cochin, Paris, France) for providing the E148Q-I692del/E148Q DNA sample, Ingen (Rungis, France) and AmpliTech (Compiegne, France) for providing reagent sets for our study, and Sean Crosson for critical reading of the manuscript.

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DOI: 10.1373/clinchem.2003.021212

Simple Sequence-specific-Primer-PCR Method To Identify the Three Main Apolipoprotein E Haplotypes, *Panagiotis Pantelidis,[1*] Michelle Lambert-Hammill,[2] and Anthony S. Wierzbicki[2]* ([1] Pan-Pathology Molecular Diagnostics Laboratory and [2] Department of Chemical Pathology, St. Thomas' Hospital, Lambeth Palace Road, London SE1 7EH, UK; * address correspondence to this author at: Pan-Pathology Molecular Diagnostics Laboratory, Department of Infection, 5th Floor North Wing, St. Thomas' Hospital, Lambeth Palace Road, London, SE1 7EH, UK; fax 44-20-79280730, e-mail panagiotis.pantelidis@gstt.sthames.nhs.uk)

Apolipoprotein E (ApoE) is involved in the binding, internalization, and catabolism of lipoprotein particles. ApoE is secreted by macrophages and hepatocytes and serves as a ligand for the LDL (Apo B/E) receptor and on hepatic tissues for the specific ApoE type 1 and 2 receptors for triglyceride-rich remnants *(1, 2)*. The gene coding for ApoE is located on chromosome 19q13.32 and contains several polymorphic loci. The three common isoforms of ApoE, termed E2, E3, and E4, are defined by two single-nucleotide polymorphisms (SNPs) at positions 2059(T/C) and 2197(C/T) of the gene, which are located in the codons that code for amino acids 112 and 158 of the mature protein. The presence of a T allele at position 2059 (2059-T) of the gene defines a cysteine at position 112 (Cys_{112}), whereas 2059-C defines an arginine for the same position (Arg_{112}). Similarly, 2197-C on the gene defines an arginine at position 158 (Arg_{158}), whereas 2197-T codes for cysteine for the same position (Cys_{158}). ApoE3 (Cys_{112}/Arg_{158}) is the most common of the three isoforms found. The ApoE2 isoform Cys_{112}/1Cys_{158} is associated with remnant hyperlipidemia because this isoform has a lower affinity for ApoE receptors. The ApoE4 (Arg_{112}/Arg_{158}) isoform is associated with increased hepatic synthesis of VLDL and is a risk factor for Alzheimer disease and other neurologic diseases. Other rare isoforms are also found and are associated with mixed hyperlipidemia *(1)*.

Several methods have been used for genotyping the three major ApoE isoforms. The problems inherent in phenotyping methods have led to the gradual adoption of genotyping methods for determination of ApoE isoforms in most laboratories *(3)*. By far the commonest method has been PCR amplification followed by restriction fragment length polymorphism (RFLP) analysis. Although many variations of this methodology have been published, the original method used *Hha*I restriction endonuclease, which cleaves at the GCGC sequence that encodes

Arg_{112} (E4) and Arg_{158} (E3, E4), but does not cut at the GTGC sequence that encodes Cys_{112} (E2 and E3) and Cys_{158} (E2) *(4)*. Other methodologies used include capillary electrophoretic detection *(5)*, Amplification Refractory Mutation System PCR (ARMS-PCR) *(6, 7)*, and "real-time" PCR detection *(8, 9)*.

The PCR-RFLP mapping approach involves a single PCR reaction followed by restriction enzymatic digestion and genotypic assignment after subsequent analyses by electrophoresis. Although the methodology requires only a single PCR reaction per sample, the restriction enzyme digestion step increases the post-PCR processing time by 2 h. Furthermore, because incomplete restriction enzyme digestion can lead to false genotyping data, many researchers prefer to perform overnight digestions, thus increasing the assay time even further. In addition, the small size fragments produced after restriction enzyme digestion are usually resolved by acrylamide gels electrophoresis, thus limiting the number of samples that can be examined at one time. The real-time PCR and capillary electrophoretic detection methods are faster but require expensive dedicated instrumentation. The several ARMS-PCR methodologies reported identify the individual alleles by amplification using specific primers in combination with a common primer either in a four- *(6)* or two-PCR *(7)* reaction set-up, with the latter requiring denaturing acrylamide gel electrophoresis or capillary electrophoresis to eliminate amplification artifacts. The fact that the reported ARMS-PCR methodologies do not establish experimentally the cis/trans chromosomal orientation of the alleles at SNP positions 2059/2197 makes identification of the ApoE isoforms rather tedious and open to interpretational errors.

We describe here an ApoE haplotype-specific sequence-specific-primer (SSP)-PCR methodology that identifies in three PCR reactions the allelic haplotypes that determine the three main ApoE isoforms. We have previously used this approach to determine the cis/trans chromosomal orientation of the individual nucleotides in SNPs located in the interleukin-13 gene and, thus, the specific allelic haplotypes of that gene *(10)*. We designed two forward primers with variations in their 3′ nucleotides such that each was specific for one of the two variants in the 2059 locus and two reverse primers whose 3′-end nucleotides identified either of the nucleotide variants in the 2197 locus (MDL PRIMERS 1–4; Table 1). The sequence-specific forward and reverse primers were then combined in three haplotype-detecting reaction mixtures "Primer Mix E2" (primers 1 and 3), "Primer Mix E3" (primers 1 and 2), and "Primer Mix E4" (primers 2 and 4). Because this genotyping system is based on the presence or absence of PCR amplification by allele-specific primers, it is imperative to ensure PCR amplification for those reactions that do not produce haplotype-specific amplicons. For this reason, each ApoE-specific Primer Mix also contained a pair of "control primers" (primers 8 and 9), which amplify two regions of chromosome 6 in the HLA-DR locus, to verify PCR amplification in the absence of haplotype-specific amplification in each PCR reaction.

For each DNA sample, 5 μL of each Primer Mix (E2, E3, and E4) was placed in the bottom of three 0.2-mL wells in a 96-well plate. To this we added a mixture of 8 μL of DNA (resuspended in DNase-free water or Tris buffer) and PCR reaction mixture containing PCR buffer [$(NH_4)_2SO_4$], $MgCl_2$, deoxynucleotide triphosphate mixture, and *Taq* DNA polymerase [Bioline (UK) Ltd.] and overlaid each reaction with 10 μL of mineral oil. Thus, each 13-μL PCR reaction contained the amount of the primers indicated in Table 1 and in addition, 1× PCR Buffer, 2mM $MgCl_2$, 0.32 U of *Taq* DNA polymerase, 150 μM each deoxynucleotide triphosphate, and 0.08–0.15 μg of genomic DNA. Amplification was performed on a PTC-200 thermal cycler (GRI) using a high-stringency touchdown-PCR protocol with high annealing temperatures to ensure specificity of amplification. The PCR cycling condition were as follows: initial denaturation for 1 min at 96 °C; followed by 5 cycles of 20 s at 96 °C, 45 s at 70 °C, and 25 s at 72 °C; 21 cycles of 25 s at 96 °C, 50 s at 65 °C, and 30 s at 72 °C; 4 cycles of 30 s at 96 °C, 60 s at 55 °C, and 120 s at 72 °C. The PCR products were analyzed by electrophoresis on 2% Tris-borate-EDTA/ethidium bromide agarose gels with 10 μL of Orange G loading dye and visualized under ultraviolet illumination.

For all PCR reactions (E2, E3, and E4), the presence of a 173-bp band indicated the presence of the specific ApoE haplotype. The control primer pair binds on two positions on chromosome 6, [on GI:20196696, MDL PRIMER-8 binds on sequences 53359–53341 and 89472–89454, whereas MDL PRIMER-9 binds on sequences 52575–52594 and 87875–87894]. Therefore, with the control primer pair, two products were expected of 785 and 1598 bp. Although the 785-bp control amplification product was always present when PCR amplification occurred, the 1598-bp product was often missing in those samples with poor-quality DNA or excessive ApoE haplotype-specific

Table 1. Primers required for ApoE haplotype detection by SSP-PCR analysis.

Primer	Primer sequence, 5′–3′	Primer identifies	Forward or reverse orientation	Final amount in each 13-μL PCR reaction, ng
MDL PRIMER-8	TGC CAA GTG GAG CAC CCA A	(Control) HLA-DRB1 region	Forward	6.5
MDL PRIMER-9	GCA TCT TGC TCT GTG CAG AT	(Control) HLA-DRB1 region	Reverse	6.5
MDL PRIMER-1	CGG ACA TGG AGG ACG TGT	APOE-112cys	Forward	50
MDL PRIMER-2	CTG GTA CAC TGC CAG GCG	APOE-158arg	Reverse	50
MDL PRIMER-3	CTG GTA CAC TGC CAG GCA	APOE-158cys	Reverse	50
MDL PRIMER-4	CGG ACA TGG AGG ACG TGC	APOE-112arg	Forward	50

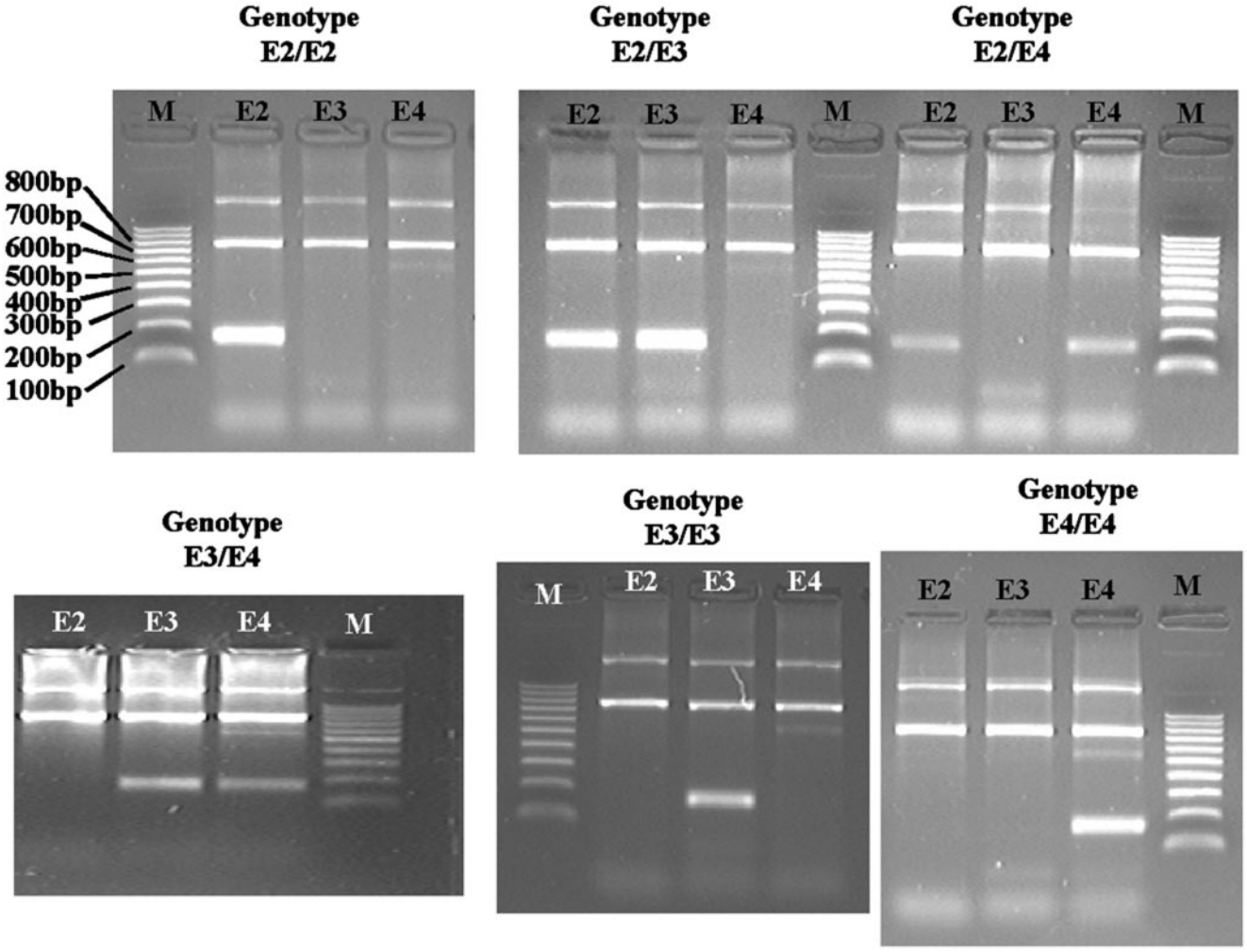

Fig. 1. Amplification products of the six common ApoE genotypes by SSP-PCR.

Primer mixture E2 contains MDL-Primers 1 and 3, which produce an ApoE-specific 173-bp product only if the nucleotides that encode for Cys_{112} and Cys_{158} are on the same chromosome. Primer mixture E3 contains MDL-Primers 1 and 2, which produce an ApoE-specific 173-bp product only if the nucleotides that encode Cys_{112} and Arg_{158} are on the same chromosome. Primer mixture E4 contains MDL-Primers 4 and 2, which produce an ApoE-specific 173-bp product only if the nucleotides that determine Arg_{112} and Arg_{158} are on the same chromosome. In all PCR reactions the control primer pairs produced the two larger PCR products as predicted, thus confirming PCR amplification in those PCR reactions that were devoid of allele-specific PCR products.

amplification. Nevertheless, a sample was considered negative for a particular ApoE haplotype when the haplotype-specific amplicon was absent and the 785-bp control amplicons was present. Absence of haplotype-specific and control amplicons in the same reaction was indicative of PCR amplification failure. Examples of ApoE haplotype-positive PCR reactions are shown in Fig. 1.

The accuracy of this genotype determination method was demonstrated by analysis of a cohort of 50 DNA samples previously typed by the PCR-RFLP method and which included all ApoE haplotype combinations, examples of which are shown in Fig. 1. For all samples, there was 100% concordance between the two methods. The SSP-PCR ApoE genotyping method performed equally well with DNA extracted by different methods, including Qiagen extraction and the high-salt method, and with variable amounts of DNA starting material. PCR amplification problems were observed when EDTA was included in the DNA resuspension/elution buffer. The methodology was easily adaptable to high-throughput genotyping by a combination of automatic DNA extraction using the QIAGEN MDX 8000 system, PCR plate technology, and 96-well-compatible agarose gel electrophoresis. With this system, the DNA of 96 samples could be extracted and the ApoE haplotype determined in a single run.

This work utilized facilities made available through a new service and innovative grant from the Guy's and St. Thomas' Charitable Foundation for setting up the Pan-Pathology Molecular Diagnostic Laboratory.

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DOI: 10.1373/clinchem.2003.021683

Genetic Testing for Familial Mediterranean Fever in Austria by Means of Reverse-Hybridization Teststrips, *Christian Oberkanins,[1*] Andreas Weinhäusel,[2] Gernot Kriegshäuser,[1] Anne Moritz,[1] Fritz Kury,[1] and Oskar A. Haas[2]* ([1] ViennaLab Labordiagnostika GmbH, Am Kanal 27, A-1110 Vienna, Austria; [2] CCRI, St. Anna Children's Hospital, A-1090 Vienna, Austria; * author for correspondence: fax 43-1-74040-199, e-mail oberkanins@viennalab.co.at)

Familial Mediterranean fever (FMF) is an autosomal-recessive, inflammatory disorder characterized by short, recurrent attacks of fever, accompanied by pain in the abdomen, chest, or joints and erysipelas-like erythema. Its most severe complication is progressive amyloidosis, leading to end-stage renal failure. FMF predominantly affects Turks, Arabs, Armenians, and Sephardic Jews, with carrier rates reported as high as 1 in 5, but it has been observed in lower frequencies throughout the Mediterranean area *(1)*. It is caused by several mutations within the marenostrin/pyrin-encoding gene *MEFV* on chromosome 16p13.3, which differently affect the severity of the disease phenotype and the risk of developing renal amyloidosis *(2–4)*. Although established clinical criteria for FMF exist *(5)*, many patients remain undiagnosed because of rather nonspecific symptoms; therefore, molecular genetic analysis could substantially improve early and correct diagnosis of FMF and allow initiation of lifelong prophylactic treatment of affected individuals with colchicine *(6)*.

Aiming at a simple but powerful first-line screening tool for FMF genotyping, we have set up a reverse-hybridization, teststrip-based assay (FMF StripAssay) for the simultaneous detection of 12 *MEFV* mutations: E148Q in exon 2, P369S in exon 3, F479L in exon 5, and M680I (G/C), M680I (G/A), I692del (2076–2078), M694V, M694I, K695R, V726A, A744S, and R761H in exon 10. For this purpose, we collected DNA samples from individuals who had previously been typed positive for one of these mutations and used them to generate recombinant plasmid clones for the 12 mutant alleles (TOPO TA Cloning Kit; Invitrogen). After confirming the presence of mutations by DNA sequencing, we used these plasmid clones as homozygous reference samples to determine suitable reverse-hybridization probes.

We synthesized a series of candidate 15- to 25mer oligonucleotides, selected from the FMF databank sequence (GenBank accession no. AF111163) and encoding all wild-type or mutant alleles, and immobilized them via 3′-poly(dT) tails on nitrocellulose membrane as an array of parallel lines *(7)*. We then amplified the 12 mutant plasmids, as well as a wild-type genomic DNA (obtained from a nonsymptomatic individual, and mutation-negative by DNA sequencing) by multiplex PCR, using four sets of biotinylated primers specific for *MEFV* exons 2, 3, 5, and 10 *(3, 8)*. Biotinylated amplification products were then hybridized under controlled stringency (45 ± 0.5 °C) to our preliminary probe arrays, and specifically bound fragments were identified at room temperature by use of streptavidin-alkaline phosphatase conjugate and enzymatic color reaction *(7)*.

After selecting the oligonucleotides that could differentiate best between wild-type and mutant alleles, we prepared a final probe array, which also included a 5′-biotinylated control oligonucleotide to allow performance control of the detection system. For two particular *MEFV* gene regions (codons 680 and 692–695), where more than one mutation is located within the standard length of our hybridization probes, a common oligonucleotide represented the wild-type sequence. The membrane-bound array was finally sliced into 3-mm, ready-to-use teststrips.

We evaluated the specificity of the FMF StripAssay by analyzing a series of amplification products obtained from mutant plasmid clones, as well as wild-type, heterozygous, or homozygous mutant genomic DNA samples (Fig. 1). Reverse-hybridization and enzymatic color detection were carried out either manually, using thin-walled plastic incubation trays (Bio-Rad) and a shaking waterbath (GFL) set to 45 °C, or in a fully automated device (profiBlot IIT; TECAN AG). We observed no difference in staining patterns between manual and automated teststrip processing (data not shown). Our results demonstrated that the assay will specifically detect the presence of any of the 12 *MEFV* mutations as well as correctly identify homozygotes by the absence of the corresponding wild-type signal (Fig. 1, strips 1–12). For amplified wild-type DNA, none of the mutant signals was visible (Fig. 1, strip 26), and for a negative PCR product obtained with water used in place of DNA, only the biotinylated control probe, which is expected to produce color irrespective of the presence of hybridizing DNA fragments, stained positively (Fig. 1, strip 27).