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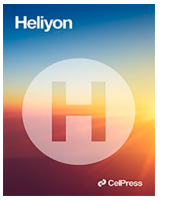

An optimized method for PCR-based genotyping to detect human APOE polymorphisms

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A R T I C L E I N F O

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A B S T R A C T

Background: Apolipoprotein E (*APOE*) is one of the most polymorphic genes at two single nucleotides (rs429358 and rs7412). The various isoforms of *APOE* have been associated with a variety of diseases, including neurodegenerative, type 2 diabetes, etc. Hence, predicting the *APOE* genotyping is critical for disease risk evaluation. The purpose of this study was to optimize the tetra amplification refractory mutation system (Tetra-ARMS) PCR method for the detection of APOE mutations.

Material and methods: Here, in our optimized Tetra-ARMS PCR method, different factors like cycle conditions, using HiFidelity enzyme instead of Taq polymerase and setting its best concentration, and the lack of using dimethylsulfoxide (DMSO) for amplifying the GC-regions were set up for all primer pairs. The sensitivity and accuracy were tested. For validation of the assay, the results were compared with known genotypes for the *APOE* gene that were previously obtained by two independent methods, RFLP and Chip-typing.

Results: Successful Tetra-ARMS PCR and genotyping are influenced by multiple factors. Our developed method enabled us to amplify the DNA fragment by 25 cycles without adding any hazardous reagent, like DMSO. Our findings showed 100 % accuracy and sensitivity of the optimized Tetra-ARMS PCR while both criteria were 95 % for RFLP and 100 % for the chip-typing method. In addition, our results showed 91 % and 100 % consistency with RFLP and chip typing methods, respectively.

Conclusions: Our current method is a simple and accurate approach for detecting *APOE* polymorphisms within a large sample size in a short time and can be performed even in low-tech laboratories.

1. Introduction

Apolipoprotein E (*APOE*) is a major plasma protein with a critical role in the transporting of various lipids, such as triglycerides and

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cholesterol, synthesized primarily in the liver and brain. *APOE* gene locates on chromosome 19q13.2 containing four exons and three introns [1,2]. The two main polymorphisms in the human *APOE* gene, rs429358, and rs7412 locate on the fourth exon of the gene, resulting in three different alleles, ε2 (Cys112/Cys158), ε3 (Cys112/Arg158), and ε4 (Arg112/Arg158), which coded ε2, ε3, and ε4 isoforms. The presence of C or T nucleotides at the position of 112 (C·526C > T) and 158 (c.388 T > C), depending on the combination of 112 and 158 amino acids, resulting six genotypes, homozygous (ε2/ε2, ε3/ε3, ε4/ε4) and heterozygous (ε3/ε4, ε2/ε3, ε2/ε4) [3–5].

Although the frequency of *APOE* genotypes varies among the different populations, wild-type ε3/ε3 is the most common isoform in all ethnicities [6,7]. Numerous experimental data have demonstrated that *APOE* gene variants are associated with many diseases, including, Alzheimer's [8], type 2 diabetes mellitus [9], cardiovascular diseases [10], obesity [11], and progressive multiple sclerosis [12], and Parkinson [13]. Although the ε2 allele is recognized as a risk factor for heart disease and type III hyperlipoproteinemia, it has a protective effect against late-onset Alzheimer's disease, in contrast to an ε4 allele that is a risk factor for this disease [14]**.** Therefore, the *APOE* genotypes play a crucial role in determining the genetic risk associated with various diseases. Hence, it is worth evaluating the allelic variation in different populations.

The primary methods for assessing the genetic variation of *APOE* isoforms were based on protein isoelectrofocusing [15]. Protein isoelectric focusing (IEF) is a method that separates proteins based on their isoelectric points (pI). The pI is the pH at which a protein has no net charge and remains stationary in an electric field. This technique involves placing proteins into a gel matrix with a pH gradient and subjecting them to an electric field, causing them to migrate toward their respective pI values. Through this process, proteins are separated based on their charge. In the context of ApoE genotyping, IEF is used to differentiate between three common isoforms of the ApoE proteinApoE2, ApoE3, and ApoE4. These isoforms result from genetic variations at positions 112 and 158 of the *APOE* gene [49].

However, at the gene level, single nucleotide polymorphisms (SNPs) are the essential variable in the human genome, comprising the valuable tool to detect the mutations at the molecular level using PCR amplification [16]. PCR restriction fragment length polymorphism (PCR-RFLP) [17], Real-time PCR [18], single base extension genotyping (SNaPshot) analysis, reverse hybridization [19], and ARMS PCR [20] are the used methods for detecting three isoforms of *APOE.* However, not all methods are equally efficient, and each method has some disadvantages [21]. PCR-RFLP is the most widely used technique for genotyping based on the digestion of PCR amplicons that consists of several steps including an electrophoretic separation step. The first step is an amplification of a fragment containing the variation that treatment of the amplified fragment with a restriction enzyme. In the next step, the presence or absence of the restriction enzyme recognition site results in the formation of restriction fragments of different sizes can be done by the electrophoretic separation step. Advantages of this approach include the lack of requirement for advanced instruments and its simple; Disadvantages include the requirement for specific endonucleases, and it is not suitable for the simultaneous analysis of a large number of different SNPs due to the requirement for a specific primer pair and restriction enzyme for each SNP. This limits its usability for high throughput analysis [22,48]. Various real-time PCR-based methods, including HRM (high-resolution melt) [23], TaqMan probe [24], and FRET (Fluorescent Resonance Energy Transfer) developed for *APOE* genotyping. But, these methods involve multiple steps and are time-consuming, and also require expensive equipment and reagents. Reports show that Tetra-ARMS PCR is an efficient and cost-effective method for simple SNP genotyping [25,26]. However, the tetra-primer ARMS PCR has not only a difficult procedure for optimization but also fails to distinguish allelic differences in some cases [27].

In the present study, we made some modifications to the PCR-based *APOE* genotyping to accurately detect SNPs of interest, followed by comparing our results with RFLP and chip-typing methods. The optimized method can be applied for cost-effective genotyping in a large-scale with high precision.

2. Subjects and methods

2.1. Sample selection

In the current study, samples were obtained from the Tehran Cardiometabolic Genetic Study participants (TCGS). TCGS is an ongoing prospective population-based longitudinal cohort study, which is conducted for the past 20 years to determine the risk factors for non-communicable diseases among a representative Tehran urban population[28–29]. Of these subjects, we selected those individuals whose *APOE* gene were previously genotyped by RFLP [30] and HumanOmniExpress-24-v1-0 bead chip methods [31]. All procedures followed the ethical standards of the ethics committee on human subject research at the Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences, which were by the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written informed consent was obtained from all participants.

2.2. Tetra-ARMS PCR

In this study, our goal is to develop an assay to detect two SNPs, rs7412 and rs429358 in AP*OE*. To this end, we selected 164 samples comprising ε3/ε3 (n = 91), ε3/ε4 (n = 38), ε2/ε3 (n = 24), ε2/ε2 (n = 3), ε2/ε4 (n = 3), and ε4/ε4 (n = 5) genotypes, from 843 samples which were previously genotyped with the RFLP method by our group [30].

2.3. Primer designing

For performing the Tetra-ARMS PCR method, three pairs of codon-specific primers were designed for selected SNPs using Gene Runner software. *APOE* genotyping by Tetra-ARMS PCR was performed with specific Cys primers (Cys112 and Cys158) as well as Arg

primers (Arg112 and Arg158), that Cys primers, containing Cys112 (115 bp) and Cys158 (253 bp) primers, or Arg primers, containing Arg112 (444 bp) and Arg158 (307 bp) primers. The primer specificity was also checked using the BLAST program at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The primer sequences designed in this study are listed in Table 1.

2.4. PCR reaction optimization

In this study, we attempt to optimize the PCR reaction by combinations of various affecting factors, including the different concentrations of primers, annealing temperature, and three kinds of DNA polymerases, to select the best mixture that can efficiently amplify the region of interest. To this end, we modified the Tetra-ARMS PCR reaction to detect the *APOE* mutations in three stages. In the multiplex Tetra-ARMS PCR, the interaction of inner and outer primers is a complex phenomenon. Hence, in the first stage, different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5 μM) of both outer and inner primers were used to select the optimum primers pair titration. In the second stage, the performance of three different enzymes, Taq DNA polymerase, Taq plus DNA polymerase, and High Fidelity (HiFid) DNA polymerase in three different concentrations (1, 1.5, and 2 units) were compared. All enzymes were purchased from Kowsar. In the third stage, the annealing temperature was checked at five different levels (61 °C, 63 °C, 65 °C, 67 °C, and 69 °C) while other PCR factors were fixed. Finally, the PCR reactions were performed using KBC Alpha PCR Mix under various conditions (different primers concentrations, annealing temperature, and the different polymerases) with 25, 30, 35, and 40 amplification cycles to obtain the best PCR product.

2.5. Assay validation

In the current study, we evaluated the optimized T-ARMS PCR efficiency for genotype determination via calculating the accuracy and sensitivity parameters [32,47]. Besides, we examined the reproducibility of optimized T-ARMS PCR by selecting 6 samples from each genotype (n = 24 samples) and conducting the PCR reaction for each sample, and comparing the acquired results [33].

2.6. Statistical analysis

Data were analyzed using SPSS (version 21.0). Kappa test agreement was applied to assess the diversity of the two genotyping methods [34]. The power of study was calculated by Monte Carlo method [35].

3. Results

3.1. Tetra-ARMS PCR

We used 164 samples out of 843 samples which were previously genotyped with the RFLP method by our group to optimize a method for detecting two key *APOE* polymorphisms. The genotypes of selected samples comprised ε3/ε3 (n = 91), ε3/ε4 (n = 38), ε2/ε3 (n = 24), ε2/ε2 (n = 3), ε2/ε4 (n = 3), and ε4/ε4 (n = 5). We obtained 91 % power with our sample size, which was statistically significant (p-value = 0.0001), suggesting the obtained results are reliable and robust. Various factors play a role in the correct genotyping of the multiplex Tetra-ARMS PCR method, necessitating extensive optimization in the early stages. Hence, the initial PCR primer pairs were designed for the two main SNPs of the *APOE* gene, rs429358, and rs7412. A pair of outer primers in combination with four inner (allele-specific) primers produced the specific PCR product of interest. The routine enzyme used in Tetra-ARMS PCR is Taq polymerase. The HiFid DNA polymerase is a combination of Taq and Pfu polymerases with both 5′–3′ polymerase and 5′–3′ exonuclease activities. We found that among the enzymes, HiFid DNA polymerase generated the best amplification. We carried out the gradient PCR, and the optimal annealing temperature was 67 °C. Dimethyl sulfoxide (DMSO) is one of the PCR enhancers reducing melting temperature and improving the amplification of GC-rich regions. Interestingly, we found that replacing HiFid DNA polymerase with Taq polymerase, enabled to amplification of the DNA fragment by 25 cycles without adding DMSO.

After optimization, PCR amplification was carried out in a total volume of 20 μL reaction for each sample containing about 50 ng DNA template. We tested all three primer pairs concentrations (0.2 μM FO, 0.15 μM RO, 0.15 μM FI-1, 0.5 μM RI-1, 0.15 μM FI-2, and 0.4 μM RI-2) in 10 μL KBC Alpha PCR Mix (containing 2 mM Mgcl2 and 0.2 mM dNTP), and 2 units of HiFid DNA polymerase using PEQLAB thermal cycler PCR. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 10 min, followed by 25

Table 1
Sequence of primers used, fragment size for rs7412 and rs429358 polymorphisms of *APOE* gene for multiplex Tetra-ARMS PCR.

Name	Sequence (5′-3′)	bp	Tm°C[a]
Common-OF	ACTGACCCCGGTGGCGGAGGA	21	69.2
Common-OR	CAGGCGTATCTGCTGGGCCTGCTC	24	72.1
rs429358-IR1	GCGGTACTGCACCAGGCGGCCTCA	24	73.8
rs429358-IF1	GGCGCGGACATGGAGGACGGGC	22	73.3
rs7412-IR2	CCCGGCCTGGTACACTGCCAGTCA	24	72.1
rs7412-IF2	CGATGCCGATGACCTGCAGACGC	23	70

[a] Tm, melting temperature.

cycles of denaturation at 95 °C for 30 s, annealing at 67 °C for 30 s, and extension at 72 °C for 30 s. The final extension step was done at 72 °C for 7 min. The amplified products were visualized on the ethidium bromide-stained 2 % agarose gel. The optimized multiplex Tetra-ARMS PCR product of six representative samples is displayed in Fig. 1. The identified genotypes related to two polymorphisms within the *APOE* gene using multiplex Tetra-ARMS PCR were shown in Table 2. Hence, our modification and optimization of the Tetra-ARMS methodology could efficiently detect the correct genotypes in the absence of DMSO. However, in contrast to using DMSO, the PCR reaction with Taq polymerase was not successful.

3.2. Assay validation

In the current study, a total of 164 samples were analyzed by their *APOE* genotype. The results showed 100 % for both sensitivity and accuracy in our optimized Tetra-ARMS PCR. We computed these criteria for RFLP and Chip typing methods. Both accuracy and sensitivity parameters were 95 % for RFLP while they were 100 % for the chip-typing method. Additionally, we found that the reproducibility of our optimized Tetra-ARMS PCR was 100 % for all tested samples. Subsequently, we compared the results of the T-ARMS PCR method with those obtained by RFLP and chip genotyping. According to Table 3, our developed Tetra-ARMS PCR could successfully detect samples with ε3/ε3, ε4/ε4, ε2/ε2, and ε2/ε4 genotypes as PCR-RFLP method, representing the complete consistency between two methods (Table 3). However, there was a partial concordance between the Tetra-ARMS PCR and PCR-RFLP methods in detecting ε2/ε3 and ε3/ε4 genotypes. Overall, there was 91 % and 100 % consistency between our optimized Tetra-ARMS PCR with RFLP and chip typing methods, respectively, in recognizing the genotypes of interest that were statistically significant (p-value <0.001).

4. Discussion

One of the distinguished genes, *APOE*, is regarded as a valuable biomarker in the identification of individuals at an elevated risk for Alzheimer's disease, which is a major concern worldwide [36–39]. Therefore, developing a reliable and precise method for *APOE* genotyping is crucial. There are various molecular methods for *APOE* genotyping [39]. Tetra-ARMS PCR is a convenient, reliable, and cost-effective method for use in clinical laboratories. On the contrary, PCR-RFLP is not appropriate for clinical sample genotyping and high-throughput assays due to the high cost, incomplete digestion of the PCR product, and a long period of multiple steps [40,41]. In comparison to HRM, FRET, TaqMan, and DNA direct sequencing, Tetra-ARMS PCR does not require expensive equipment, making it applicable in even low-tech laboratories [42]. Although we could successfully amplify the high GC content gene (*APOE*) using our modified method, some studies reported that Tetra-ARMS PCR might not be useful for SNP genotyping in GC-rich regions [39,43].

In the present study, we have optimized an efficient genetic test for *APOE* genotyping based on Tetra-ARMS PCR that was also validated by RFLP and chip typing techniques. The current method embraces some additional advantages compared to the original Tetra-ARMS PCR developed by Young et al., in 2007. In the original method, DMSO, a hazardous chemical, was applied in the PCR reaction buffer to facilitate the GC-rich region amplification, however, it has a detrimental influence on subsequent applications [44]. It has been reported that DMSO can disrupt base pairing. Therefore the imposed mismatched base-pairing during the annealing step of PCR could result in an increased mutation rate at the priming site [45]. Hence, due to the mutagenesis effect of DMSO, it is preferable to eliminate the DMSO for sensitive applications, like DNA sequencing and genotyping, which has been achieved with the present modified method. In our protocol, we used the High Fidelity (HiFid) DNA polymerase, instead of DMSO, for amplifying our region rich in GC, which is in line with previous studies [44]. The HiFid DNA polymerase is a combination of Taq and Pfu polymerases with both 5′–3′ polymerase and 3′–5′exonuclease activities. As a result, it has a low error rate during the amplification process and can amplify the GC-rich regions [46]. Additionally, contrary to similar studies [35], we can obtain an accurate PCR product in a relatively short time (only 25 PCR cycles) and costs, which is another advantage of our method, especially for large-scale investigations.

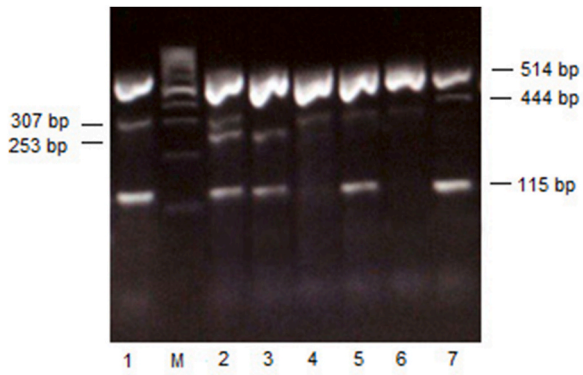

Fig. 1. Validation of *APOE* variants using Tetra-ARMS PCR. PCR products from representative samples were analyzed on a 2 % agarose gel. Outer primers (FO and RO) amplified the 514-bp fragment and the combinations of each inner primer (FI-1 and RI-1 at codon 112, and FI-2 and RI-2 at codon 158) and outer primer in two SNP sites. Lanes are numbered from left to right. Lane M is a 100 base pair (bp) ladder size.

Table 2
The amplified products resulted from *APOE* gene polymorphisms using multiplex Tetra-ARMS PCR.

Genotype	rs429358 (codon 112)		rs7412 (codon 158)		Common
	AA[a]	Product size	AA	Product size	Product size
ϵ2/ϵ2	Cys	115	Cys	253	
ϵ3/ϵ3	Cys	115	Arg	307	
ϵ4/ϵ4	Arg	444	Arg	307	
ϵ2/ϵ3	Cys	115	Cys	253	
			Arg	307	514
ϵ2/ϵ4	Cys	115	Cys	253	
	Arg	444	Arg	307	
ϵ3/ϵ4	Cys	115	Arg	307	
	Arg	444			

[a] Amino Acid.

Table 3
The number of homozygous and heterozygous genotypes for *APOE* locus identified by three methods (n = 164).

Method	Genotype					
	ε3/ε3 (91)	ε3/ε4 (38)	ε2/ε3 (24)	ε2/ε2 (3)	ε2/ε4 (3)	ε4/ε4 (5)
Tetra-ARMS PCR	91	38	24	3	3	5
PCR-RFLP	91	42	20	3	3	5
Chip-Typing	91	38	24	3	3	5

Measure of Agreement (Kappa), Kappa coefficient (SE) = 0.909(0.029), p-value <0.001. The sample size for each genotype was shown in parentage.

In summary, in the present study, using various modifications in PCR setup for the GC-rich region, we can optimize the rapid and cost-effective method for massive *APOE* genotyping that has the potential to become a ready-to-use kit applicable for both socio-economic and clinical purposes.

Author contribution statement

Leila Najd-Hasaan-Bonab: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper. Mehdi hedayati: Conceived and designed the experiments; Contributed reagents, materials. Seyed Abolhassan Shahzadeh Fazeli:Contributed reagents, materials. Maryam S Daneshpour: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Data availability statement

Data included in article/supplementary material/referenced in article.

Ethics approval and consent to participate

Written informed consent was obtained from all participants. All procedures followed the ethical standards of the ethics committee on human subject research at the Research Institute for Endocrine Sciences, Shahid Beheshti University of Medical Sciences(ethics approval number: 31ECRIES94/02/15).

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

APOE Apolipoprotein E
Tetra-ARMS PCR Tetra-Primer Amplification Refractory Mutation System PCR
PCR-RFLP PCR restriction fragment length polymorphism
HiFid DNA polymerase HiFidelity DNA polymerase
TCGS Tehran Cardiometabolic Genetic Study
TLGS Tehran Lipid and Glucose Study
T2D Type 2 diabetes
DMSO Dimethyl sulfoxide

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