Closed-tube genotyping of apolipoprotein E by isolated-probe PCR with multiple unlabeled probes and high-resolution DNA melting analysis

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Isolated-probe PCR (IP-PCR) is a method that combines asymmetric PCR, unlabeled probes, and high-resolution DNA melting while maintaining a closed tube system. A double-stranded DNA (dsDNA) dye LCGreen® I was used to detect the unlabeled probes. LCGreen I is also used to detect the 277-base pair PCR product peak as an internal amplification control. To accomplish this, IP-PCR separates the asymmetric PCR amplification step and the detection step of the unlabeled probes. This prevents the probes from interfering with the amplification of the DNA target. The samples are then melted using a high-resolution DNA melting instrument: the HR-1™. The closed tube system virtually eliminates PCR product contamination or sample carryover. The target apolipoprotein E (APOE) was chosen to test the IP-PCR technique. APOE contains two single nucleotide polymorphisms (SNPs) located 139 base pairs apart in a GC-rich region of the human genome. The results from this study show that the IP-PCR technique was able to determine the correct APOE genotype for each of the 101 samples. The IP-PCR technique should also be useful in detecting SNPs in other high-GC regions of the human genome.

INTRODUCTION

Many PCR techniques have been used to genotype apolipoprotein E (APOE). These include real-time PCR (1), capillary electrophoresis with laser-induced fluorescence (2), denaturing high-performance liquid chromatography (3), TaqMan® assays (4), matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and homogeneous mass-extend technology (5), allele-specific PCR (6), and real-time PCR combined with fluorescence resonance energy transfer hybridization probes (7).

Isolated-probe PCR (IP-PCR) is a new technique that separates unlabeled probes from asymmetric PCR. The unlabeled probes are placed in the top of the capillary tubes. This prevents the probes from interfering with the asymmetric amplification step. After amplification, the LightCycler® capillary tubes are inverted and centrifuged using a desktop centrifuge, forcing the PCR mixture into the cap to mix the unlabeled probes and the PCR mixture. An additional denaturing and annealing cycle is needed for the probes to hybridize to the template to create a detectable signal. The samples are then melted using a high-resolution melting instrument to determine the six different APOE genotypes.

Asymmetric PCR selectively amplifies one strand of the genomic DNA (8). This is accomplished by using unequal concentrations of forward and reverse primers. The optimum asymmetric ratio of forward to reverse primers needs to be determined experimentally for each target (9). The PCR mixture contains a double-stranded dye LCGreen® I (Idaho Technology, Salt Lake City, UT, USA) that fluoresces when it is bound to double-stranded DNA (dsDNA) (10).

In conventional asymmetric PCR, the probes need to have a melting temperature lower than the extension temperature so that they do not hybridize to the DNA template when the polymerase is extending the primers (9). This limits the probe design and makes it difficult to design probes that are long enough to produce a strong fluorescent signal, especially in high-GC targets like APOE. Primer and probe design for asymmetric PCR often requires several attempts to obtain a set of compatible primers and unlabeled probes that will amplify the specific target and produce an adequate signal.

Optimization of IP-PCR is straightforward because there are no probes in the PCR to interfere with the primers or the DNA polymerase. Designing probes for IP-PCR is also straightforward; the only restriction being that the probe melting temperature must be distinguishable from the PCR product peak. IP-PCR also allows multiple unlabeled probes to be multiplexed in one reaction as long as they have different melting temperatures.

MATERIALS AND METHODS

DNA Isolation

Previously genotyped whole blood samples of the six APOE genotypes were obtained from the molecular pathology laboratory at ARUP (Salt Lake City, UT, USA). These samples were genotyped at ARUP using a LightCycler ApoE Mutation Detection kit (Roche Applied Science, Indianapolis, IN, USA). The samples were then deidentified for use in this experiment. The DNA was isolated using the PUREGENE® Genomic DNA Purification kit from Gentra Systems (Minneapolis, MN, USA).

Primer and Probe Design

Both the forward and reverse primers were designed using a DNA melting temperature calculator program that was written using LabVIEW (National Instruments, Austin, TX, USA). The sequence of the forward primer is 5′-ACGCGGGCACGGC TGTCCAAGG-3′. The reverse primer sequence is 5′-GGCGCTCGCGGAT GGCGCTGA-3′. The forward and reverse primers amplify a PCR product 277-base pairs-long with a melting temperature of 93.1°C and a GC content of 75%. The GenBank® accession no. for APOE is K00396.

The APOE 112 probe sequence is 5′-ACATGGAGGACGTG**T**GCGGC CGCCTG-P-3′. The APOE 158 probe sequence is 5′-GCGGCTCCTCCGC GATGCCGATGACCTGCAGAAG**C** GCCTGGC-P-3′. The SNP position is indicated by the underlined and bold text.

All primers and probes were synthesized by the Core Facility at the University of Utah (Salt Lake City, UT, USA).

Asymmetric PCR with Isolated Probes

PCR was performed in 10-µL reaction volumes with 50 mM Tris buffer, pH 8.3, 500 µg/mL bovine serum albumin (BSA), 2 mM $MgCl_2$, 200 µM each deoxynucleotide triphosphate, 0.4 U KlenTaq1™ polymerase (Ab Peptides, St. Louis, MO, USA), l× LCGreen I, 7% dimethyl sulfoxide (DMSO), 0.05 µM forward primer, 0.5 µM reverse primer, and 5 ng/µL human DNA. This mixture was pipeted into the capillary tube. It was next centrifuged down to the bottom of the capillary tube using a desktop centrifuge at 2000× *g* for 3–4 s. Then, 2 µL probe mixture containing 0.25 µM both 112 and 158 probes were pipeted into the top plastic well of the capillary tube. Finally, the capillary tube was capped.

LightCycler 2.0 Amplification Protocol

PCR was performed in a LightCycler 2.0 instrument (Roche Applied Science) with an initial denaturing step at 95°C for 15 s; followed by 50 cycles of denaturation at 95°C for 2 s; annealing at 68°C for 3 s; and extension at 72°C for 10 s. Transition rates between temperatures were programmed at 20°C/s. Fluorescence was acquired once each cycle at the end of the extension step.

Combining the Isolated Probes and the Asymmetric PCR Products

Following the PCR, the LightCycler capillary tubes were removed from the intrument's capillary carousel, inverted, and centrifuged for 2–3 s at approximately 1000–2000× *g*. The capillary tubes were placed back into the capillary carousel. The carousel was then placed into an LC Carousel Centrifuge 2.0 (Roche Applied Science) and centrifuged. Prior to high-resolution melting, the samples were denatured at 95°C for 5 s and renatured at 45°C for 5 s for 2 cycles in the LightCycler 2.0. This allowed the probes to hybridize to their specific targets.

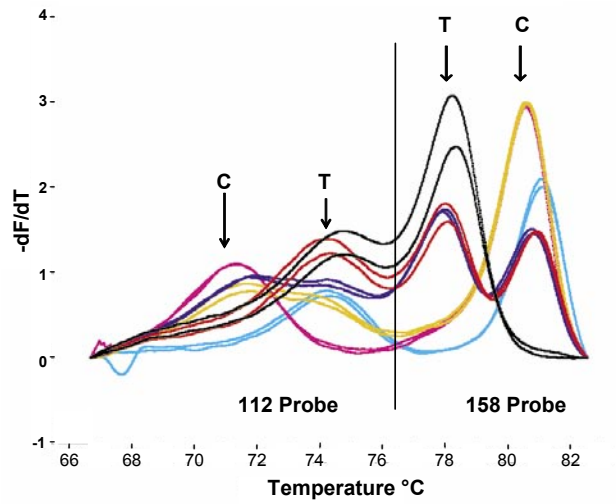

Figure 1. Graph showing the six different melting curve plots that are produced by the two unlabeled probes of the apolipoprotein E gene (*ApoE*) samples. The *ApoE* 2–2 (black curve) genotype is T/T at both the 112- and 158-single nucleotide polymorphism (SNP) positions. The *ApoE* 3–3 (light blue curve) genotype is T/T at SNP position 112, and C/C at SNP position 158. The *ApoE* 4–4 (pink curve) genotype is C/C at both the 112- and 158-SNP positions. The *ApoE* 2–3 (red curve) genotype is T/T at SNP position 112, and T/C at SNP position 158. The *ApoE* 2–4 (dark blue curve) genotype is T/C at both the 112- and 158-SNP positions. The *ApoE* 3–4 (orange curve) genotype is T/C at SNP position 112, and C/C at SNP position 158.

High-Resolution DNA Melting

Following the LightCycler 2.0 protocol, each sample was melted in the HR-1™. The HR-1 instrument is a single-sample, high-resolution DNA melting instrument (Idaho Technology). The HR-1 is capable of recording temperature and fluorescence data at a rate of 50–100 data points per degree Celsius. The starting temperature was 65°C, and ending temperature was 95°C with a ramp rate of 0.3°C/s. The light-emitting diode (LED) power was auto adjusted to 90% fluorescence.

Data Analysis

The data for each sample was analyzed by a melting curve analysis program written by R. Palais (University of Utah Department of Mathematics) using LabVIEW. The APOE genotypes of each sample were then scored according to the number of peaks and the melting temperatures of those peaks.

DNA Sequencing of PCR Samples

The asymmetric PCR samples were prepared by first diluting the sample 1:2 with distilled water. Three microliters of the diluted DNA from each sample were added to a 1.6-mL centrifuge tube. Next, 0.5 µM reverse primer was added. Three microliters of distilled water were added to each centrifuge tube to reach the 7 µL required for sequencing. The symmetric PCR samples were prepared by first diluting the sample 1:4 with distilled water. Three microliters of the diluted DNA sample were added to a 1.6-mL centrifuge tube. Next, 0.5 µM either forward or reverse primer was added. Then 2.5 µL distilled water were added to reach 7 µL needed for sequencing. The samples were sequenced by the Core Facility at the University of Utah. The sequencing data for all samples were analyzed using the SEQUENCHER™ 4.5 software program (Gene Codes, Ann Arbor, MI, USA).

RESULTS AND DISCUSSION

The data from each high-resolution melt was displayed using -dF/dT versus temperature plots or melting peak plots. The two melting peaks for the SNP 112 unlabeled probe showed up at 72°C for the mismatched base C and 75°C for the perfectly matched base T. The two melting peaks for the SNP 158 unlabeled probe were 78°C for the mismatched T and 81.5°C for the perfectly matched C. The apparent genotypes were assigned for each sample using the unique melting peak profiles produced by the 112 and 158 unlabeled probes (see Supplementary Figure S1 available online at www.BioTechniques.com).

The results obtained from ARUP and the LightCycler ApoE Mutation Detection kit and IP-PCR were concordant in 96 out of 101 samples. According to ARUP, samples 101, 102, 103, 105, and 106 were genotyped as the *ApoE* 2–3. The IP-PCR method assigned genotypes to these samples as follows: sample 101, *ApoE* 3–3; sample 102, *ApoE* 3–4; sample 103, *ApoE* 4–4; sample 105, *ApoE* 3–3; and sample 106, *ApoE* 3–4. These five samples were retested using IP-PCR and high-resolution DNA melting. The results from the five retested samples confirmed the previous IP-PCR results.

To determine the exact genotype, the five discordant samples were deidentified and retested at ARUP. Also the original IP-PCR amplification products were sequenced. The retested samples and the DNA sequencing results concurred with the IP-PCR results in all five cases.

The data shows that the IP-PCR technique combined with high-resolution melting was 100% correct in determining the APOE genotype in these 101 DNA samples. IP-PCR provides a simple, cost effective, single tube system that is able to distinguish the six different genotypes of APOE (Figure 1). The region covered by the two unlabeled probes is between 76%–80% GC. This high-GC content presents challenges for both designing primers and for amplification using real-time PCR.

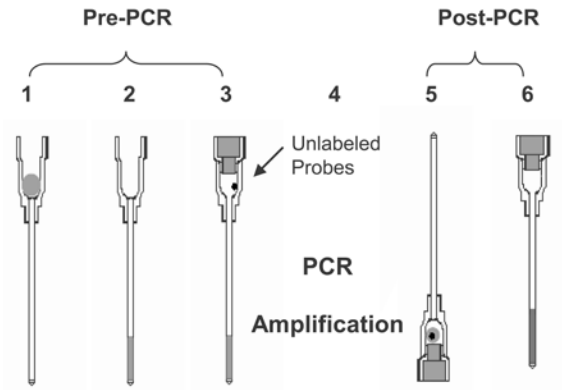

Figure 2. Depiction of the steps involved in isolating the unlabeled probes from the amplification step of PCR using the IP-PCR method. Step 1: pipet 9 µL PC master mix and 1 µL DNA into the tube. Step 2: centrifuge the mixture at 2000× *g* for 1-2 s to the bottom of the tube. Step 3: add 0.5 µM each unlabeled probe and cap the tube. Step 4: amplify samples in the LightCycler. Step 5: invert tube and centrifuge PCR mixture into the lid. Step 6: centrifuge the unlabeled probe and PCR mixture to the bottom of the tube. The samples are then denatured and renatured in the LightCycler. This allows the unlabeled probes to anneal to their single-stranded DNA targets. The samples are then melted in the HR-1.

High-GC segments are known to form secondary structures when single-stranded and, in some instances, the secondary structures are able to form at a higher melting temperature than the primer-DNA duplex (11). The presence of these secondary structures in the PCR inhibits the ability of the primer to bind to the single-stranded DNA and may reduce amplification efficiency or inhibit PCR completely (12).

To overcome these difficulties, DMSO, a PCR enhancer, was added to the PCR (13). DMSO interacts with dsDNA by disrupting the hydrogen bonds between the base pairs of the different DNA strands. This disruption caused by DMSO effectively lowers the melting temperatures of the primers, probes, and the PCR product (14). The addition of DMSO to the PCR improved the amplification and cleaned up some background noise in the high-resolution melting curve plots.

IP-PCR separates the unlabeled probes from the amplification process. In IP-PCR, the unlabeled probes are placed in the plastic well of the LightCycler capillary tube after the PCR mix and DNA have been centrifuged down to the bottom of the tube (Figure 2). This eliminates the potential problems of the probes interfering or inhibiting the PCR (9,15). Isolating the unlabeled probes from PCR allows

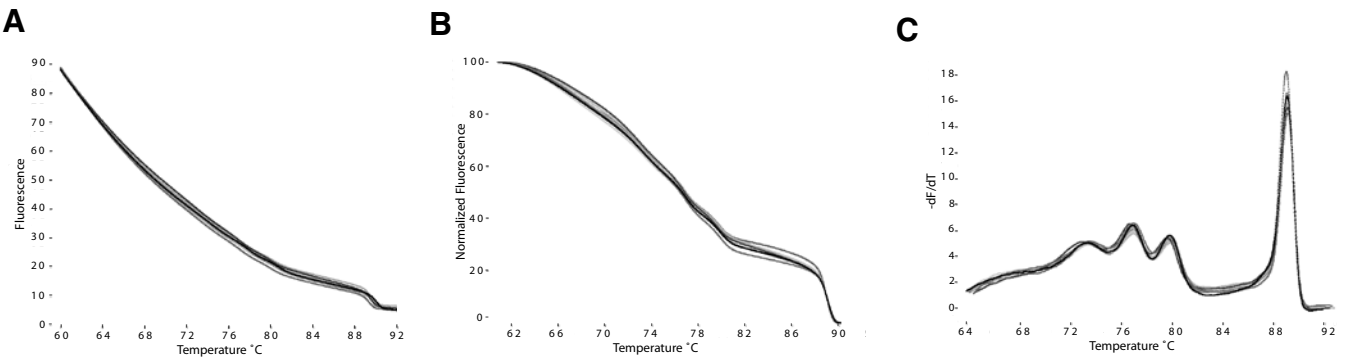

Figure 3. Graphs showing how the raw data from the HR-1 instrument are processed. (A) The raw data from nine apolipoprotein E (APOE) 2–3 samples are imported from the HR-1 instrument. (B) The raw fluorescence data are normalized to 100%, and the background fluorescence is subtracted. (C) The sample data are temperature shifted or corrected by selecting the PCR product melting portion of each sample and forcing them to overlay the selected sample graph.

a 5′ to 3′ exonuclease-negative DNA polymerase to be used to extend the primers. The unlabeled probes are blocked using a 3′ phosphate group to prevent the probes from being extended by the polymerase during the high-resolution DNA melting step. The unlabeled probes used in IP-PCR can also have a melting temperature that is higher than the extension temperature of the PCR. This greatly reduces the amount of time and effort spent on optimizing the PCR conditions.

Isolating the unlabeled probes from the amplification step of PCR lends itself to using unlabeled probes in multiplexing experiments. Multiplexed unlabeled probes could be designed to cover the entire DNA target region to scan for unknown DNA mutations over a greater area than currently used unlabeled probe PCR techniques.

The fluorescent dsDNA dye LCGreen I was used to detect the different APOE genotypes. LCGreen I can be used in saturating concentrations without inhibiting PCR (9,16). When compared with other fluorescent DNA dyes, such as SYBR® Green I, SYBR Gold, ethidium bromide, and others, LCGreen I exhibits a unique capability of detecting heteroduplexes that form when a heterozygous sample is amplified (17–19).

High-resolution DNA melting currently requires a separate instrument. The high-resolution DNA melting instrument used in this study was the HR-1. The ability of the HR-1 to precisely control the temperature of the sample and record both temperature and fluorescence data gives the HR-1 the capability to produce accurate and reproducible data (20). A high-resolution melting run can vary between 30 s to 3 min/sample depending on the ramp rate and the temperature range required.

The high-resolution DNA melting data for each sample is first normalized using an exponential best-fit algorithm (21). This eliminates the background noise. Next, the normalized data are temperature corrected or x-axis adjusted. The melting curve data from the deidentified samples are x-axis adjusted or overlaid onto a known or normal sample (17). In this experiment, the 277-base pair PCR product melting curve from the positive control APOE 2–3 sample was used. This forces the melting curves together at the high-temperature portion of the melting curve where the 277-base pair PCR product melts and magnifies the differences in the melting curves at the lower temperatures where the unlabeled probes melt (Figure 3). When x-axis adjusting multiple samples, it is important to remember to select the portion of the melting curves that have the most similar melting curve characteristics. In most cases this occurs at the end of the PCR product melt, in which only the most stable DNA homoduplexes remain double-stranded.

IP-PCR can be used with any difficult or high-GC targets and/or PCR techniques that require asymmetric PCR. It is particularly well-suited for use with unlabeled probes. The idea of placing reagents in the lid of the capillary tube lends itself to options that were previously not possible.

The DNA amplification step of IP-PCR does not need to be run in an expensive real-time PCR instrument such as the LightCycler. A relatively inexpensive rapid cycle PCR instrument can be used for the amplification step, because all of the fluorescence and temperature data are collected by the high-resolution DNA melter instrument. This would allow researchers to obtain the highest degree of precision of DNA melting without having to acquire a real-time PCR instrument. There is a great need for a simple, fast, and accurate method for genotyping targets found in high-GC regions of the human genome.

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COMPETING INTERESTS STATEMENT

Aspects of melting analysis are covered by issued and pending patents owned by the University of Utah, licensed to Idaho Technology, and sublicensed to Roche Applied Science. C.T.W. holds equity in Idaho Technology.

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