

Detection of *ApoE* E2, E3 and E4 alleles using MALDI-TOF mass spectrometry and the homogeneous mass-extend technology

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ABSTRACT

Apolipoprotein (Apo) E is one of the five main types of blood lipoproteins (A–E). It is synthesized primarily in the liver and brain and helps in transporting lipids from one place to another as well as facilitates the clearing of dietary fats, such as triglycerides, from the blood. The *ApoE* gene exists in three different forms: E2, E3 and E4. E3 is considered to be the normal form. Variants of the *ApoE* gene have been associated with various diseases. Developing an assay for the genotyping of *ApoE* variants for use both in clinical and large cohort based association settings would be extremely valuable and would require the use of a platform that has high-throughput capabilities and is highly accurate. Here we describe an assay for the simultaneous genotyping of the *ApoE* variants in a single bi-plex reaction and a single well using the matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and the homogeneous mass-extend (hME) technology. The assay is robust, highly accurate and suitable for both clinical applications and for the genotyping of large disease cohorts. Moreover, the prevalence of *ApoE* variants in a cohort of Caucasians from the central Wisconsin area is outlined.

INTRODUCTION

The apolipoprotein E (*ApoE*) gene is located on the long (q) arm of chromosome 19 and codes for a protein that exists in several different forms (1). ApoE, which is synthesized primarily in the liver and brain, is one of the five main types of blood lipoproteins (A–E) and is a major component of specific lipoproteins called very low-density lipoproteins. A major function of very low-density lipoproteins is to remove excess cholesterol from the blood and carry it to receptors on the

surface of liver cells for processing. ApoE helps in transporting lipids from one place to another and facilitates the clearing of dietary fats, such as triglycerides, from the blood. Maintaining normal levels of cholesterol is essential for the prevention of cardiovascular diseases, including heart attacks and strokes. Defects in the ApoE protein could diminish its ability to bind to the receptors, which leads to an elevated blood cholesterol level.

The *ApoE* gene exists in three different forms, E2, E3 and E4, with E3 being the most common form that is found in more than half the population. The ApoE E3 allele is differentiated by a cysteine at position 112 and an arginine at position 158 in the receptor-binding region of ApoE. The ApoE E2 allele (Cys-112 and Cys-158) has significantly less binding ability. In particular, individuals with an E2/E2 combination may clear dietary fat from their body at a slower rate and are at a higher risk for early vascular disease and type III hyperlipoproteinemia (2). According to a recent study, 94.4% of type III hyperlipoproteinemia patients are homozygous for E2 (3). Inversely, only ~2% of patients homozygous for ApoE E2 develop the clinical phenotype of hyperlipoproteinemia type III (4). It is, therefore, thought that further genetic or environmental factors must be involved in the development of the disease. The ApoE E4 allele (Arg-112 and Arg-158) has been linked to atherosclerosis (5). Individuals with E4/E4 genotype are at a higher risk of developing the disease (5).

ApoE E4 has also been associated with an increased risk of late onset of Alzheimer's disease (developing after the age of 65). Although one copy of E4 constitutes a risk (E2/E4 or E3/E4), two copies of E4 (E4/E4) indicate a greater risk of developing Alzheimer's disease (6). In itself, the ApoE E4 is neither sufficient nor necessary for the development of Alzheimer's disease. However, *ApoE* genotyping can increase the specificity of the clinical diagnosis of Alzheimer's disease.

Several approaches were used previously to type *ApoE* alleles. The most commonly used approaches are based on PCR–restriction fragment length polymorphism (PCR–RFLP) (7–9). These approaches, although simple, are not suitable for the high-throughput analysis and alleles are

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sometimes difficult to be discerned because of the incomplete digestion of the PCR product that is often seen with the use of restriction enzymes. Developing an assay for use in both clinical and large cohort based association settings would be extremely valuable and would require the use of a platform that has high-throughput capabilities and is highly accurate. Mass spectrometry is known for its accuracy and high-throughput potential and was used for *ApoE* genotyping (10–14); however, the analysis either involved the use of restriction enzymes, expensive Locked Nucleic Acid® probes, and/or was not carried out in a single tube. In recent years, matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry combined with the homogeneous mass-extend (hME) reaction has proven very useful for the high-throughput mutation and single nucleotide polymorphism (SNP) analysis in a single tube format (15–17). In a wide scale application of SNP genotyping utilizing 3738 SNPs from the HapMap project using the MALDI-TOF MassARRAY system and the hME technology, Gabriel *et al.* (17) reported genotyping accuracy of 99.6% at 4–7 plex level of design. This system has also been used as a powerful tool for genetic testing in clinical settings, as recently demonstrated by Buysse *et al.* (18) by the simultaneous screening of as many as 51 known mutations in the cystic fibrosis gene, in the molecular diagnosis of other genetic and infectious diseases (19–24), and has the potential to become a routine method for both laboratory and clinical applications (25–27).

We describe a novel bi-plex assay for *ApoE* genotyping based on the MALDI-TOF mass spectrometry and the hME technology, and report the distribution of the *ApoE* variants in a Caucasian based cohort from central Wisconsin.

MATERIALS AND METHODS

DNA samples

A total of 120 anonymized genomic DNA samples were tested, 41 of which were cross-validated using PCR-RFLP. Seven samples were previously tested and kindly provided to us by the Mayo Clinic reference laboratory. The majority of the samples were of Caucasian ancestry (108 samples), 3 of Asians, 2 of Hispanics and 7 samples were of unknown ethnicity (Mayo Clinic samples).

Primer design

Both the amplification and extension primers were designed using the Assay Designer 2.05-software from Sequenom. This software designs the primers used for amplification and the base extension reactions. It also identifies the appropriate termination mixture to use in the hME reaction. The amplification primers were designed with a 10mer tag sequence to increase their mass so that they would fall outside the range of detection of the MALDI-TOF mass spectrometry. The primers, the expected extended products, and their corresponding masses are presented in Tables 1 and 2.

PCR for the MALDI-TOF analysis

For the bi-plex PCR amplification, a total of 25 ng of genomic DNA was amplified in a 5 µl reaction mixture containing 0.1 U HotStar Taq enzyme (Qiagen, Valencia, CA), 1× HotStar

Table 1. *ApoE* extension primers and products, with their calculated masses

Primer	Sequence/extension	M (Da)	Variation
ApoE112-UE	CGGACATGGAGGACGTG	5300.5	
ApoE112-C	CGGACATGGAGGACGTG <u>ddC</u>	5573.6	C
ApoE112-T	CGGACATGGAGGACGTG <u>TddC</u>	5917.9	T
ApoE158-UE	CCGATGACCTGCAGAAG	5204.4	
ApoE158-C	CCGATGACCTGCAGAAG <u>ddC</u>	5477.6	C
ApoE158-T	CCGATGACCTGCAGAAG <u>TddC</u>	5821.8	T

The extended products are underlined. 'UE' denotes 'unextended.'

Table 2. Primers encompassing the *ApoE112* and *ApoE158* variants

Primer	Sequence
ApoE112(F)	5'- <u>ACGTTGGATGCTGTCCAAGGAGCTGCAGG</u> -3'
ApoE112(R)	5'- <u>ACGTTGGATGTCGCCGCGGTACTGCACCA</u> -3'
ApoE112E	5'-CGGACATGGAGGACGTG-3'
ApoE158(F)	5'- <u>ACGTTGGATGTCGCCGATGCCGATGACCTG</u> -3'
ApoE158(R)	5'- <u>ACGTTGGATGTCGCCGATGGCGCTGAGG</u> -3'
ApoE158E	5'-CCGATGACCTGCAGAAGC-3'
ApoE(F)	5'-CTGGGCGCGGACATGGAGGAC-3'
YWL-ApoE(R)	5'-CACATGACCATGTCCCTTATGCCCGGCC TGGTACACTGC-3'

The 10mer tag sequence is italicized and underlined. The shaded primers were used for the PCR-RFLP analysis. The YWL-tail sequence is in boldface and underlined.

buffer, 2.5 mM (total) MgCl₂, 0.2 mM (each) deoxynucleotide triphosphate, and 150 nM of each of the *ApoE112* and *ApoE158* forward and reverse primers. The PCR step was initiated with a 95°C soak for 15 min, followed by 45 cycles, consisting of 95°C for 20 s, 56°C for 30 s, 72°C for 60 s, and a final extension of 3 min at 72°C.

Shrimp alkaline phosphatase (SAP)

After PCR, the remaining unincorporated dNTPs were dephosphorylated by adding 2 µl of the SAP cocktail, containing 1.53 µl of water, 0.17 µl of hME reaction buffer (Sequenom) and 0.3 µl of SAP (Sequenom). The 384-well plate was then sealed and placed in a thermal cycler with the following conditions: 37°C for 20 min, 85°C for 5 min and 4°C indefinitely.

hME reaction, nanodispensing and mass spectrometry

After the SAP treatment, a 2 µl cocktail, consisting of 1.728 µl water; 0.2 µl hME EXTEND mixture, containing 10× buffer and appropriate d/ddNTPs (Sequenom); 0.54 µl of 10 µM each extension primer mixture and 0.018 µl of 32 U/µl ThermoSequenase (Sequenom) was added. After the hME cocktail addition, the plate was again sealed and placed in a thermal cycler with the following program: 94°C for 2 min followed by 55 cycles of 94°C for 5 s, 52°C for 5 s and 72°C for 5 s. The reaction mixture was then desalted by adding 20 µl of a cationic resin mixture (6 mg), SpectroCLEAN (Sequenom). The plate was then sealed and placed in a rotating shaker for 10 min to desalt the hME solution. Completed genotyping reactions were spotted in nanoliter volumes onto a matrix arrayed silicon chip with 384 elements (Sequenom SpectroCHIP) using the MassARRAY Nanodispenser. SpectroCHIPS were analyzed using the Bruker Autoflex MALDI-TOF mass spectrometer and the spectra were processed using the SpectroTYPER software (Sequenom).

PCR-RFLP

A total of 40–50 ng of DNA was amplified in a 25 μ l reaction mixture containing a final concentration of 1.5 mM MgCl₂, 1 \times buffer (Qiagen), 200 μ M of each dNTP, 0.4 μ M of each of the ApoE (F) and YWL-ApoE (R) primers (shaded rows in Table 2), and 0.65 U HotStarTaq DNA polymerase (Qiagen). The PCR was initiated with a 95°C soak for 15 min followed by amplification for 35 cycles (94°C for 30 s, 65°C for 30 s and 72°C for 30 s), and a final extension for 10 min at 72°C. The YWL-tail attached to the YWL-ApoE primer was added to the primer so that the PCR products, if required to be, can be fluorescently labeled using a fluorescently labeled universal primer that has a primer sequence exactly similar to the tail. Nevertheless, for the validation purposes, the universal primer was never added to the reaction mixture and the PCR product was simply resolved on a regular 3% agarose gel as shown in Figure 1.

A volume of 5 μ l of the 209 bp PCR product was digested with 2.5 U of AFLIII or 3 U of HaeII enzymes for the

genotyping of the *ApoE112* and the *ApoE158* polymorphisms, respectively, in a 25 μ l reaction containing 1 \times NE buffer 2 and 80 μ g/ml BSA. All digestions were incubated at 37°C for a minimum of 3 h. The digested PCR product was resolved on a 3% agarose gel (NuSeive 3:1 agarose) containing 5 μ l of 10 mg/ml ethidium bromide. The gel was electrophoresed for 55 min at 90 V and visualized under ultraviolet light.

RESULTS

The hME reaction from Sequenom is outlined in Figure 2. Briefly, the assay involves PCR amplification, SAP treatment to remove excess dNTPs, minisequencing using a mixture of dideoxy and deoxy NTPs, cleanup of the extension reaction to remove salt, spotting of the extension product into 384 SpectroCHIPS and the analysis of the spotted product using the MALDI-TOF mass spectrometry. The entire procedure requires only a single tube or well into which reagents are dispensed in successive reaction steps. An example of the spectra obtained from the genotyping of an individual who is compound heterozygous for both the *ApoE112* and *ApoE158* polymorphisms is shown in Figure 3.

A total of 120 unique and anonymized DNA samples were tested using the MALDI-TOF mass spectrometry. Scored alleles were assigned a confidence score based on the call (conservative, moderate, aggressive and low probability). More than 99% of the calls had a conservative score and did not require manual checking. Moreover, 57 samples were tested in duplicates in different experiments and the results were always 100% concordant.

For assay validations, out of the 120 samples tested 7 were of known genotypes, provided to us by Mayo Clinic, and 41 samples were genotyped using the PCR-RFLP method using AFLIII and HaeII enzymes, which are specific for the *ApoE112* (T/C) and the *ApoE158* (C/T) alleles, respectively. Both enzymes were used previously and were validated for the analysis of *ApoE* genotypes (7,28–30). This method has an advantage over the traditional method utilizing the HhaI enzyme that was developed by Hixson and Vernier (8) in that the product is large enough that it can be visualized in an agarose gel, whereas in the method of Hixson and Vernier the HaeI fragments that are obtained are of very small size that requires to be analyzed by acrylamide gel electrophoresis and the use of radioactivity (8). The AFLIII cuts the *ApoE112* 'T' allele and results in 185 bp, but does not cut the 'C' allele, whereas HaeII cuts the *ApoE158* 'C' allele and results in 165 bp, but does not cut the 'T' allele. An example of the PCR-RFLP is shown in Figure 1. The data derived from the MALDI-TOF hME assay were compared with the data derived from the PCR-RFLP assay and the samples from Mayo Clinic. There was 100% concordance between the two assays and with the samples obtained from Mayo Clinic.

We have also studied the prevalence of the *ApoE* genotypes in a cohort from central Wisconsin. Of the 216 chromosomes from the 108 Caucasian individuals from central Wisconsin who were typed in this study, the frequency of *E2*, *E3* and *E4* was 5.1% (11 chromosomes), 81.5% (176 chromosomes) and 13.4% (29 chromosomes), respectively. The frequency of each of the *ApoE* genotypes from this study is presented in Table 3.

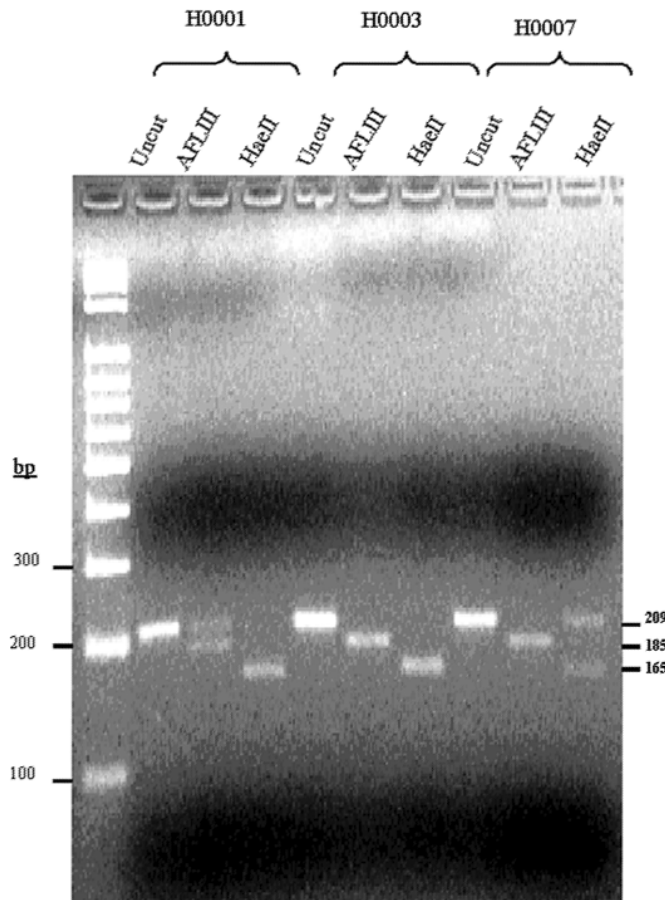


Figure 1. Validation of *ApoE* variants using PCR-RFLP. PCR products from representative samples were analyzed on a 3% agarose gel. From left to right, lane 1 is the 1 kb ladder size, standard; lanes 2–4, H0001; lanes 5–7, H0003; and lanes 8–10, H0007. Uncut PCR products are loaded in lanes 2, 5 and 8. AFLIII digestion products specific for the *ApoE112* site are loaded in lanes 3, 6 and 9. HaeII digestion products specific for the *ApoE158* site are loaded in lanes 4, 7 and 10. H0001 is (T/C) at position 112 and (C/C) at position 158, resulting in a genotype of *E3/E4*; H0003 is (T/T) at position 112 and (C/C) at position 158, resulting in a genotype of *E3/E3*; and H0007 is (T/T) at position 112 and (C/T) at position 158, resulting in a genotype of *E2/E3*.

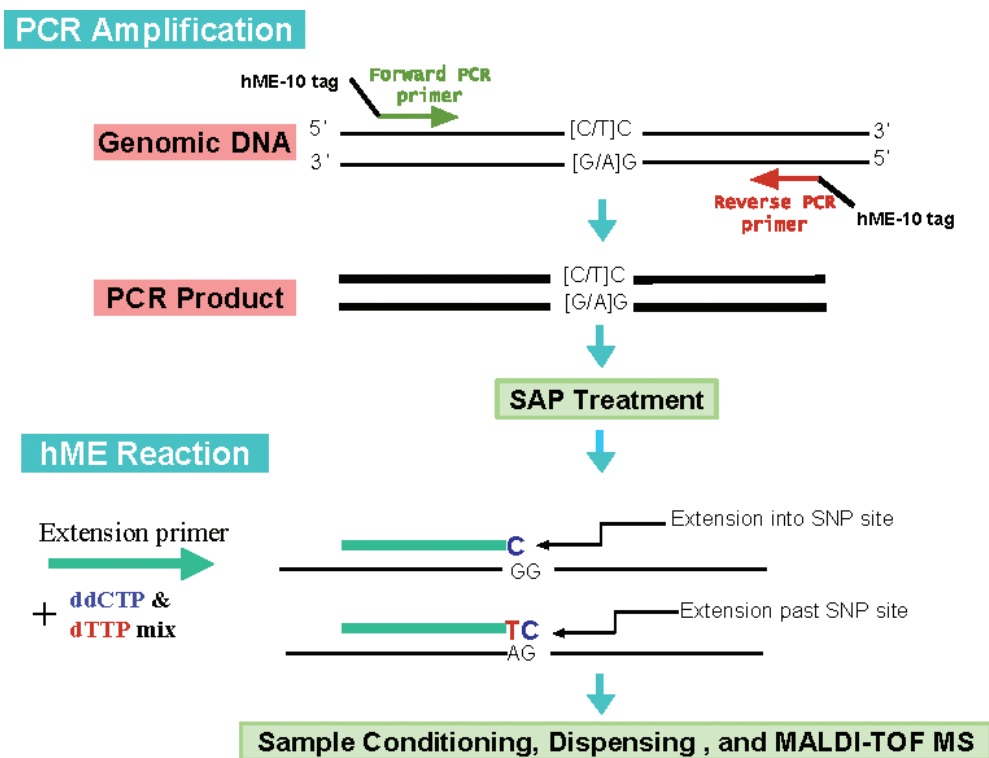


Figure 2. Outline of the homogeneous mass-extend (hME) technology. The ApoE detection involves the amplification and detection of the two variants, simultaneously. For simplicity, only one variant is illustrated in this diagram.

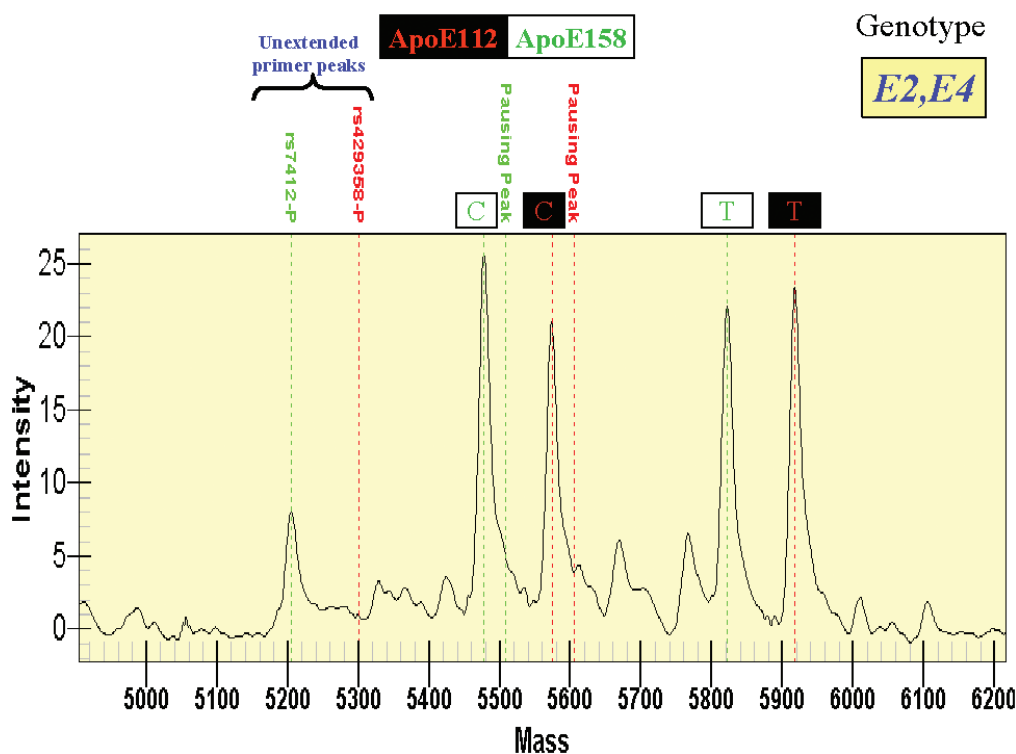


Figure 3. Simultaneous detection of the hME products for ApoE112 and ApoE158 variants using MALDI-TOF mass spectrometry. An example of the MALDI-TOF detection is shown in this figure. The x-axis shows the mass of the analytes in Daltons, the y-axis shows the intensity of the peak. The ApoE112 calls are shown in open boxes, whereas the ApoE158 are shown in solid boxes. The positions of pausing peaks that may result from incomplete extension of the extending primer are shown by dotted lines and are distinguishable from the fully extended product. For this assay we have not seen any detectable pausing peaks. The genotype call is shown in the upper right of the figure.

Table 3. Genotypes and frequency of *ApoE* variants

Call	<i>ApoE</i> 112	<i>ApoE</i> 158	Frequency (%)
<i>E2, E2</i>	TT	TT	0.9
<i>E3, E3</i>	TT	CC	66.7
<i>E4, E4</i>	CC	CC	0.9
<i>E2, E3</i>	TT	TC	6.5
<i>E2, E4</i>	TC	TC	1.9
<i>E3, E4</i>	TC	CC	23.1

DISCUSSION

We have developed an efficient assay for the simultaneous genotyping of *ApoE* variants in a single bi-plex–single well reaction utilizing the MassARRAY system and the hME technology. The MassARRAY system distinguishes molecules based solely on molecular weight. This feature adds so much importance, particularly for genetic testing, because of the extreme high accuracy of the mass spectrometer. In all the testing that was performed with the *ApoE* assay, we found that this system is extremely accurate and highly reproducible. Moreover, the direct detection of analytes solely based on their molecular weights eliminates the need for labeling or any separation steps. This particular feature when combined with the high multiplexing capability, the wide range of mass detection and the very small reaction volumes makes this system in the long run very cost-effective for high-throughput SNP and mutation analysis. Another important feature of this system is that the actual analytes can be detected directly in real-time, and therefore if a reaction failed, the cause of failure can be easily identified by comparing the size of the peaks of the unextended with the extended primers. Finally, the single tube format, with all the steps needed for PCR amplification, phosphatase treatment, primer extension and desalting of the extension product, performed in the same well without the need for transferring the reaction products for purification outside the well considerably enhances the genotyping throughput and eliminates potential errors associated with the transfer of reaction products.

It has been proposed that the *ApoE E4* allele may act as a thrifty allele. The exposure of *ApoE E4* to the contemporary environmental conditions (western diet and longer lifespan) could have rendered it a susceptibility allele for coronary artery disease and Alzheimer's disease (31). We identified the frequency of the *ApoE* alleles, and specifically *ApoE E4* allele, in a cohort from central Wisconsin. A recent self-reported ethnic origin survey on a large cohort from Marshfield Clinic Personalized Medicine research program, one of the largest DNA biobanks in the world with more than 18 000 participants from central and northern Wisconsin, has found that 76.7% of all Caucasians in central Wisconsin are of German origin (32).

Two separate large cohort studies reported frequencies of the *ApoE* alleles in Caucasians from the US and Europe. Using a cohort of US Caucasians of non-Hispanic origin, Mastana *et al.* (33) reported allele frequencies of 8, 78 and 14% for *E2*, *E3* and *E4*, respectively. Recently, a more relevant larger study that investigated *ApoE* allele distribution in the world, including Germany, reported a 7.7, 77.8 and 14.5% for *E2*, *E3* and *E4*, respectively, for the German population ($n = 2031$). This particular study also found that the *ApoE E2* frequency

fluctuates with no apparent trend ranging from 2% to 14.5% in the world (31).

Despite the relatively small size of our cohort ($n = 108$), we observed an overall allele distribution similar to previous studies, with no significant difference in allele distribution (Chi-squared test, $P > 0.56$).

In summary, we have developed a highly accurate genotyping assay for *ApoE* 112 and 158 polymorphisms using the MALDI-TOF mass spectrometry and the hME technology that is suitable for both clinical and large-scale research applications.

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Conflict of interest statement. None declared.

REFERENCES

- Mahley, R.W. (1988) Apolipoprotein E: cholesterol transport protein with expanding role in cell biology. *Science*, **240**, 622–630.
- Breslow, J.L., Zannis, V.I., SanGiacomo, T.R., Third, J.L., Tracy, T. and Glueck, C.J. (1982) Studies of familial type III hyperlipoproteinemia using as a genetic marker the apoE phenotype E2/2. *J. Lipid Res.*, **23**, 1224–1235.
- Feussner, G., Feussner, V., Hoffmann, M.M., Lohrmann, J., Wieland, H. and Marz, W. (1998) Molecular basis of type III hyperlipoproteinemia in Germany. *Hum. Mutat.*, **11**, 417–423.
- Civeira, F., Pocovi, M., Cenarro, A., Casao, E., Vilella, E., Joven, J., Gonzalez, J., Garcia-Otin, A.L. and Ordovas, J.M. (1996) Apo E variants in patients with type III hyperlipoproteinemia. *Atherosclerosis*, **127**, 273–282.
- Davignon, J., Gregg, R.E. and Sing, C.F. (1988) Apolipoprotein E polymorphism and atherosclerosis. *Arteriosclerosis*, **8**, 1–21.
- Corder, E.H., Saunders, A.M., Strittmatter, W.J., Schmechel, D.E., Gaskell, P.C., Small, G.W., Roses, A.D., Haines, J.L. and Pericak-Vance, M.A. (1993) Gene dose of apolipoprotein E type 4 allele and the risk of Alzheimer's disease in late onset families. *Science*, **261**, 921–923.
- Chapman, J., Estupinan, J., Asherov, A. and Goldfarb, L.G. (1996) A simple and efficient method for apolipoprotein E genotype determination. *Neurology*, **46**, 1484–1485.
- Hixson, J.E. and Vernier, D.T. (1990) Restriction isotyping of human apolipoprotein E by gene amplification and cleavage with HhaI. *J. Lipid Res.*, **31**, 545–548.
- Addya, K., Wang, Y.L. and Leonard, D.G. (1997) Optimization of apolipoprotein E genotyping. *Mol. Diagn.*, **2**, 271–276.
- Little, D.P., Braun, A., Darnhofer-Demar, B. and Koster, H. (1997) Identification of apolipoprotein E polymorphisms using temperature cycled primer base extension and mass spectrometry. *Eur. J. Clin. Chem. Clin. Biochem.*, **35**, 545–548.
- Srinivasan, J.R., Kachman, M.T., Killeen, A.A., Akel, N., Siemieniak, D. and Lubman, D.M. (1998) Genotyping of apolipoprotein E by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom.*, **12**, 1045–1050.
- Komiyama, M., Ye, S., Liang, X., Yamamoto, Y., Tomita, T., Zhou, J.M. and Aburatani, H. (2003) PNA for one-base differentiating protection of DNA from nuclease and its use for SNPs detection. *J. Am. Chem. Soc.*, **125**, 3758–3762.
- Ren, B., Zhou, J.M. and Komiyama, M. (2004) Straightforward detection of SNPs in double-stranded DNA by using exonuclease III/nuclease S1/PNA system. *Nucleic Acids Res.*, **32**, e42.

14. Jacobsen,N., Bentzen,J., Meldgaard,M., Jakobsen,M.H., Fenger,M., Kauppinen,S. and Skouv,J. (2002) LNA-enhanced detection of single nucleotide polymorphisms in the apolipoprotein E. *Nucleic Acids Res.*, **30**, e100.
15. Jurinke,C., van den Boom,D., Cantor,C.R. and Koster,H. (2002) Automated genotyping using the DNA MassArray technology. *Methods Mol. Biol.*, **187**, 179–192.
16. Bray,M.S., Boerwinkle,E. and Doris,P.A. (2001) High-throughput multiplex SNP genotyping with MALDI-TOF mass spectrometry: practice, problems and promise. *Hum. Mutat.*, **17**, 296–304.
17. Gabriel,S.B., Schaffner,S.F., Nguyen,H., Moore,J.M., Roy,J., Blumenstiel,B., Higgins,J., DeFelice,M., Lochner,A., Faggart,M. *et al.* (2002) The structure of haplotype blocks in the human genome. *Science*, **296**, 2225–2229.
18. Buyse,I.M., McCarthy,S.E., Lurix,P., Pace,R.P., Vo,D., Bartlett,G.A., Schmitt,E.S., Ward,P.A., Oermann,C., Eng,C.M. and Roa,B.B. (2004) Use of MALDI-TOF mass spectrometry in a 51-mutation test for cystic fibrosis: evidence that 3199del6 is a disease-causing mutation. *Genet. Med.*, **6**, 426–430.
19. Hung,K., Sun,X., Ding,H., Kalafatis,M., Simioni,P. and Guo,B. (2002) A matrix-assisted laser desorption/ionization time-of-flight based method for screening the 1691G(A mutation in the factor V gene. *Blood Coagul. Fibrinolysis*, **13**, 117–122.
20. Humeny,A., Bonk,T., Berkholz,A., Wildt,L. and Becker,C.M. (2001) Genotyping of thrombotic risk factors by MALDI-TOF mass spectrometry. *Clin. Biochem.*, **34**, 531–536.
21. Bonk,T., Humeny,A., Sutter,C., Gebert,J., von Knebel Doeberitz,M. and Becker,C.M. (2002) Molecular diagnosis of familial adenomatous polyposis (FAP): genotyping of adenomatous polyposis coli (APC) alleles by MALDI-TOF mass spectrometry. *Clin. Biochem.*, **35**, 87–92.
22. Meyer,K., Fredriksen,A. and Ueland,P.M. (2004) High-level multiplex genotyping of polymorphisms involved in folate or homocysteine metabolism by matrix-assisted laser desorption/ionization mass spectrometry. *Clin. Chem.*, **50**, 391–402.
23. Harksen,A., Ueland,P.M., Refsum,H. and Meyer,K. (1999) Four common mutations of the cystathionine beta-synthase gene detected by multiplex PCR and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. *Clin. Chem.*, **45**, 1157–1161.
24. Ilina,E.N., Malakhova,M.V., Generozov,E.V., Nikolaev,E.N. and Govorun,V.M. (2005) Matrix-assisted laser desorption ionization-time of flight (mass spectrometry) for hepatitis C virus genotyping. *J. Clin. Microbiol.*, **43**, 2810–2815.
25. Marvin,L.F., Roberts,M.A. and Fay,L.B. (2003) Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry in clinical chemistry. *Clin. Chim. Acta*, **337**, 11–21.
26. Corona,G. and Toffoli,G. (2004) High throughput screening of genetic polymorphisms by matrix-assisted laser desorption ionization time-of-flight mass spectrometry. *Comb. Chem. High Throughput Screen.*, **7**, 707–725.
27. Tost,J. and Gut,I.G. (2005) Genotyping single nucleotide polymorphisms by MALDI mass spectrometry in clinical applications. *Clin. Biochem.*, **38**, 335–350.
28. Hoffmann,M.M., Scharnagl,H., Koster,W., Winkler,K., Wieland,H. and Marz,W. (2001) Apolipoprotein E1 Baden (Arg(180)→Cys). A new apolipoprotein E variant associated with hypertriglyceridemia. *Clin. Chim. Acta*, **303**, 41–48.
29. Francoual,J., Audibert,F., Trioche,P., Chalas,J., Capel,L., Lindenbaum,A., Labrune,P. and Frydman,R. (2002) Is a polymorphism of the apolipoprotein E gene associated with preeclampsia? *Hypertens. Pregnancy*, **21**, 127–133.
30. Zivelin,A., Rosenberg,N., Peretz,H., Amit,Y., Kornbrot,N. and Seligsohn,U. (1997) Improved method for genotyping apolipoprotein E polymorphisms by a PCR-based assay simultaneously utilizing two distinct restriction enzymes. *Clin. Chem.*, **43**, 1657–1659.
31. Corbo,R.M. and Scacchi,R. (1999) Apolipoprotein E (APOE) allele distribution in the world. Is APOE?4 a ‘thrifty’ allele? *Ann. Hum. Genet.*, **63**, 301–310.
32. McCarty,C.A., Wilke,R.A., Giampietro,P.F., Westbrook,S.D. and Caldwell,M.D. (2005) Marshfield Clinic Personalized Medicine Research Project (PMRP): design, methods and recruitment for a large population-based biobank. *Personalized Med.*, **2**, 49–79.
33. Mastana,S.S., Calderon,R., Pena,J., Reddy,P.H. and Papiha,S.S. (1998) Anthropology of the apolipoprotein E (*apo E*) gene: low frequency of apo E4 allele in Basques and in tribal (Baiga) populations of India. *Ann. Hum. Biol.*, **25**, 137–143.