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Establishing a cost-effective tetra-primer amplification refractory mutation system PCR for genotyping the apolipoprotein-A5 -1131T>C polymorphism: a preliminary study for genetic association analysis in the Jambi Malay population

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Abstract

Background: The Apolipoprotein A5 (APOA5) gene's -1131T>C polymorphism is a significant genetic marker for dyslipidemia, with effects varying across ethnicities. Investigating this in specific populations like the Jambi Malay requires genotyping methods that are both reliable and feasible for large-scale studies, often in limited-resource settings. **Methods:** DNA was extracted from blood samples using a solid-phase column extraction method and subsequently diluted. Four specific primers (two outer and two inner allele-specific) for the T-ARMS PCR assay were designed. The PCR protocol was optimized by varying the annealing temperature, while other parameters like initial denaturation (95°C) and extension (72°C) were kept constant based on standard literature. Amplified products were visualized using gel electrophoresis. **Results:** An optimized T-ARMS PCR protocol was successfully established. The assay produced clear and distinct banding patterns on a 2.0% agarose gel, allowing for unambiguous genotype calling. Homozygous wild-type (TT) samples showed two bands (512 bp control and 355 bp T-allele), homozygous variant (CC) samples showed two bands (512 bp control and 194 bp C-allele), and heterozygous (TC) samples showed three bands (512 bp, 355 bp, and 194 bp). The assay demonstrated high specificity with no non-specific amplification at an optimal annealing temperature of 59°C. **Conclusion:** The developed T-ARMS PCR assay is a robust, cost-effective, and reliable method for genotyping the APOA5 rs662799 polymorphism. This tool is well-suited for large-scale molecular epidemiological studies to investigate the genetic architecture of dyslipidemia in the Jambi Malay and other resource-limited settings.

Keywords: APOA5 -1131T>C Polymorphism; T-ARMS PCR; Genotyping; Annealing Temperature; Optimization; Cost-Effective; Jambi Malay.

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INTRODUCTION

The landscape of human health in the 21st century is dominated by complex, multifactorial diseases [1,2]. Conditions such as dyslipidemia, metabolic syndrome, and atherosclerotic cardiovascular disease (ASCVD) arise not from a single genetic defect but from an intricate interplay between multiple genetic predispositions and a lifetime of environmental exposures [3–5]. Unlike Mendelian disorders, in which a single gene mutation often leads to a predictable phenotype, the etiology of these diseases is far more complex. Their development is influenced by a constellation of genetic variants, each typically conferring a small amount of risk, which is then modulated by factors such as diet, physical activity, and other lifestyle choices [6–9]. Understanding the genetic architecture of these conditions is a paramount challenge in modern medicine and public health, as it holds the key to identifying at-risk individuals and developing targeted prevention strategies.

Among many candidate genes, the Apolipoprotein A5 (APOA5) gene, located on chromosome 11q23, has emerged as a key regulator of triglyceride (TG) homeostasis. ApoA5 protein acts as a potent activator of lipoprotein lipase (LPL) and facilitates the clearance of triglyceride-rich lipoproteins (TRLs), making it a powerful determinant of plasma TG levels [10,11]. One of the most studied variants of this gene is the promoter polymorphism -1131T>C (rs662799) [12,13]. The presence of the minor 'C' allele is consistently associated with reduced APOA5 expression, leading to higher TG levels, lower HDL-C levels, and an increased risk of metabolic syndrome [14–16].

Crucially, the frequency and effect size of the rs662799 C-allele exhibited significant ethnic variability. Its frequency is substantially higher in East Asian populations (>25%) than in Europeans (10-16%), and the association with metabolic syndrome appears stronger in East Asians [15,17–19]. However, there is a conspicuous lack of data from genetically diverse populations in Maritime Southeast Asia, including the various ethnic groups of Indonesia. The Jambi Malay of Sumatra, with their unique ancestral history stemming from the Austronesian expansion and subsequent admixtures, represent a critical, unstudied population regarding this genetic marker [20,21].

Large-scale genetic association studies are necessary to determine the role of this polymorphism in Jambi Malays. However, such studies are often hindered in resource-limited settings by the high cost of gold-standard genotyping technologies, such as DNA sequencing or microarrays. These financial and technical barriers effectively preclude locally relevant genetic epidemiological studies. Therefore, the development of a cost-effective, accessible, and reliable genotyping method is a prerequisite for investigating the genetic epidemiology of dyslipidemia in this region [22–24]. The Tetra-Primer Amplification Refractory Mutation System (T-ARMS) PCR is an ideal

candidate method, as it allows for the determination of all three genotypes (homozygous wild-type, heterozygous, and homozygous variant) in a single reaction with minimal equipment requirements [22–25]. This preliminary study aimed to develop, optimize, and validate a T-ARMS PCR assay for the rapid genotyping of the APOA5 rs662799 polymorphism, providing a crucial tool for future population-based research in the Jambi Malay population.

METHODS

Genomic DNA

Genomic DNA was extracted from whole blood samples collected from volunteer donors of Jambi Malay ethnicity using asolid phase-proteinase K methods provided by Favorprep®. DNA concentration and purity were determined using spectrophotometry. Samples with a 260/280 ratio between 1.8 and 2.0 were used for assay development. All participants provided written informed consent, and the study was approved by the ethics committee (approval number 2080/UN21.8/PT.01.04/2025).

Primer design

The DNA sequence flanking the APOA5 rs662799 polymorphism was obtained from the NCBI dbSNP database. Four primers were designed using the Primer3 web tool, following the principles of T-ARMS PCR. The set consisted of two outer primers (Outer Forward/Reverse) and two inner, allele-specific primers (Inner Forward T-allele / Inner Reverse C-allele). To enhance specificity, a deliberate mismatch was introduced at the -2 position from the 3' end of both inner primers. The primers were designed to produce amplicons of sufficiently different sizes for clear separation via gel electrophoresis. The sequences of the designed primers are listed in Table 1.

Table 1. Primer sequences for the APOA5 rs662799 T-ARMS PCR assay.

Primer Name	Sequence (5’ ☐ 3’)	Target Allele
Outer Forward	GCT-TCA-CTA-CAG-GTT-CCG-CAG	-
Outer Reverse	GCC-GTG-GTT-TTA-TGT-GGC-AGC	-
Inner Forward	CAG-GAA-CTG-GAG-CGA-AAG-TG	G
Inner Reverse	GCT-TTT-CCT-CAT-GGG-GCA-AAT-CTT	A

T-ARMS PCR optimization

PCR reactions were performed in a total volume of 25 µL in GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The reaction mixture contained approximately 50 ng of genomic DNA, 1X PCR Master Mix (containing Taq DNA polymerase, dNTPs, and reaction buffer), and the primer mix. Optimization was carried out systematically: (1) Annealing Temperature: A gradient PCR was performed across a temperature range of 58°C to 61°C to determine the optimal annealing temperature that yielded specific and robust amplification of all expected products; and (2) Primer Concentration: The molar ratio of outer to inner primers was adjusted to achieve balanced amplification intensity of the allele-specific products, ensuring reliable identification of heterozygous samples.

Optimized PCR protocol

The final optimized PCR reaction mixture (25 µL) contained: 12.5 µL of 2X PCR Master Mix, 2.5 µL of 10X Primer Mix, 8.0 µL of nuclease-free water, and 2.0 µL of genomic DNA template (~50 ng). Thermal cycling was performed under the following

conditions: initial denaturation at 95°C for 5 minutes; followed by 35 cycles of 95°C for 30 seconds, 59°C for 30 seconds, and 72°C for 45 seconds; and a final extension at 72°C for 7 minutes.

Genotype analysis

PCR products were analyzed by electrophoresis on a 2.0% agarose gel in 1X TBE buffer, stained with a safe DNA dye. A 100 bp DNA ladder was used as a size marker. The gel was run at 50V for 75 minutes, and the banding patterns were visualized under UV illumination (Lonza, Basel, Switzerland).

RESULTS

Systematic optimization of the annealing temperature using gradient PCR identified 59°C as the optimal temperature, providing the best balance of specificity and amplification efficiency. At this temperature, the assay produced bright, distinct bands with equal intensity for heterozygous samples and no visible non-specific products or primer-dimers (Figure 1).

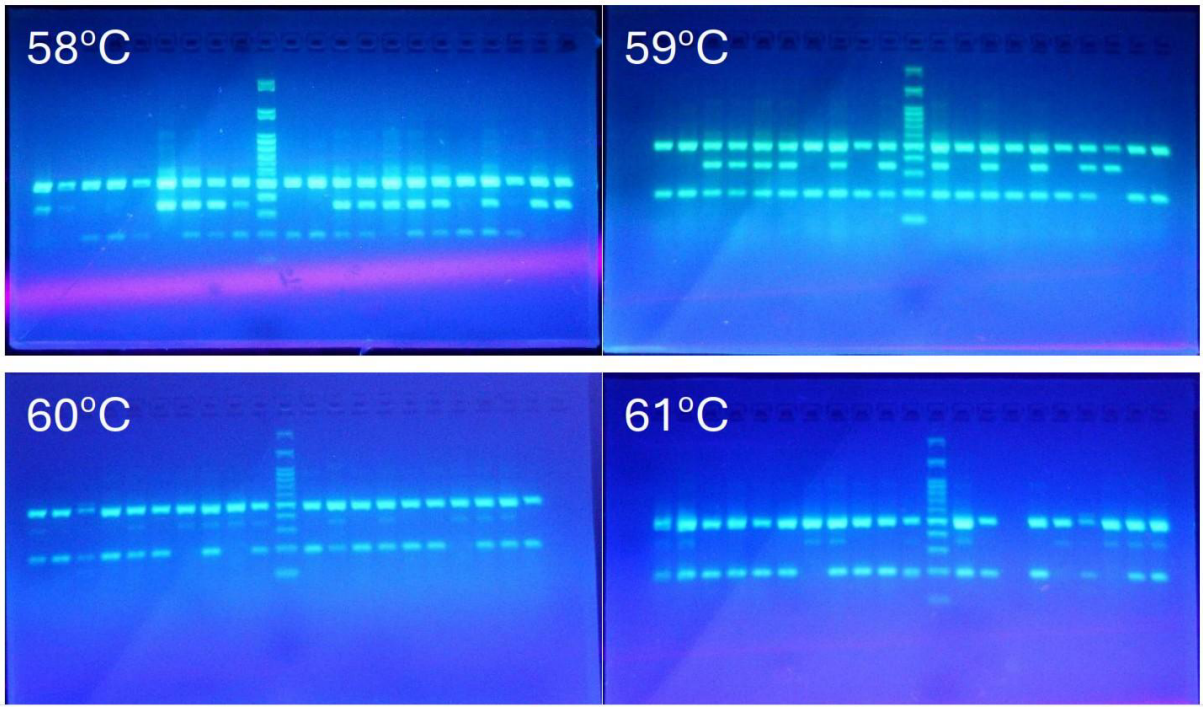

Figure 1. Visualization of 2.0% agarose gel electrophoresis of T-ARMS PCR products for APOA5 rs662799 using 58-610C annealing temperature.

The optimized T-ARMS PCR assay successfully distinguished the three genotypes of the APOA5 rs662799 polymorphism. The outer primers generated a 512 bp control band in all reactions, confirming successful amplification. The allele-specific primers produced fragments of unique sizes, allowing for direct genotype interpretation from the gel image (Figure 2).

DISCUSSION

This study reports the successful development, optimization, and preliminary validation of a Tetra-Primer Amplification Refractory Mutation System (T-ARMS) PCR assay for genotyping the APOA5 rs662799 (-1131T>C) polymorphism. The primary achievement of this study was the establishment of a protocol that is simple, rapid, cost-effective, and highly reliable. These attributes make the developed assay an

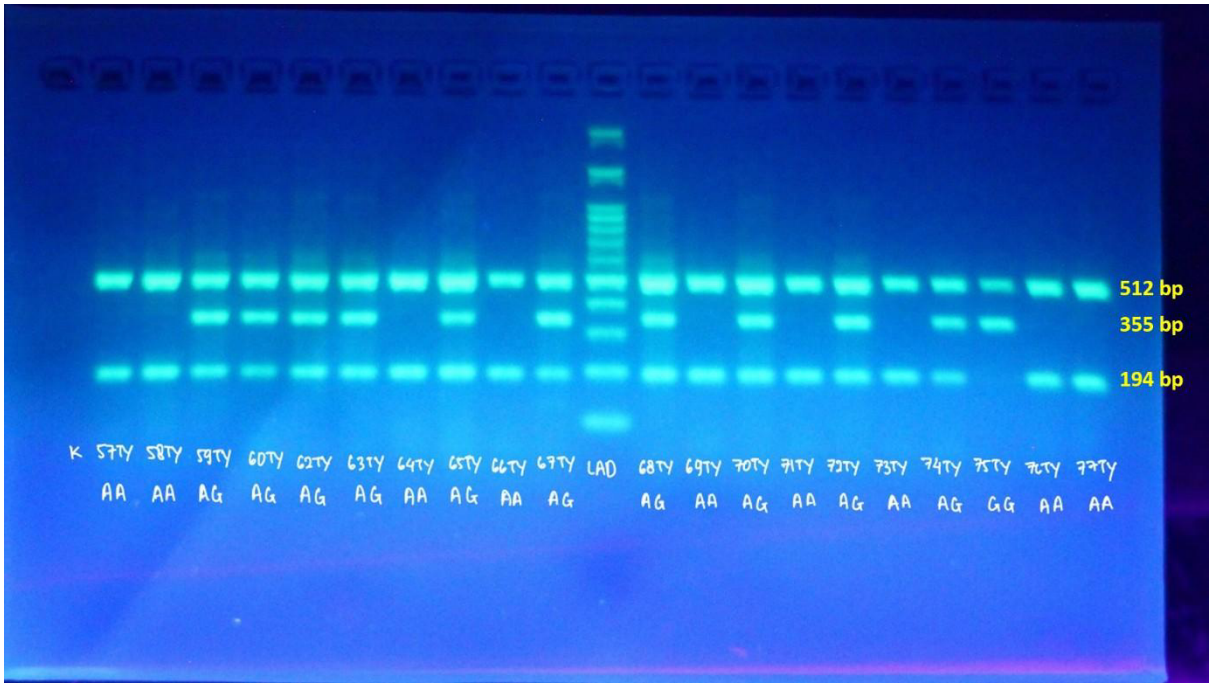

Figure 2. Representative 2.0% agarose gel electrophoresis of T-ARMS PCR products for APOA5 rs662799 using 59oC annealing temperature. Lane LAD: 100 bp DNA ladder. The assay clearly distinguishes the three genotypes: Homozygous AA (Lane 57TY, 58TY, 64TY, 66TY, 69TY, 71TY, 73TY, 76TY, 77TY); Heterozygous AG (Lane 59TY, 60TY, 62TY, 63TY, 65TY, 67TY, 68TY, 70TY, 72TY, 74TY), and Homozygous GG (Lane 75TY), based on the presence of the 512 bp control band, the 355 bp T-allele band, and the 194 bp C-allele band. Lane K: No Template Control.

ideal and much-needed tool for large-scale genetic epidemiological studies, particularly in resource-limited settings (LRS), such as those often encountered in Jambi, Indonesia, and similar regions [22–25]. The challenge of studying the genetic basis of complex diseases, such as dyslipidemia, in diverse, underrepresented populations (such as the Jambi Malay ethnicity) is often logistical and financial [26–28]. High-throughput genotyping technologies, such as microarrays or Next-Generation Sequencing (NGS), while comprehensive, often entail high initial investment and per-sample costs, as well as sophisticated laboratory infrastructure. These factors present significant barriers to implementation in many research institutions in developing nations [29–31].

Our study directly addresses this barrier by developing an "appropriate technology" that leverages standard laboratory equipment to answer a locally relevant health question in a low-resource setting. The T-ARMS PCR method offers distinct advantages over other common alternatives, such as PCR-RFLP (Restriction Fragment Length Polymorphism). Although PCR-RFLP is cost-effective, it requires an additional post-PCR step: enzymatic digestion. This not only adds to the workflow time but also increases costs (for restriction enzymes and buffers) and introduces a potential point of failure due to incomplete digestion [32–34]. In contrast, our developed T-ARMS assay consolidates allele detection and an internal control into a single PCR reaction. Genotype determination can be performed directly via a single gel electrophoresis, significantly reducing the time, per-sample cost, and potential for contamination or handling errors [25,35,36].

The success of a T-ARMS PCR assay is highly dependent on meticulous primer design and precise optimization of the reaction conditions [37]. In this study, we designed four primers and systematically optimized the key parameter, annealing

temperature (Ta). Our data demonstrate that a Ta of 59◦C is optimal, providing the best balance of specificity and amplification efficiency. As shown in Figure 1, slightly lower temperatures (58◦C) began to show smearing or non-specific products, whereas higher temperatures (60–61◦C) could potentially decrease amplicon yield. At 59◦C, the assay produced sharp, bright, and distinct bands with no visible primer dimers or non-specific amplification.

Another critical aspect of our design was the introduction of a deliberate mismatch at the -2 position from the 3' end of both the inner primers. This is a well-established strategy in ARMS design for dramatically enhancing assay specificity. This additional mismatch exploits the inability of DNA polymerase to efficiently extend a primer with a double mismatch near its 3' end, ensuring that each inner primer only amplifies its target allele [38,39]. The success of this strategy was validated by the clear and unambiguous banding patterns on the 2.0% gel, where all three genotypes (TT, TC, and CC) were easily distinguishable based on the unique combination of the three fragments (512 bp control, 355 bp T-allele, and 194 bp C-allele).

The true significance of this tool's development lies in its potential to fill critical knowledge gaps. The APOA5 rs662799 polymorphism is one of the most studied and validated genetic markers associated with triglyceride metabolism. It has been established that the frequency and effect size of the 'C' (risk) allele vary significantly across global populations. Its frequency is reportedly high in East Asian populations (>25%) but much lower in European populations (10-16%) [15,17–19]. Currently, there is a conspicuous lack of data from genetically diverse populations in Maritime Southeast Asia, including Indonesia. The Jambi Malays of Sumatra, with their unique ancestral history stemming from the Austronesian expansion and subsequent admixtures, represent a critical, unstudied population regarding this genetic marker [20–24]. Therefore, this assay is a fundamental tool for the first characterization of the APOA5 rs662799 allele and genotype frequencies in Jambi Malay.

Subsequent association studies using this tool can accurately quantify the impact of this polymorphism on lipid profiles (TG and HDL-C) and dyslipidemia risk within this specific ethnic group. Data generated from future population studies using this assay have important implications for public health in Jambi and Indonesia. If the 'C' risk allele frequency is found to be high in the Jambi Malay population and its association with dyslipidemia is confirmed, this could inform population-specific risk stratification strategies. Individuals identified as carriers of high-risk genotypes (TC or CC) may benefit from earlier lipid screening or more intensive lifestyle interventions (such as diet and physical activity), aligning with the shift towards ethnically tailored precision medicine.

It is important to acknowledge the limitations of this study. First, as inherent to the T-ARMS methodology, this assay is designed to genotype a single known SNP (rs662799) and cannot identify novel variants within the APOA5 gene that may also be relevant to lipid metabolism in the Jambi Malay population. Second, although agarose gel electrophoresis is highly accessible and cost-effective, it can become a bottleneck for very high-throughput applications (e.g., thousands of samples) because of its manual and semiquantitative nature. Third, although the banding patterns produced were highly clear and unambiguous, formal validation against a gold standard, such as Sanger sequencing, on a subset of samples would further strengthen the assay's accuracy in a full-scale population study.

The future directions are clear. The most immediate next step is to deploy this validated tool in a large-scale population study to achieve the primary research goal of determining the prevalence and clinical significance of rs662799 in the Jambi Malay

population. Furthermore, as suggested, this assay has the potential for future refinements, such as multiplexing with primers for other relevant SNPs associated with dyslipidemia, or adapting it to a gel-free detection platform (like capillary electrophoresis) to increase throughput and automation, albeit at a higher cost.

CONCLUSIONS

We successfully developed and optimized a specific, reliable, and cost-effective T-ARMS PCR assay for genotyping the APOA5 rs662799 (-1131T>C) polymorphism. Through systematic annealing temperature optimization, we established that 59◦C yielded the clearest and most specific genotype band separation, enabling the unambiguous identification of homozygous (TT and CC) and heterozygous (TC) individuals in a single reaction. The primary achievement of this methodological study is the provision of a validated "appropriate technology" tool that directly addresses the cost barriers that often hinder genetic epidemiological research in resource-limited regions. This tool is now ready for deployment in a large-scale population study to investigate, for the first time, the prevalence and clinical significance of the APOA5 risk variant in the Jambi Malay population. The resulting data will be crucial for filling a critical knowledge gap regarding the genetic architecture of dyslipidemia in Indonesia and will pave the way for a deeper understanding of the genetic determinants of cardiovascular risk in this unique Maritime Southeast Asian population.

CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

FUNDING

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DECLARATION OF ARTIFICIAL INTELLIGENCE USE

AI-based language models, such as Gemini AI and Paperpal, were employed to language refinement (improving the grammar, sentence structure, and readability of the manuscript) and content summarization (assisting in summarizing the findings and conclusions concisely). We confirm that all AI-assisted processes were critically reviewed by the authors to ensure the integrity and reliability of the results. The final decisions and interpretations presented in this article were solely made by the authors.

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