Optimization of PCR Condition: The First Study of High Resolution Melting Technique for Screening of *APOA1* Variance

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ABSTRACT

Background High resolution melting (HRM) is a post-PCR technique for variant screening and genotyping based on the different melting points of DNA fragments. The advantages of this technique are that it is fast, simple, and efficient and has a high output, particularly for screening of a large number of samples. *APOA1* encodes apolipoprotein A1 (apoA1) which is a major component of high density lipoprotein cholesterol (HDL-C). This study aimed to obtain an optimal quantitative polymerase chain reaction (qPCR)-HRM condition for screening of *APOA1* variance.

Methods Genomic DNA was isolated from a peripheral blood sample using the salting out method. *APOA1* was amplified using the RotorGeneQ 5Plex HRM. The PCR product was visualized with the HRM amplification curve and confirmed using gel electrophoresis. The melting profile was confirmed by looking at the melting curve.

Results Five sets of primers covering the translated region of *APOA1* exons were designed with expected PCR product size of 100–400 bps. The amplified segments of DNA were amplicons 2, 3, 4A, 4B, and 4C. Amplicons 2, 3 and 4B were optimized at an annealing temperature of 60 °C at 40 PCR cycles. Amplicon 4A was optimized at an annealing temperature of 62 °C at 45 PCR cycles. Amplicon 4C was optimized at an annealing temperature of 63 °C at 50 PCR cycles.

Conclusion In addition to the suitable procedures of DNA isolation and quantification, primer design and an estimated PCR product size, the data of this study showed that appropriate annealing temperature and PCR cycles were important factors in optimization of HRM technique for variant screening in *APOA1.*

Key words *APOA1*; HRM; optimization

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Abbreviations: *APOA1*, apolipoprotein class A member 1 gene; apoA1, apolipoprotein class A member 1 protein; dsDNA, double stranded DNA; HDL-C, high density lipoprotein cholesterol; HRM, high resolution melting; LCAT, lecithin cholesterolacyl transferase; qPCR, quantitative polymerase chain reaction; RCT, reverse cholesterol transport; SR-B1, scavenger receptor class B member1; ssDNA, single stranded DNA

High resolution melting (HRM) is a post-PCR technique for variant screening and genotyping based on different melting points of DNA fragments.[1] The main principle of HRM is the integration of intercalating dye to double stranded DNA (dsDNA) and disintegration of the fluorescence dye when the dsDNA are separated to single stranded DNA (ssDNA). By using a real time measurement of the fluorescence level, a melting curve of fluorescence level against time is formed. The melting profile is mainly influenced by the length of DNA fragment, the GC content and heterozygosity.[1–3] Thus, the advantages of this technique are that it is fast, simple, efficient and high throughput result, particularly for screening of a large number of samples.[1, 3, 4]

APOA1 is located in chromosome 11q23 and encodes apolipoprotein A1 (apoA1). *APOA1* spans about 2.2 kb and contains 4 exons.[6] ApoA1 composes up to 70% of HDL-C.[7] In the structure of HDL, apoA1 is a group of proteins arranged in an anti-parallel double-belt structure.[8] It is a cofactor for lecithin cholesterolacyl transferase (LCAT), substrate for scavenger receptor class B member 1 (SR-B1).[9] Deletions in the *APOA1* result in a decrease of HDL-C level to less than 40 mg/dL.[10] The monogenic disorder caused by *APOA1* mutation comprises about less than 5% of low HDL-C level cases,[11] but it accelerates the atherosclerosis process in contrast to other mutations in genes which encode the main protein of reverse cholesterol transport (RCT).[12]

This study aimed to obtain an optimal quantitative polymerase chain reaction (qPCR)-HRM condition for screening of *APOA1* variance. So far, it is the first study which screens variance in *APOA1* using HRM technique; therefore optimization of the technique is very

important in order to get a reliable result.[13] In addition to a suitable procedure for DNA isolation and quantification, primer design and an estimated PCR product size, this study found that appropriate annealing temperature and PCR cycles were important factors in optimization of HRM technique for variant screening in *APOA1*.

MATERIALS AND METHODS

Primer design

The first step in this study was designing primers for *APOA1*. The referent sequences were taken from the database of UCSC Genome Bioinformatics Human Genome Browser Gateway (http://genome.ucsc.edu/cgi-bin/hgGateway?redirect=manual&source=genome.ucsc.edu) and Ensembl (www.ensembl.org) with the accession numbers were GRCh38/hg38[14] and ENSG00000118137,[6] respectively. The primers were designed with PCR product sizes 100–400 bps using the *Lightcycler Primer Design Software* V.1.0.R.84 (Idaho Tech, Salt Lake City, UT). The concentration of primers both forward and reverse primers was 10 pmol/µL.

Based on the gene sequence database, the primers were designed from the start codon at exon 2. Exon 1 is an untranslated region, and thus it was not included in the analysis. The primers for amplicon 2 and amplicon 3 have a PCR product size of less than 250 bps as an ideal. They were located in intron with the purpose of covering the entire exon area. Exon 4 was divided into 3 amplicons, *i.e*. 4A, 4B and 4C in order to get optimum HRM results. The details are listed in Table 1. The primers were designed to cover all exons from 2 to 4 and their flanking regions.

DNA isolation and quantification

The genomic DNA was isolated using the salting out method. Then, the DNA concentration was quantified using the Nanovue Plus (GE Healthcare Bio Sciences AB, Buckinghamshire, UK). The DNA samples were diluted using distilled water to a concentration of 10 ng/µL. The genomic DNA from normal healthy donors was used in the optimization of qPCR-HRM. The criteria of normal healthy donors were that their HDL-C level > 40 mg/dL, cholesterol total level < 200 mg/dL, LDL-C level < 160 mg/dL, triglyceride level < 150 mg/dL, and their DNA sequence were similar to the reference sequence in the NCBI database. Informed consent for participation in this study was obtained. The investigation was approved by the Institutional Review Board of the Diponegoro University Faculty of Medicine and Dr. Kariadi Hospital (number 190/EC/FK/RSDK/2013) and conformed to the principles outlined in the Declaration of Helsinki.[15]

HRM technique and its data analysis

APOA1 was amplified using qPCR RotorGeneQ 5Plex HRM (Qiagen, Valencia, CA) with total volume 10 µL in each tube. Each tube consisted of 5 µL Type-It HRM Master Mix (Qiagen, Cat. No. 206542), 1 µL of forward and reverse primers each with a concentration of 10 pmol/µL, 2 µL of DNA with a concentration of 10 ng/µL and 2 µL of H_2O. The curve analysis was done with

Table 1. List of primers, nucleotide positions, PCR products size and their GC content

Exon	Amplicon	Primer Sequences	Nucleotide Positions	Primer GC content (%)	Product size (bp)	Amplicon GC content (%)
2	2	F: GCCAGGCCCTTCTTCTC	GRCh38/hg38: chr11: 116836271-116836287	64.7	116	59.0
		R: GGTTGGCTCCCTAGGTT	GRCh38/hg38: chr11: 116836370-116836386	58.8		
3	3	F: GGCCTGATCTGGGTCTC	GRCh38/hg38: chr11: 116836512-116836528	64.7	246	58.6
		R: TCATCCCACAGGCCTCT	GRCh38/hg38: chr11: 116836741-116836757	58.8		
4	4A	F: CCAGCCCTCAACCCTTC	GRCh38/hg38: chr11: 116837258-116837274	64.7	290	60.3
		R: CCCTCTTGGAGCTCTGC	GRCh38/hg38: chr11: 116837531-116837547	58.8		
4	4B	F: GCAGGAGGAGATGGAGCTCTA	GRCh38/hg38: chr11: 116837485-116837505	57.1	327	69.7
		R: CCTTGGCGGAGGTCCTC	GRCh38/hg38: chr11: 116837795-116837811	64.7		
4	4C	F: TCAGCGAGAAGGCCAAG	GRCh38/hg38: chr11: 116837769-116837785	58.8	273	63.4
		R: AGGAAGTCCCTGCTCCA	GRCh38/hg38: chr11: 116838025-116838041	58.8		

bp, base pair; F, forward primer; R, reverse primer; GC, guanine and cytosine.

Rotor Gene–Pure Detection version 2.1.0 (build 9) Software (Qiagen). The PCR product was visualized with the amplification curve and confirmed with1.5% agarose gel for 30 minutes. The melting profile was analyzed through melting curve.

RESULTS

Optimization of *APOA1* was done in exon 2 to 4 which were divided into 5 amplicons. They were amplified by using 5 pairs of primers. Because of its length, exon 4 was divided into 3 amplicons, *i.e*. 4A, 4B and 4C. Exon 1 were excluded. At 60 °C annealing temperature and 40 PCR cycles, the HRM result of amplicon 2, 3, 4B and 4C showed a crossing point less than cycle 30th and single peak of melting curve (Fig. 1A). Clear bands matched with the estimated PCR product sizes were seen in the agarose gel. However, double peaks of melting curve (Fig. 1B) and double bands on the agarose gel (Fig. 1C) were seen for amplicon 4A and 4C.

In order to get better HRM curves which represent correct product for amplicon 4A and 4C, we modified the annealing temperature and number of PCR cycle. For amplicon 4A, we tried to increase the temperature to 62 °C and 40 cycles. Although the PCR result showed one correct band in gel electrophoresis, the relative thin band and the amplification curve pattern (Fig. 2) indicated that PCR could be further optimized. PCR with temperature 62 °C and 45 cycles produced better results, i.e. the amplification curve increased after the 30th cycle (Fig. 3A), a single peak of melting curve (Fig. 3B), and a thick band matched with the estimated PCR product size was seen in the agarose gel (Fig. 3C). However, a smear was seen at the upper end of the estimated PCR product size. Further increasing the temperature to 63 °C and 50 cycles resulted in no amplification in the raw material of qPCR-HRM (data not shown).

Optimization of amplicon 4C was done with higher annealing temperature and PCR cycle than that in Fig. 2, i.e. 63 °C and 50 times, respectively. This condition resulted in an increase of amplification curve after the 30th cycle (Fig. 4A), a single peak of melting curve (Fig. 4B), and a clear single band matched with the estimated PCR product size in the agarose gel (Fig. 4C).

The summary of optimum qPCR-HRM condition of amplicons 2, 3, 4A, 4B and 4C are listed in Table 2.

DISCUSSION

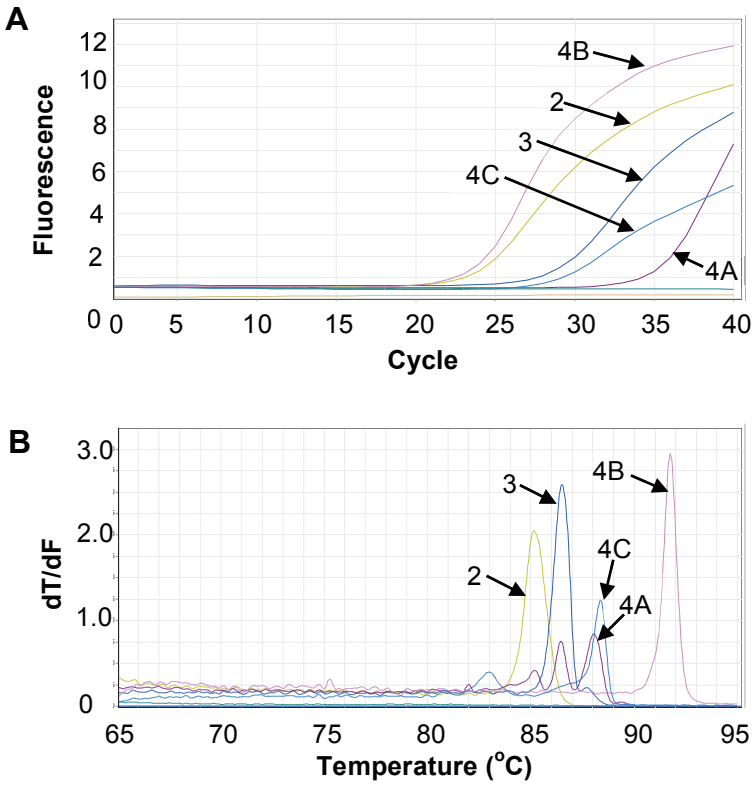
It has long been known that the important factors in the optimization of HRM technique for genetic variant screening are a suitable procedure of the DNA isolation and quantification, primer design and an estimated PCR product size,[13] and data from this study clearly supports the idea that appropriate annealing temperature and PCR cycles were also important factors in optimization of HRM technique for variant screening in *APOA1.*

The primers covering exons 2, 3 and 4 were designed using a well-established software, the *Lightcycler Primer Design Software* V.1.0.R.84 (Idaho Tech). The maximum PCR product size was 327 bps in exon 4B which was qualified for maximum PCR product size to achieve 100% sensitivity and 100% specificity of the HRM technique.[1, 13] The primers of amplicons 2 and 3 were ideal primers with PCR product sizes of less than 250 bps,[13] while the size of amplicons 4A and 4C were 290 bps and 273 bps, respectively. These primer designs and the estimated PCR product size were clearly important in the optimization of PCR-HRM technique for variant screening in *APOA1.* In addition to target length, primer sequences should be carefully checked for known polymorphisms. Failed identification of any SNP in the primer sequence will reduce the success rate of HRM analysis since the HRM melting profile is influenced by DNA heterozygosity.[1–3]

Initially, the experiment used annealing temperature 60 °C and 40 PCR cycles and it showed that amplicons 2, 3, 4B and 4C have crossing points of less than 30 (Fig. 1A). It means that the sample was adequate and the amplification was efficient.[13] On the other hand, amplicon 4A had a crossing point more than 30 cycles, meaning that the sample may not be adequate and the amplification may not efficient.[13] Amplicons 4A and 4C had double bands on agarose gel. These could be caused by unspecific PCR products, and thus required an increase in annealing temperature to obtain optimal qPCR-HRM conditions. Other possible causes for results of amplicon 4A which had a crossing point more than 30, double peak melting curve and double band on agarose gel were less efficient DNA amplification, a low annealing temperature leading to unspecific PCR products, and a CG rich content (60.3%) in the DNA sequence.[13]

As shown in Fig. 2, an annealing temperature of 62 °C and 40 PCR cycles also failed to show an optimal condition for amplicon 4C. Thus, further increases of annealing temperature and PCR cycle, i.e. 63 °C and 50 cycles were performed to get a single peak of HRM curve and single band on agarose gel (Fig. 4).

Although a single peak of HRM curve could be obtained for amplicon 4A by increasing the annealing temperature from 60 to 62 °C, the amplification curve did not reach a plateau pattern (Fig. 2) suggesting that the optimum temperature was obtained but an additional PCR cycle was needed. Although a PCR with a temperature of 62 °C and 45 cycles produced better results, a thin smear remained at the upper the estimated PCR



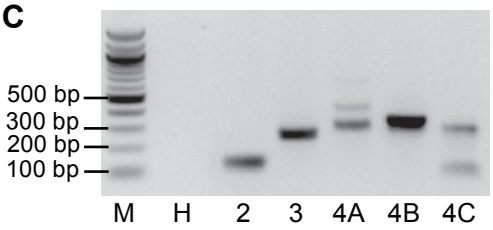
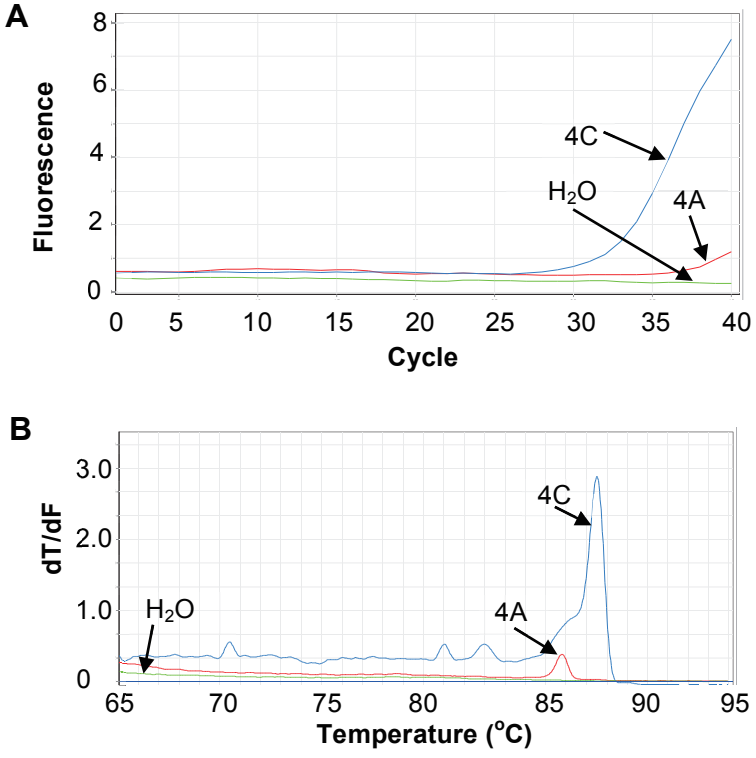


Fig. 1. The result of optimization of *APOA1* using annealing temperature 60 °C and 40 times of PCR cycles.
(**A**) qPCR amplification curve of amplicons 2, 3, 4B and 4C showed crossing point less than 30, while amplicon 4A showed that of more than 30. (**B**) HRM curve of amplicons 2, 3 and 4B had single peak, while amplicon 4A and 4C had double peak of melting. (**C**) Amplicons 2, 3 and 4B had clear single band on the agarose gel matched with the estimated PCR product size, but amplicons 4A and 4C had nonspecific band; the temperature of qPCR was set for starting at 65 °C. bp, base pair; dT/dF, delta temperature per delta fluorescence; H, H_2O; HRM, high resolution melting; M, molecular weight marker; qPCR, quantitative polymerase chain reaction; 2, 3, 4A, 4B, and 4C are names of amplicons.



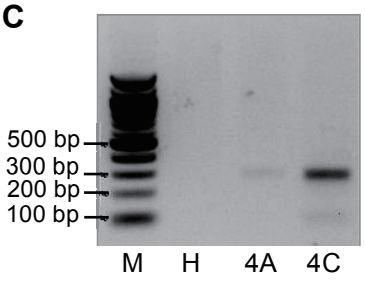


Fig. 2. The result of optimization of amplicon 4A and 4C *APOA1* using annealing temperature 62 °C and 40 times of PCR cycles.
(**A**) qPCR amplification curve of amplicons 4A and 4C showed crossing point more than 30. (**B**) HRM curve of amplicons 4A and 4C had single peak of melting. (**C**) Amplicon 4A had clear single band on the agarose gel matched with the estimated PCR product size, but amplicon 4C had a nonspecific band. bp, base pair; dT/dF, delta temperature per delta fluorescence; H, H_2O; HRM, high resolution melting; M, molecular weight marker; qPCR, quantitative polymerase chain reaction; 4A and 4C are names of amplicons.

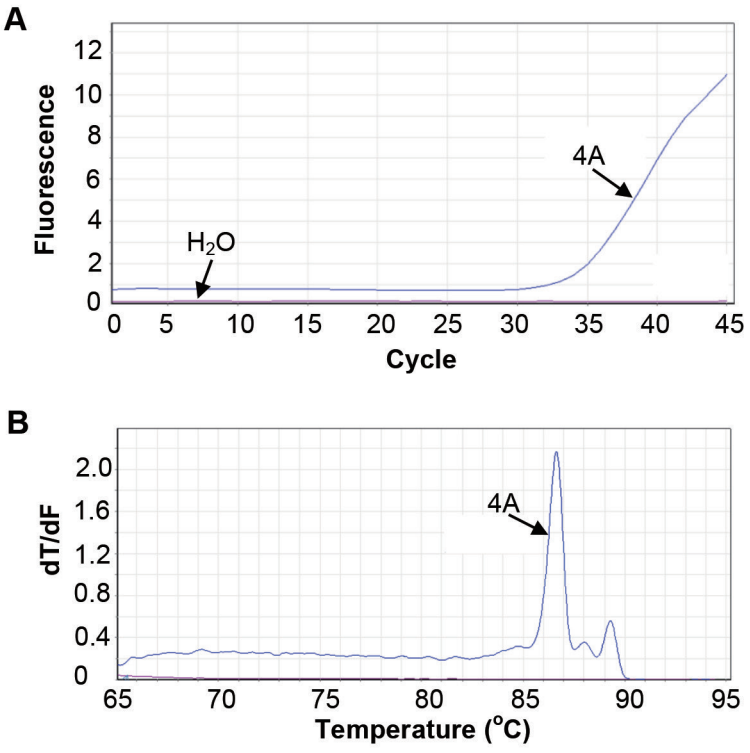


Fig. 3. The result of optimization of amplicon 4A *APOA1* using annealing temperature 62 °C and 45 times of PCR cycles.
(**A**) qPCR amplification curve of the amplicons showed that amplification was started at the 30th cycle. (**B**) Its HRM curve had single peak of melting. (**C**) The amplicon had a clear single band on the agarose gel matched with the estimated PCR product size, with very thin smear at the upper of the correct band. bp, base pair; dT/dF, delta temperature per delta fluorescence; H, H_2O; HRM, high resolution melting; M, molecular weight marker; qPCR, quantitative polymerase chain reaction; 4A is a name of an amplicon.

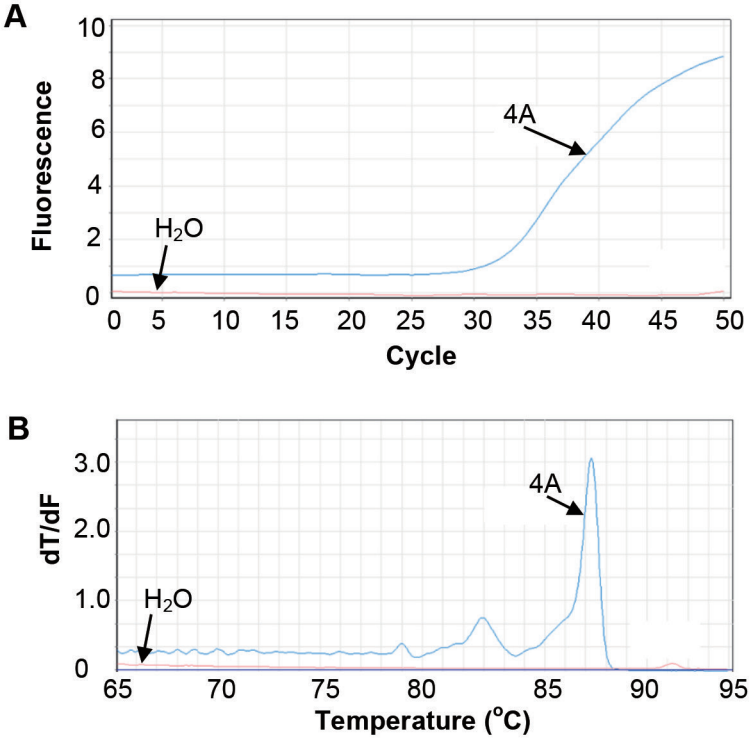


Fig. 4. The result of optimization of amplicon 4C *APOA1* using annealing temperature 63 °C and 50 times of PCR cycles.
(**A**) qPCR amplification curve of the amplicon 4C showed crossing point more than 30. (**B**) Its HRM curve had single peak of melting. (**C**) The amplicon had a clear single band on the agarose gel matched with the estimated PCR product size. bp, base pair; dT/dF, delta temperature per delta fluorescence; H, H_2O; HRM, high resolution melting; M, molecular weight marker; qPCR, quantitative polymerase chain reaction; 4A is a name of an amplicon.

Table 2. Profile of qPCR-HRM of *APOA1*

Amplicon	Program	Temperature (°C)	Time	Cycle
2, 3 and 4B	Initial denaturation	95	5 min	1
	Denaturation	95	10 s	
	Annealing	60	10 s	40
	Elongation	72	10 s	
	HRM	65–95 ºC, 0.1 ºC temperature increment Wait for 2 s for each steps afterwards		1
	HRM final step	40	1 min	1
4A	Initial denaturation	95	5 min	1
	Denaturation	95	10 s	
	Annealing	62	20 s	45
	Elongation	72	30 s	
	HRM	65–95 ºC, 0.1 ºC temperature increment Wait for 2 s for each steps afterwards		1
	HRM final step	40	1 min	1
4C	Initial denaturation	95	5 min	1
	Denaturation	95	10 s	
	Annealing	63	10 s	50
	Elongation	72	10 s	
	HRM	65–95 ºC, 0.1 ºC temperature increment Wait for 2 s for each steps afterwards		1
	HRM final step	40	1 min	1

HRM, high resolution melting; qPCR, quantitative polymerase chain reaction.

product size (Fig. 3), and further increasing of temperature to 63 °C and 50 cycles resulted in no amplification detected in the raw material of qPCR-HRM. Thus, at this point, considering that the amplification curve after the 30th cycle and a single peak of the melting curve could be achieved by using an annealing temperature of 62 °C and 45 cycles, this method could be accepted.

Our results indicated that there are primers which have similar PCR conditions for HRM, and thus facilitate multiple primers for a qPCR-HRM running. In contrast, some primers may need different conditions to achieve optimum conditions as in *APOA1*. To evaluate whether methods of *APOA1* variant screening showed in this study are appropriate for detection of mutant *APOA1*, further qPCR-HRM analysis using either genomic DNA or plasmid DNA harboring *APOA1* mutation is warranted.

In conclusion, in addition to a suitable procedure of DNA isolation and quantification, primer design and an estimated PCR product size, the data in this study showed that appropriate annealing temperature and PCR cycle were important factors in optimization of HRM technique for variant screening in *APOA1*.

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The authors declare no conflict of interest.

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