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Original Article

A simple and rapid method for detection of single nucleotide variants using tailed primer and HRM analysis

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ABSTRACT

Background: Single nucleotide polymorphisms (SNPs) are widely used genetic markers with applications in human disease diagnostics, animal breeding, and evolutionary studies, but existing genotyping methods can be labor-intensive and costly. The aim of this study is to develop a simple and rapid method for identification of a single nucleotide change.

Methods: A modified Polymerase Chain Reaction Amplification of Multiple Specific Alleles (PAMSA) and high resolution melt (HRM) analysis was performed to discriminate a bovine polymorphism in the *NCAPG* gene (*rs109570900*, 1326T > G).

Results: The inclusion of tails in the primers enabled allele discrimination based on PCR product lengths, detected through agarose gel electrophoresis, successfully determining various genotypes, albeit with some time and labor intensity due to the use of relatively costly high-resolution agarose gels. Additionally, high-resolution melt (HRM) analysis with tailed primers effectively distinguished the GG genotype from the TT genotype in bovine muscle cell lines, offering a reliable way to distinguish SNP polymorphisms without the need for time-consuming AS-PCR.

Conclusions: Our experiments demonstrated the importance of incorporating unique mismatched bases in the allele-specific primers to prevent cross-amplification by fragmented primers. This efficient and cost-effective method, as presented here, enables genotyping laboratories to analyze SNPs using standard real-time PCR.

Keywords: allele specific, high-resolution melt, genotype, single nucleotide polymorphism

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are the most prevalent genetic markers in the genome and find diverse applications, such as human disease diagnostics, marker-assisted selection in animal breeding, and evolutionary studies (Shastry, 2002; Sermyagin et al., 2018; Gao et al., 2023). Numerous techniques for SNS genotyping are presently accessible; nonetheless, these methods can frequently involve labor-intensive processes or be cost-prohibitive (Mamotte, 2006). For instance, the TaqMan assay is less labor-intensive but necessitates the acquisition of allele-specific hybridization probes labeled with distinct fluorescent dyes. Moreover, access to expensive

instrumentation is crucial for real-time monitoring of PCR amplification (Bernard et al., 1998). Recently, precise genome editing techniques like prime editing have made it possible to replace single nucleotides (Scholefield and Harrison, 2021). Therefore, the development of modified methods is essential for the rapid and cost-effective analysis and identification of single variants.

Among the methods commonly used for single nucleotide polymorphism (SNP) genotyping, allele-specific PCR, also known as PCR allele-specific amplification (PASA), is a popular choice for detecting amplification products through agarose gel electrophoresis (Sommer et al., 1992). However, PASA often necessitates two separate reactions, one for each allele, as a single base-pair change near the 3' primer end may not reliably distinguish between the two SNP alleles (Ahmadian et al., 2001). To address these challenges, PCR amplification of multiple specific alleles (PAMSA) has been developed (Dutton and Sommer, 1991; Okimoto and Dodgson, 1996). PAMSA involves using at least two allele-specific primers in a single reaction, enabling the detection of all SNP alleles within a sample. While PAMSA simplifies the process into a single-tube reaction, it may require more expensive equipment and reagents, including fluorescence detection, real-time fluorescence detection, or a sequencer (Takatsu et al., 2004).

A cost-effective PAMSA method has been devised, which employs only three unlabeled primers for allele detection via agarose gel electrophoresis. This method utilizes allele-specific primers with a destabilizing mismatch within five bases of the 3' end and a 5' tail for amplifying PCR products of varying lengths. These additional features enhance allele specificity, eliminate the need for extensive optimization, and enable PCR product detection. The method has successfully genotyped variants, although it remains somewhat time-consuming and laborious due to the use of high-resolution agarose gels. In this study, we have devised a novel real-time PCR-based method employing high-resolution melting (HRM) analysis. The method utilizes tailed or extended allele-specific primers designed to match only one of the potential allele sequences at the 3'-terminal nucleotide. Notably, this technique eliminates the need for electrophoresis and expensive agarose, thereby reducing both the experimental procedures and the required time.

MATERIALS AND METHODS

Genomic DNA isolation, PCR amplification and electrophoresis

Total genomic DNA was extracted from bovine muscle satellite cells using a DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA). PCR was performed using the BioFACT™ H-Star Taq DNA Polymerase Kit (BIOFACT, Daejeon, Korea). PCR conditions included an initial denaturation step at 95℃ for 15 minutes, followed by denaturation at 95℃ for 20 seconds, annealing at 55℃ for 40 seconds, and extension at 72℃ for 3 minutes, cycled 45 times. A final extension was carried out at 72℃ for 4 minutes. Following the manufacturer's instructions, the PCR products were subjected to electrophoresis on a 3% high-resolution agarose gel (MetaPhor® Agarose, LONZA, Basel, Switzerland) in TBE (Tris/Borate/EDTA) Buffer (BIOFACT) at a constant voltage of 6 V. Visualization was accomplished by staining with ethidium bromide.

Allele specific PCR primers design

We utilized a SNP (*rs109570900* T > G) located on bovine chromosome 6 (NCAPG) for this study. Three primer sets were designed using the PrimerQuest™ Tool (https://sg.idtdna.com/pages) with modifications to enable allele-specific (AS) amplification. The control forward and reverse primer set encompasses the amplicon region of AS-specific primers. To identify the allele-specific variant, the 3' terminal bases matched the AS, but the 3rd nucleotide from the 3' end was intentionally mismatched to increase allele specificity. The primers for the minor allele included an additional 5 base pairs. Additionally, a common reverse primer was designed to amplify the AS-specific PCR product (Fig. 1 and Table 1).

High-resolution melting (HRM) analysis

The high-resolution melting (HRM) analysis was conducted using the Type-it® HRM PCR Kit (QIAGEN, Hilden, Germany) and the Rotor-Gene Q machine (QIAGEN, Hilden, Germany). The HRM protocol consisted of an initial PCR activation step at 95℃ for 5 minutes, followed by denaturation at 95℃ for 10 seconds, annealing at 55℃ for 30 seconds, and extension at 72℃ for 10 seconds for a total of 40 cycles. Subsequently, the HRM stage included a 90-second hold at 65℃, followed by a gradual temperature increase of 0.1℃ every 2 seconds until reaching 95℃,

while measuring the change in fluorescence.

RESULTS

We first conducted polymerase chain reaction (PCR) amplification of multiple specific alleles (PAMSA) to discriminate a bovine polymorphism in the *NCAPG* gene (*rs109570900*, 1326T > G) that significantly associated to an increase in bovine carcass weight at puberty (Setoguchi et al., 2009; Lindholm-Perry et al., 2011). We found that the inclusion of tails within the primers facilitated the discrimination of alleles based on the PCR product lengths, enabling their detection through agarose gel electrophoresis. The method has successfully determined the genotypes of various variants. However, it remains somewhat time-consuming and labor-intensive, primarily due to the requirement of using high-resolution agarose gels, which can be relatively more costly compared to standard agarose gels.

We carried out HRM assay for a SNP, *rs109570900* as derived above using five primers; major allele forward (red; 24 bp), minor allele forward (blue; 29 bp), and common reverse (27 bp) (Fig. 2A), and two control forward and reverse primers. We detected amplification of major allele (red line) and control region of NCAPG (black line) in bovine muscle cell lines 22 containing homo TT, but not minor allele GG. In the 23 cell line, genotype GG was amplified (blue), suggesting HRM using tailed primers successfully and rapidly distinguished a single base change (Fig. 2B). Furthermore, we combined two cell lines (22 and 23) to create heterozygous alleles (GT) to test the efficacy of our new method in detecting heterozygous genotypes. Our observations revealed that the melting curve and peak were positioned between GG and TT, suggesting the annealing of two distinct-sized amplicons (Fig. 2C).

DISCUSSION

High-resolution melt analysis (HRM) is a powerful and widely used technique that enables the precise examination of DNA sequence variations. Through the analysis of the melting profiles of amplified DNA fragments, HRM provides a sensitive and high-resolution method for distinguishing nucleic acid species based on their specific melting characteristics. This approach allows for the detection of even minor sequence variations, making HRM particularly valuable for a diverse range of applications within the field of molecular biology. In the context of mutation scanning, HRM is employed to identify and characterize sequence alterations, including single nucleotide polymorphisms (SNPs) and small insertions or deletions. By subjecting PCR-amplified DNA products to controlled temperature changes, HRM generates distinct

Table 1. Oligonucleotide sequences for bovine SNP *rs109570900*

Primer	Sequence (5' to 3)	Product size
HRM primer (*NCAPG*)	GATTCTTACTCTACCTACCACACC (Forward) TTCTATCACCAATGAAGAAAATTACTT (Reverse)	126 bp
Major allele (*NCAPG*)	CCTACCACACCAATATCCCTATTT (Forward) TTCTATCACCAATGAAGAAAATTACTT (Reverse)	113 bp
Minor allele (*NCAPG*)	CTCTACCTACCACACCAATATCCCTATTG (Forward) TTCTATCACCAATGAAGAAAATTACTT (Reverse)	118 bp

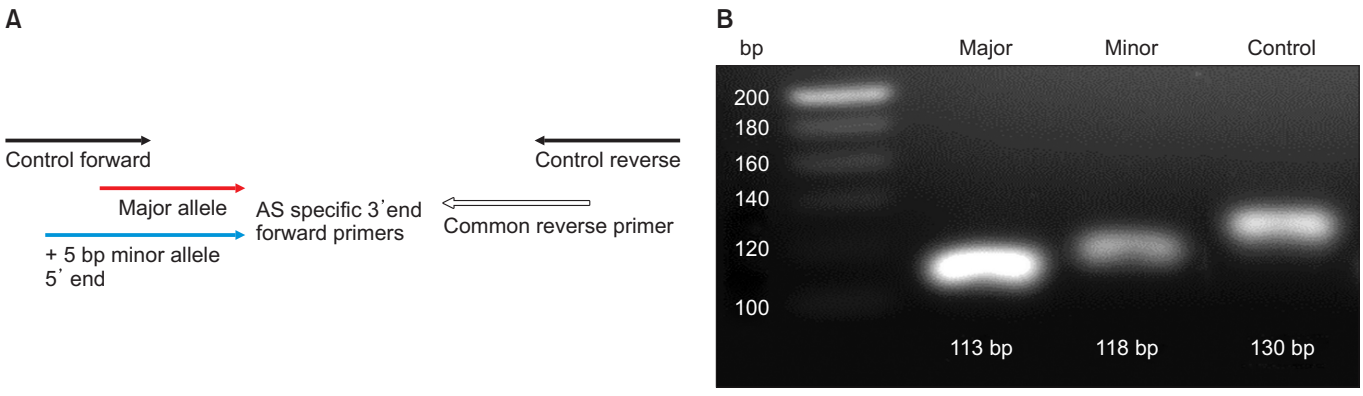

Fig. 1. PCR amplification of multiple specific alleles (PAMSA). (A) Scheme view of designed primer sets for single nucleotide variants (B) PAMSA amplification products of two allele-specific primers.

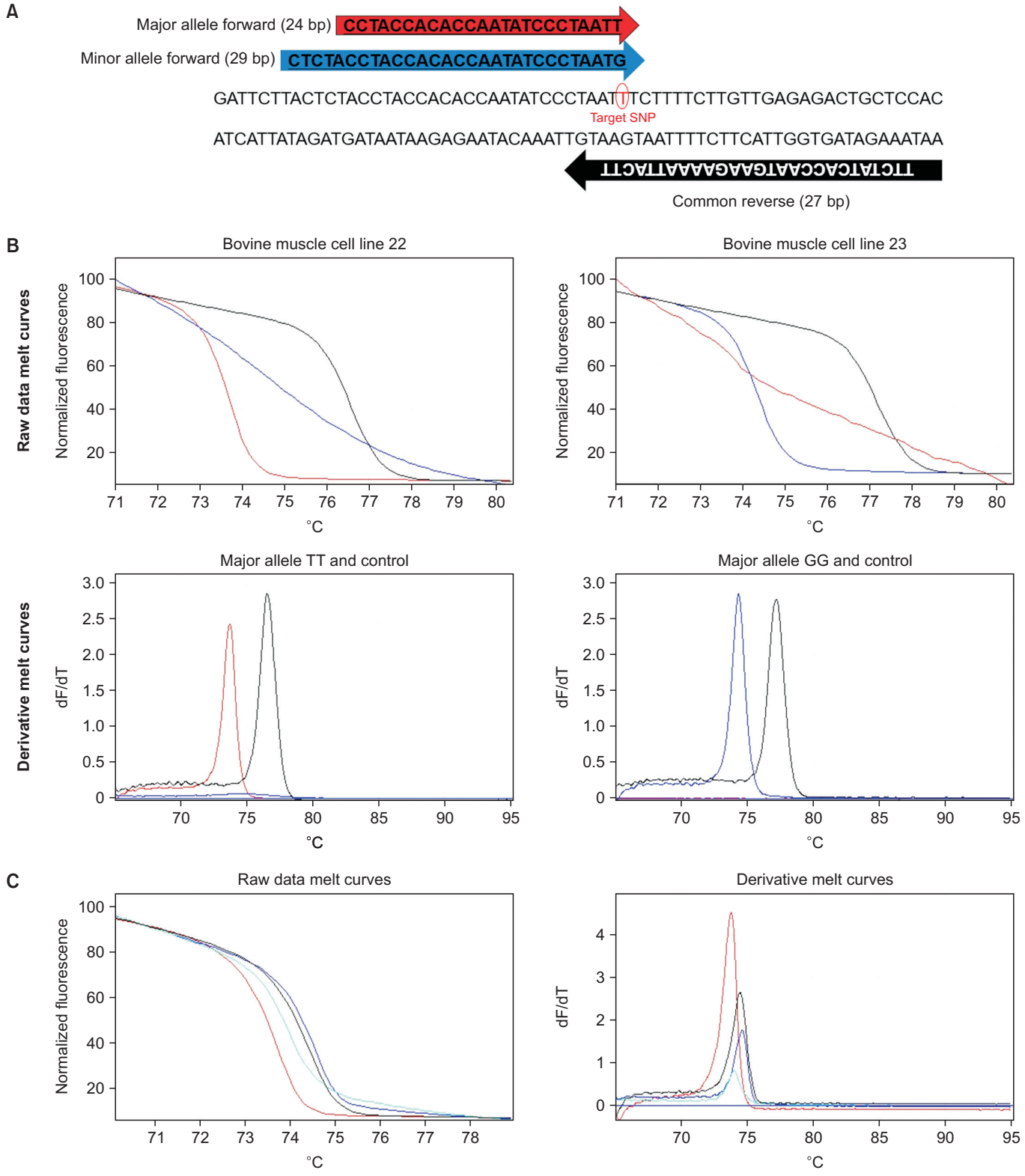

Fig. 2. Allele specific HRM using tailed primer set. (A) Tailed and 3' end modified AS specific primers location for *rs109570900*. (B) Results of high-resolution melting curve analysis of two homozygous alleles (TT or GG). Raw data melt curves: the plot demonstrates the sharp decrease in fluorescence. (C) Results of high-resolution melting curve analysis of hetero genotype (TG). Homozygous TT (red line), homozygous GG (blue line), heterozygous GT (aqua line) and control (black line); derivative melt curves: the differences between the three genotypes are also discriminated by changes in temperature values of melting peak (℃).

melting curves that can reveal variations in the DNA sequence. These variations are manifested as alterations in the melting behavior of the DNA fragments, enabling the identification of specific mutations or polymorphisms within the target DNA region. (Garritano et al., 2009).

While high-resolution melt analysis (HRM) offers exceptional sensitivity and specificity in detecting nucleic acid sequence variations, certain challenges can arise due to the influence of various factors on the analysis of melting temperatures. These factors can affect the accuracy and reliability of HRM-based assays, particularly when discerning subtle sequence differences, such as single nucleotide changes. One of the critical factors that can impact HRM results is the composition of the elution buffer used during DNA extraction or purification. Contaminants or components in the elution buffer may interfere with the DNA melting process, leading to alterations in the melting temperature profiles. Similarly, the choice of elution buffer can affect the stability and integrity of the DNA template, potentially influencing the reproducibility and consistency of the HRM data. Moreover, the concentration of the DNA template itself can significantly influence the melting behavior observed during HRM analysis. Variations in the DNA template concentration can affect the kinetics of DNA denaturation, potentially leading to shifts in the melting temperature curves. In the case of single nucleotide changes, even slight deviations in the DNA template concentration can impact the precision of HRM-based genotyping or mutation scanning assays, potentially leading to erroneous interpretations of the results (Jeong et al., 2019). To address these issues, we employed tailed primers with a minimum of 5 base pairs and a mismatched site located 2 base pairs away from the 3' end. These conditions helped us prevent genotyping errors caused by subtle changes and reduced the risk of cross-amplification due to fragmented or degraded oligomers. Additionally, utilizing desalted or cartridge-grade oligos, rather than HPLC or PAGE-purified ones, can result in cost savings.

CONCLUSION

The tailed primer HRM method developed in this study proved to be highly effective, providing a dependable means of discriminating SNP polymorphisms without the need for time-consuming AS-PCR. Our experiments underscored the significance of introducing distinct mismatched bases in the allele-specific primers to prevent cross-amplification by fragmented primers. This efficient and cost-effective method, as presented here, empowers genotyping laboratories to analyze SNPs using standard real-time PCR.

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