Integrated Single-Label Liquid-Phase Assay of APOE Codons 112 and 158 and a Lipoprotein Study in British Women, Mohammad Reza Abdollahi,1* Philip A.I. Guthrie,1 George Davey Smith,2 Debbie A. Lawlor,2 Shah Ebrahim,3 and Ian N.M. Day1,4 (1 Bristol Genetic Epidemiology Laboratory and 2 Department of Social Medicine, University of Bristol, Bristol, United Kingdom; 3 Department of Epidemiology & Population Health, London School of Hygiene & Tropical Medicine, London, United Kingdom; 4 Human Genetics Division, Southampton General Hospital, Southampton, United Kingdom; 4 address correspondence to this author at: Bristol Genetic Epidemiology Laboratory, University of Bristol, No. 24 Tyndall Ave., Bristol, United Kingdom BS8 1TQ; 4 address correspondence to this author at: Bristol Genetic Epidemiology Laboratory, University of Bristol, No. 24 Tyndall Ave., Bristol, United Kingdom BS8 1TQ; 4 address correspondence to this author at: Bristol Genetic Epidemiology Laboratory, University of Bristol, No. 24 Tyndall Ave., Bristol, United Kingdom BS8 1TQ; e-mail r.abdollahi@bristol.ac.uk and rabdollahi@gmail.com)

Background: Apolipoprotein E (APOE) is an important element of lipid metabolism and, hence, cardiovascular disorders. APOE has 3 main allelic variants: ε3, ε4, and ε2. Of these, ε3 is the most common, followed by ε4 and ε2. The associations of these isoforms with cardiovascular disorders and Alzheimer disease have been widely studied in different populations. Most of the genotyping in these studies has been performed with gel-based methods, which have important limitations, particularly for large epidemiologic studies. We therefore developed an integrated “one-tube” liquid-phase assay.

Methods: To measure APOE isoforms, we developed an integrated single-label liquid-phase fluorescence assay containing 2 PCR primers, 2 probes, and 2 quencher oligonucleotides. We used a 384-well LightTyper, but the assay would be generically applicable for use with any fluorescence detector with thermal ramp control. We validated this method and applied it in the British Women’s Heart and Health Study.

Results: There were 4 melting peaks, at 41, 56, 61, and 69 °C, which generated 6 distinctive patterns representing genotypic combinations of ε3, ε4, and ε2. The magnitude and direction of the associations found with total cholesterol, HDL-cholesterol, triglycerides, and estimated LDL-cholesterol were consistent with previous reports.

Conclusion: The one-tube LightTyper assay presented here enables accurate, convenient, and economical genotyping of APOE and can be used for large epidemiologic studies.

© 2006 American Association for Clinical Chemistry

Apolipoprotein E (APOE) has a pivotal role in the metabolism of chylomicrons, chylomicron remnants, VLDL-cholesterol, and HDL-cholesterol (HDL-C), and as a ligand, it binds both LDL-cholesterol (LDL-C) and APOE receptors (1). The APOE gene is located on chromosome 19q13.2 and has 3 main allelic variants: ε3, ε4, and ε2. ε3 is the most common, followed by ε4 and ε2. These 3 allelic variants differ at 2 single-base variations located in exon 4 and 7.21 mmol/L (both based on 20 replicates), and between-imprecision (CV) was 0.96% at 5.11 mmol/L and 0.81% at 0.45). Venous blood was taken after a fast of at least 6 h and was used to determine total cholesterol, LDL-C, HDL-C, and triglycerides (TGs).

Total cholesterol, HDL-C, and TGs were measured on frozen serum samples (maximum time frozen, 6 weeks) with a Hitachi 757 analyzer (Roche Diagnostics) and standard reagents. LDL-C was estimated using the Friedewald equation [LDL-C = total cholesterol − (HDL-C + TGs × 0.45)] (8). For total cholesterol, the within-batch imprecision (CV) was 0.96% at 5.11 mmol/L and 0.81% at 7.21 mmol/L (both based on 20 replicates), and between-
batch imprecision was 1.3% at 5.24 mmol/L and 2.1% at 7.21 mmol/L (both based on 13 replicates).

For the LightTyper assay, a 495-bp sequence (GenBank accession no. AF261279) of APOE spanning 112T>C and 158C>T was amplified with 2 primers (MWG-Biotech): forward, 5'-GCCTACAAATCGGAACTGGA-3'; reverse, 5'-ACCTGCTCCTCACCTCGT-3'. The probes for the APOE single-base variations 112T>C and 158C>T were as follows: APOE 112F, 5'-fluorescein-GGACGTGCGCGGC-phosphate-3'; APOE 158F, 5'-fluorescein-TGCAAGCGCCCTGGCAGTACC-phosphate-3'. The quencher oligonucleotides were as follows: APOE

![Fig. 1. Pattern of the APOE genotypes. Peaks A and B represent the T and C alleles of APOE 112T>C, respectively; peaks C and D represent alleles T and C of APOE 158C>T, respectively.](image)
templates contained 20 ng of genomic DNA and were plated on 384-well PCR plates [cat. no. TF-0384/W; ABgene (www.abgene.com)] in 2 μL of water and dried at 80 °C for 10 min for storage. The PCR mixture contained 0.5 μL of 10× PCR buffer, 0.05 mM deoxynucleotide triphosphates, 0.05 μM forward primer and 0.25 μM reverse primer (i.e., asymmetric PCR), 0.04 μM each probe, 1.3 M betaine, 2 mM MgCl₂, 0.025 U/μL Taq DNA polymerase (Promega), and H₂O to 5 μL. Thermal cycling, performed on a DNA Engine Tetrad® 2 (MJ Research), was as follows: 94 °C for 2 min, 94 °C for 45 s, 61 °C for 45 s, and 72 °C for 45 s (last 3 steps repeated for 90 cycles), followed by 72 °C for 2 min. PCR products were covered with 5 μL of Chillout 14™ liquid wax (MJ Research), and then centrifuged at 3000 rpm for 3 min before plate analysis in the LightTyper instrument (cat. no. 03 357414001; Roche Diagnostics GmbH).

Genotypes were determined automatically with LightTyper software, Ver. 1 (Roche Diagnostics GmbH), and validated in-house software (9). Genotyping using HhaI restriction digestion (5) was also performed as a validation test on 96 random samples representing all 6 genotypes.

As shown in Fig. 1, the 6 APOE genotypes had distinctive patterns. The peaks at 41 and 56 °C represented the T and C alleles of APOE 112T–C, respectively, and the peaks at 61 and 69 °C corresponded to the T and C alleles of APOE 158C>T, respectively. The characteristic peaks for the different genotypes were as follows: e3/e3 gave 2 peaks at 41 and 69 °C; e4/e4 gave 2 peaks at 56 and 69 °C; e2/e2 gave 2 peaks at 41 and 61 °C; e3/e4 gave 3 peaks at 41, 56, and 69 °C; e3/e2 gave 3 peaks at 41, 61, and 69 °C; and e4/e2 gave 4 peaks at 41, 56, 61, and 69 °C (Fig. 1). The relative peak heights also showed characteristic quantitative differences according to genotype. The frequencies of e3, e4, and e2 were 0.78, 0.14, and 0.08, respectively. We genotyped 94% of samples successfully, and genotype frequencies were in Hardy–Weinberg equilibrium (χ² = 5.722; P = 0.13; df = 3).

As a further validation of this assay, we examined the association of APOE with lipids in the participants (see Table 1 in the Data Supplement that accompanies the online version of this Technical Brief at http://www.clinchem.org/content/vol52/issue7/). Total cholesterol, HDL-C, TGs, and estimated LDL-C were all associated with APOE genotype. The magnitudes and directions of these associations were consistent with those reported in the literature for similar populations (10). Thus, total cholesterol and LDL-C were lower in individuals with the e2 allele and higher in those with the e4. For HDL-C, e2 allele associations were more notable, and for TGs, the effect of e4 homozygosity was most notable. For LDL-C and total cholesterol, but not for HDL-C, the effect of the e2 allele dominated that of the e4 allele in e2/e4 heterozygotes.

We have established a one-tube LightTyper assay for genotyping APOE alleles 2, 3, and 4 that could be performed in a 384-well LightTyper instrument. This assay is suitable for epidemiologic studies. To maximize resolution of alleles carrying single-base variations, it is preferable to choose the most destabilizing base pair mismatch to maximize the difference in melting temperature. The difference in melting temperature between the quencher nucleotide and the probe for the single-base variation is also of major importance.

In our initial development (data not shown), we used both separate short amplicons spanning codons 112 and 158, respectively, and subsequently, separate binding assays on one larger amplicon. The former was used to genotype the same initial core plate of 360 samples. The peak heights were somewhat higher for the 112 assay when we used the separate short amplicons, but all genotype calls were the same with the 2-assay format, the integrated assay, and the gel-based assay.

Integrated assays of e2, e3, and e4 have included molecular haplotyping using single-strand conformational polymorphism analysis (11), denaturing gradient gel electrophoresis (12), multiple-primer single-base extension capillary electrophoresis Snapshot assays (13), and a commercial dual-wavelength fluorescence assay (Roche LightCycler). The first 3 are gel-based methods that are not economical for use in high-throughput assays, and the latter is a dual-label approach that incompletely differentiates the 4 relevant melt peaks by temperature and uses proprietary design and reagents. The combination of 2 probes for codons 112 and 158 that give 4 well-separated melting temperatures for respective mismatch and match binding in conjunction with simple end-point fluorescence reading using 1 label and wavelength has enabled the integrated liquid-phase assay design described here.

Studies of e2/e4 heterozygotes have revealed that the E4 protein isoform is catabolized from all lipoprotein fractions ~3-fold faster than the E2 protein isoform (14), whereas E3 is catabolized at an intermediate rate (15–17). In e2/e4 heterozygotes, E2 is found predominantly in HDL-C, whereas E4 is equally distributed in VLDL and HDL (14). The APOE isoforms differ in metabolic pathways through the classes and sizes of lipoproteins underpinning the observed associations with total cholesterol, LDL-C, HDL-C, and TGs. The consistency of associations observed for each genotype group with other studies further validates this new integrated APOE assay. The same oligonucleotide mixture and PCR conditions may be suitable for adaptation to other PCR instruments and fluorescence readers.

M.R.A. and P.A.I.G. were supported by the University of Bristol. BWHHS is funded by the UK Department of Health. Genetic studies in BWHHS have been supported by the British Heart Foundation (BHF). D.A.L. is funded by a UK Department of Health career scientist award.
Insulin assays have been widely used to provide diagnostic information for diabetes mellitus and rare hypoglycemic syndromes (1–3) and to quantify insulin for pharmacokinetic evaluations. The ARCHITECT® insulin assay (Abbott Laboratories) is a 1-step chemiluminescent immunoassay that uses paramagnetic microparticles coated with anti-insulin monoclonal antibody and acridinium-labeled anti-insulin monoclonal antibody conjugate. Samples, microparticles, and conjugate are added to a reaction vessel to form a particle–insulin–conjugate sandwich. After incubation, washing removes materials not bound in the 1st step. Addition of the pretrigger reagent, which includes hydrogen peroxide, and the trigger reagent, which includes sodium hydroxide, leads to acridinium-produced chemiluminescence, measured as relative light units (RLUs). The RLUs are proportional to the insulin concentration in the sample.

Methods: We used Clinical and Laboratory Standards Institute protocols to assess the analytical performance of the ARCHITECT insulin assay and compared its accuracy with that of the E-test TOSOH II (IRI) from TOSOH Corporation. We used 3 recombinant insulin analogs (lispro, aspart, and glargine) to evaluate the cross-reactivity of insulin analogs with the ARCHITECT immunoassay reagent.

Results: The total CV for the ARCHITECT assay was <5%. Correlation between the ARCHITECT insulin assay and the E-test TOSOH II (IRI) was satisfactory in the measured range, but we detected a slope deviation between the assays. The ARCHITECT insulin assay showed low cross-reactivity to the insulin analog aspart, whereas it detected the other insulin analogs, lispro and glargine, in concentrations as high as the theoretical dilution linearity, detection limit, and effects of interfering substances. When interpreting results, clinicians and laboratory pathologists should be aware of the cross-reactivity of the ARCHITECT and other immunoassays to specific insulin analogs prescribed to diabetes patients.

© 2006 American Association for Clinical Chemistry