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...
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 (MALDI-TOF) mass spectrometry combined with the homogeneous mass-extend (hME) reaction has proven very useful.

In a wide scale application of SNP genotyping utilizing 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 (MALDI-TOF) mass spectrometry combined with the homogeneous mass-extend (hME) reaction has proven very useful.

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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 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).

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

| Primer       | Sequence/extension | M (Da) | Variation |
|--------------|--------------------|--------|-----------|
| ApoE112-UE  | CGGACATGGAGGACGGT  | 5300.5 |           |
| ApoE112-C   | CGGACATGGAGGACGTC4C| 5573.6 | C         |
| ApoE112-T   | CGGACATGGAGGACGTT2C| 5917.9 | T         |
| ApoE158-UE  | CCGATGACCTGCAAGAGA| 5204.4 |           |
| ApoE158-C   | CCGATGACCTGCAAGAG4C| 5477.6 | C         |
| ApoE158-T   | CCGATGACCTGCAAGAG2C| 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'-ACGGTTGAGCTGTCAGCTGGACTGACCTG-3' |
| ApoE112(R)   | 5'-ACGGTTGAGCTGTCAGCTGGACTGACCTG-3' |
| ApoE112E     | 5'-CGGACATGGAGGACGTC4C |
| ApoE158(F)   | 5'-ACGTGGATGCTCCGATGGATGCCTG-3' |
| ApoE158(R)   | 5'-ACGTGGATGCTCCGATGGATGCCTG-3' |
| ApoE158E     | 5'-CCGTGACCTGCAAGAGA-3' |
| ApoE(F)      | 5'-CTGGCCCGCGGACATGGAGGACAG-3' |
| WYL-ApoE(R)  | 5'-CACATGACCATTCCTGTATGCACCCGGCC-3' |
PCR–RFLP
A total of 40–50 ng of DNA was amplified in a 25 μl reaction mixture containing a final concentration of 1.5 mM MgCl₂, 1x buffer (Qiagen), 200 μM of each dNTP, 0.4 μM 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 μl 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 μl reaction containing 1× NE buffer 2 and 80 μg/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 μl 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 E2/E2; 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   | ApoE112 | ApoE158 | Frequency (%) |
|--------|---------|---------|--------------|
| E2, E2 | TT      | TT      | 0.9          |
| E3, E3 | TT      | CC      | 66.7         |
| E4, E4 | CC      | CC      | 0.9          |
| E2, E3 | TT      | TC      | 6.5          |
| E2, E4 | TC      | TC      | 1.9          |
| E3, E4 | 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.

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