



OPEN Rapid SNP genotyping detection method based on PCR-lateral flow dipstick detection technique

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This study established a polymerase chain reaction–lateral flow dipstick (PCR-LFD) method for the visual detection of SNP genotypes. Targeting the *MC4R* gene SNP g.732 C>G, highly specific primers were designed for the mutation site, incorporating a Locked Nucleic Acid (LNA) modification at the 3' terminal nucleotide of the SNP, a BIOTIN modification at the 5' end of the upstream primer, and a fluorescein isothiocyanate (FITC) modification at the 5' end of the downstream primer. The detection primers were used for PCR amplification with the sample, and the reaction system was optimized. The amplification products were subsequently detected using LFD. The results demonstrated that the optimized reaction system and modified primers effectively distinguished among CC, CG, and GG genotypes at the g.732 C>G. Blood samples from 24 Hu sheep were analyzed using the PCR-LFD assay specific to this SNP. The genotyping results from PCR-LFD were completely consistent with those obtained from the mutation analysis of the same blood samples. The PCR-LFD method established in this study did not require genomic DNA extraction; whole blood could be directly used as a template for PCR amplification combined with LFD, enabling on-site visual detection. This positions PCR-LFD as a rapid, simple, and visually interpretable tool for on-site SNP genotyping.

Keywords *MC4R*, SNP, Polymerase chain reaction (PCR), Lateral flow dipstick (LFD), Detection

SNP (Single Nucleotide Polymorphism) refers to DNA sequence polymorphisms caused by single nucleotide variations at the genomic level¹. There are mainly the following detection methods: PCR-based methods: Cleaved Amplified Polymorphic Sequence (CAPS): Based on base variations at restriction sites, with fragment sizes analyzed by electrophoresis². Competitive Allele-Specific PCR (KASP): Detects SNPs through specific base pairing at the primer's terminal nucleotide³. High-Resolution Melting Analysis (HRM): Differentiates DNA sequences based on their melting characteristics, including length, GC content, and base complementarity⁴. TaqMan probe method: Tracks PCR products using fluorescent dyes and specific probe markers⁵. Sequencing-based methods: Sanger sequencing: Based on the ddNTP chain termination method⁶. Single-cell SNP detection: Involves amplification and sequencing of the genome from a single cell⁷. Gene chip: Utilizes probe hybridization with DNA, with fluorescence signal intensity used for genotyping⁸. High-throughput sequencing/Next-generation sequencing (NGS): Enables large-scale SNP genotyping and novel SNP site discovery through targeted or genome-wide library construction⁹.

The polymerase chain reaction-lateral flow dipstick (PCR-LFD) assay is an emerging method suitable for rapid, on-site detection of PCR products^{10,11}. In this assay, the PCR amplification product is directly applied to a colloidal gold test strip, with the entire process completed within 1.5 h¹². This method eliminates the need for time-consuming agarose gel electrophoresis (AGE) and offers advantages such as simplicity, speed, and low cost¹³.

Currently, lateral flow dipstick (LFD) assays have been reported for the detection of pathogenic bacteria and viruses, including *Cronobacter*¹⁴, *Staphylococcus haemolyticus*¹⁵, *Influenza A virus*¹⁶, and *Hepatitis A virus*¹⁷. Yi et al. established a rapid detection method for *African swine fever virus* nucleic acid by combining PCR amplification with a lateral flow immunoassay¹⁸. Blažková et al. developed a rapid immunochromatographic strip test for detecting *Cronobacter spp*¹⁹. Saetang et al. established a multiplex PCR-LFD assay with high sensitivity and specificity for detecting pathogenic *V. parahaemolyticus*²⁰. Song et al. developed a sensitive and specific PCR-LFD assay for the efficient detection of *Mycoplasma bovis* nucleic acid¹⁰.

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Genetic typing of functional SNP loci is critical for early individual selection in animal breeding, as rapid and straightforward genotyping enhances breeding efficiency. This study aimed to develop a rapid SNP-locus detection method using PCR-LFD. During nucleic acid amplification, primers were modified with specific antigens and antibodies, which were then labeled on the LFD to enable detection of amplification products. This approach simplified the procedure and significantly improved detection efficiency.

The *MC4R* gene is a key candidate gene associated with feeding, fattening, and growth-related traits in mammals²¹. Multiple SNPs within this gene have been linked to growth traits^{22–25}. Previous studies revealed a significant association between the *MC4R* SNP g.732 C > G and body height in adult Hu sheep²⁶. The g.732 C > G locus is located in the coding region and exhibits three genotypes: CC, CG, and GG. Highly specific primers with high amplification efficiency could be designed for this mutation site. Furthermore, the direct amplification capability of current PCR reagents using whole blood, combined with the simplicity and rapidity of LFD, enabled the development of a PCR-LFD method for rapid genotyping at this SNP locus.

Materials and methods

Ethics statement

All procedures involving animals were conducted in accordance with the Guidelines for the Care and Use of Animals of the Zhejiang Academy of Agricultural Sciences. The study was approved by the Ethical Committee of the Zhejiang Academy of Agricultural Sciences and complied with the ARRIVE guidelines.

Sample collection

Hu sheep used in this study were sourced from Hangzhou Pangda Agricultural Development Co., Ltd., under uniform feeding standards and environmental conditions. A 5 mL sample of jugular vein blood was collected from each animal, placed in EDTA anticoagulant tubes, and stored at -20°C for subsequent experiments. This study was approved by the Experimental Animal Welfare and Ethics Committee of the Zhejiang Academy of Agricultural Sciences.

Design of PCR primers

Based on the *MC4R* gene sequence, primers were specifically designed for the g.732 C > G mutation locus using DNAMAN (version 8.0) after sequence alignment. The 3' end of the forward or reverse primer corresponded to the SNP site. A Locked Nucleic Acid (LNA) modification was applied to the nucleotide at the 3' end of the SNP locus. The primer design followed these principles:

- (1) The annealing temperatures of the upstream and downstream primers were matched, with amplification products ranging from 200 to 350 bp.
- (2) The last or second-to-last nucleotide at the 3' end of the forward or reverse primer corresponded to the SNP site.
- (3) BIOTIN and fluorescein isothiocyanate (FITC) modifications were introduced at the 5' ends of the upstream and downstream primers, respectively. Primers were synthesized by General Biosystems (Anhui, China), as shown in Table 1.

Primer specificity verification and PCR reaction system establishment

Modified and synthesized primers were reconstituted from dry powder and diluted accordingly. A Marathon DNA Polymerase reaction solution (Tiosbio, China) was prepared following the specifications outlined in Table 2. PCR amplification was performed using whole blood templates from *MC4R* g.732 C > G homozygous GG and CC genotypes, and corresponding genotype groups were established as follows:

The *MC4R*-732-F-C + *MC4R*-R primer pair was used with a homozygous CC blood template. The *MC4R*-732-F-G + *MC4R*-R primer pair was used with a homozygous GG blood template. For mismatch controls: The *MC4R*-732-F-C + *MC4R*-R primer pair was used with a homozygous GG blood template. The *MC4R*-732-F-G + *MC4R*-R primer pair was used with a homozygous CC blood template. PCR conditions were as follows: Pre-denaturation at 94°C for 2 min; denaturation at 98 °C for 10 s; annealing with a temperature gradient from 55 to 65 °C for 30 s; extension at 68 °C for 30 s; 32 cycles in total; and storage at 4°C.

Detection of PCR products using the LFD assay

PCR reactions were performed using the *MC4R*-732-F-C and *MC4R*-R primers. The following templates were prepared individually: blood from an individual homozygous for the CC genotype; genomic DNA from an individual homozygous for the CC genotype; blood from an individual homozygous for the GG genotype; genomic DNA from an individual homozygous for the GG genotype; and double-distilled water as a negative control, using the same volumes as the blood and DNA templates. Simultaneously, a separate reaction was conducted using the *MC4R*-732-F-G and *MC4R*-R primers with the same set of templates. The reaction system

Primers	Primer sequence (5' → 3')	Modification method	Length
<i>MC4R</i> -732-F-C	CGATCACCATCAGTGCCATCT	5' BIOTIN, LNA	254 bp
<i>MC4R</i> -732-F-G	CGATCACCATCAGTGCCATGT	5' BIOTIN, LNA	
<i>MC4R</i> -R	GATCAGGATGGTCAGCGTGATC	5' FITC	

Table 1. Primer system for the detection of *MC4R* gene SNP 732 locus. Italics represent LNA-modified bases.

Components	Volume (μL)
Sheep whole blood	2.00
2 \times Marathon buffer	25.00
dNTP mixture (2.5 μM)	10.00
PCR forward primer (10 μM)	0.40
PCR reverse primer (10 μM)	0.40
Marathon DNA polymerase (1 U/ μL)	1.00
ddH ₂ O	11.20
Total volume	50

Table 2. PCR amplification primer system.

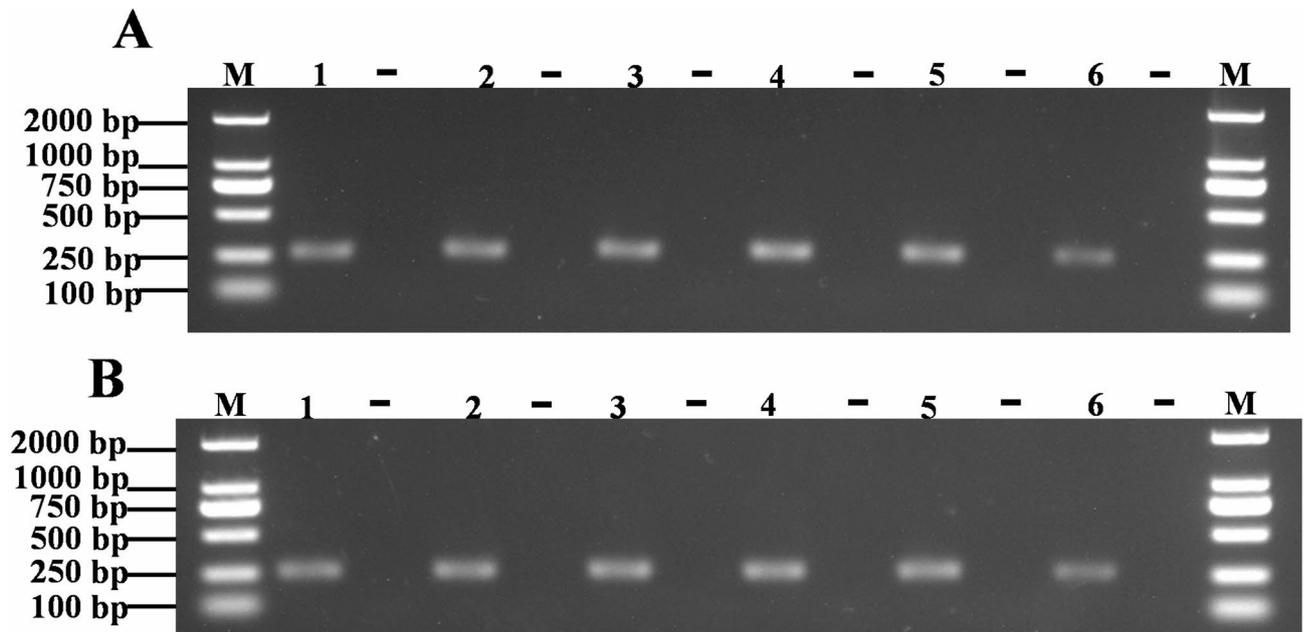


Fig. 1. PCR amplification of SNP detection primers (marker DL2000). **(A)** Template is the CC individual. –: Negative control. **(B)** Template is the GG individual. –: Negative control.

is detailed in Table 2. The PCR conditions were as follows: pre-denaturation at 94 °C for 2 min; denaturation at 98 °C for 10 s; annealing at 63 °C for 30 s; extension at 68 °C for 30 s; 32 cycles in total; and storage at 4 °C.

Following amplification, the PCR tube was opened, and the binding pad end of the detection LFD (Tiosbio, Beijing) was inserted into the tube. Once the reading area was fully infiltrated, the LFD was laid flat for one minute to allow for color development. The results were then read directly based on the appearance of red bands.

Result comparison between PCR-LFD and mutation results

To assess the practicality and accuracy of the detection system, blood samples from 24 randomly selected Hu sheep were tested. Results obtained via LFD were compared with mutation analysis results to verify the reliability of this SNP-locus detection system in identifying unknown random samples.

Results

Primer specificity verification and PCR reaction system establishment

Based on the principles described in the “Design of PCR primers” section, primers targeting the SNP locus were designed and synthesized. PCR amplification was conducted using whole blood templates from individuals homozygous for either the GG or CC genotype at *MC4R* g.732 C > G. In the reaction system using a whole blood template from a homozygous CC individual, single, bright amplification bands were observed with the MC4R-732-F-C and MC4R-R primers at annealing temperatures of 55 °C, 57 °C, 59 °C, 61 °C, and 63 °C, with product sizes matching those listed in Table 1. However, the bands became faint at 65 °C. The results are shown in Fig. 1A. Similarly, in the reaction system using a whole blood template from a homozygous GG individual, single, bright amplification bands were observed with the MC4R-732-F-G and MC4R-R primers at annealing temperatures of 55 °C, 57 °C, 59 °C, 61 °C, and 63 °C, with product sizes consistent with those in Table 1. At 65 °C, the bands also became faint. The results are presented in Fig. 1B.

In the reaction system using a whole blood template homozygous for CC, amplification bands appeared with the MC4R-732-F-C and MC4R-R primers at annealing temperatures of 55°C, 57°C, 59°C, 61°C, 63°C, and 65°C. Subsequently, PCR was performed with the same primers (MC4R-732-F-C + MC4R-R) on a whole blood template homozygous for GG at the same annealing temperatures. After agarose gel electrophoresis, no amplification bands were detected under UV light, as shown in Fig. 2A. Similarly, PCR was performed with the MC4R-732-F-G and MC4R-R primers on a whole blood template homozygous for CC across the same annealing temperatures. No amplification bands were observed after electrophoresis, as shown in Fig. 2B.

Establishment of a PCR- LFD assay for SNP detection

Reactions were performed with the MC4R-732-F-C + MC4R-R primers using the following templates: blood from individuals with homozygous CC genotype; genomic DNA from individuals with homozygous CC genotype; blood from individuals with homozygous GG genotype; genomic DNA from individuals with homozygous GG genotype; and double-distilled water as a negative control, with volumes matched to those of blood and DNA templates.

The PCR-LFD detection results are shown in Fig. 3. The reaction products of the MC4R-732-F-C + MC4R-R primers with blood and genomic DNA from homozygous CC individuals displayed blue bands at the control line (C line) and red bands at the test line (T line), indicating positive detection. This demonstrated that the MC4R-732-F-C + MC4R-R primer specifically detected the C allele in both blood and genomic DNA. In contrast, reactions with blood and genomic DNA from homozygous GG individuals, as well as the negative control (double-distilled water), produced only a single blue band at the control line without a test line, indicating negative detection. This confirmed that the MC4R-732-F-C + MC4R-R primer did not detect the G allele and was unaffected by its presence.

Reactions were performed with the MC4R-732-F-G + MC4R-R primers using the following templates: blood from individuals with homozygous CC genotype; blood from individuals with homozygous GG genotype; genomic DNA from individuals with homozygous CC genotype; genomic DNA from individuals with homozygous GG genotype; and double-distilled water as a negative control, with volumes matched to those of the blood and DNA templates. The PCR-LFD detection results are shown in Fig. 4. Reactions using MC4R-732-F-G + MC4R-R primers with blood and genomic DNA from homozygous CC individuals, as well as the negative control, produced a single blue band at the control line (C line) without a test line (T line), indicating a negative result. This demonstrated that the MC4R-732-F-G + MC4R-R primer did not detect the C allele and was unaffected by its presence. In contrast, reactions with blood and genomic DNA from homozygous GG individuals produced both a blue band at the control line and a red band at the test line, indicating positive detection. This confirmed that the MC4R-732-F-G + MC4R-R primer specifically detected the G allele in blood and genomic DNA.

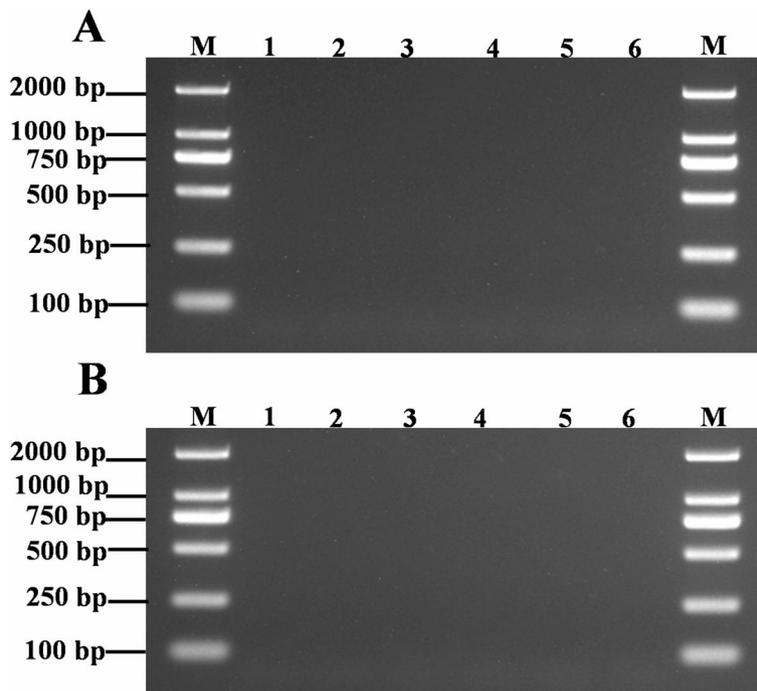


Fig. 2. PCR amplification of SNP detection primers in mismatched genotype individuals (marker DL2000). (A) Template is the GG individual. (B) Template is the CC individual.

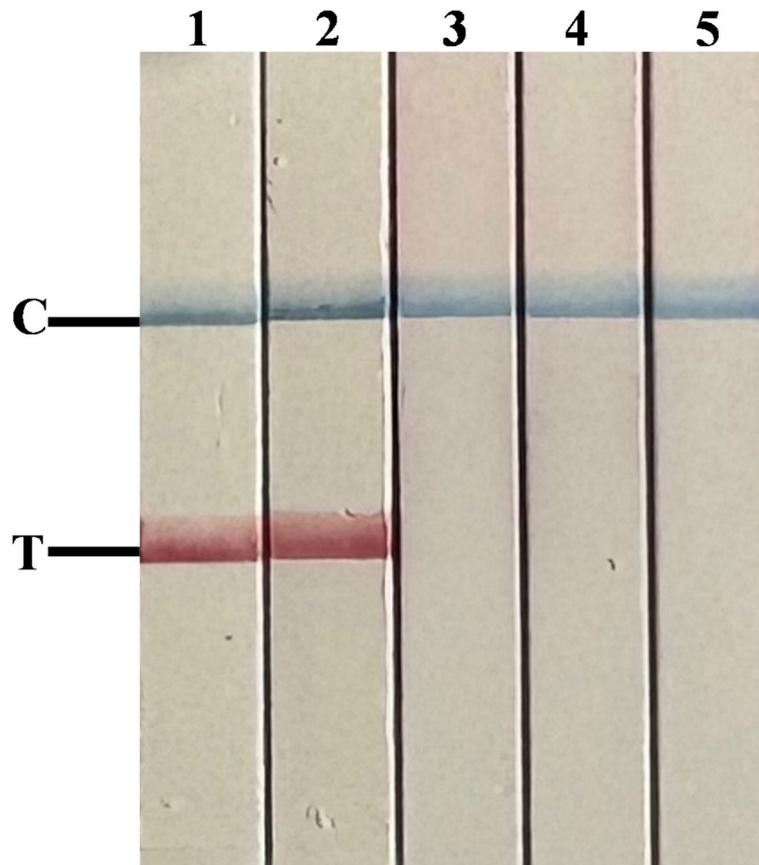


Fig. 3. SNP-locus PCR-LFD detection with the MC4R-732-F-C + MC4R-R primer. Templates 1–5 correspond to blood from homozygous CC individuals, genomic DNA from homozygous CC individuals, blood from homozygous GG individuals, genomic DNA from homozygous GG individuals, and double-distilled water as negative control, respectively.

Result comparison between PCR-LFD and mutation results

Blood samples from 24 Hu sheep were randomly selected for testing. Genotyping results obtained from PCR-LFD detection were compared with mutation analysis results to evaluate the reliability of this SNP-locus detection system for unknown samples. Tests were performed on individual sheep blood samples using both primer sets, MC4R-732-F-G + MC4R-R and MC4R-732-F-C + MC4R-R, with results summarized in Supplementary Table S1. For samples yielding positive results with both MC4R-732-F-G + MC4R-R and MC4R-732-F-C + MC4R-R primers, the corresponding genotype was identified as heterozygous (GC). Samples positive only with MC4R-732-F-C + MC4R-R and negative with MC4R-732-F-G + MC4R-R were classified as homozygous CC. Conversely, samples positive only with MC4R-732-F-G + MC4R-R and negative with MC4R-732-F-C + MC4R-R were classified as homozygous GG. The PCR-LFD detection results are illustrated in Fig. 5. The results of PCR-LFD were completely consistent with the mutation results, as shown in Supplementary Table S1.

Discussion

In theory, PCR could only be successfully carried out if the single-stranded template correctly paired with the primer strand, based on the base-pairing principle²⁷. A set of PCR primers differing by a single base was designed targeting regions with base variations and amplified using conventional PCR instruments. The base types at the target loci were inferred from the presence or absence of amplification products of the expected length.

Locked nucleic acid (LNA) is a RNA derivative containing a methylene bridge between the 2'-O and 4'-C positions of the nucleotide sugar ring. LNAs pair with DNA or RNA following general base-pairing rules. This bridged structure enhances nucleic acid backbone stability, increases melting temperature, and improves base-pairing specificity, significantly reducing mismatches. Consequently, LNA is widely used in applications such as gene chips and RNA interference²⁸.

In this study, LNA modification was applied to design amplification primers for SNP loci. The 3' ends of both forward and reverse primers corresponded to the target SNP loci, with LNA modification at these 3' terminal nucleotides. This modification significantly enhanced primer binding specificity to the template DNA, ensuring correct amplification according to the base-pairing principle. Thus, nucleotide types at the target loci were determined based on the presence or absence of amplification products, enabling genetic typing at SNP loci. LNA-modified primers exhibited higher annealing temperatures than unmodified primers. Therefore, an annealing temperature gradient test was performed during primer selection to identify the optimal reaction temperature.

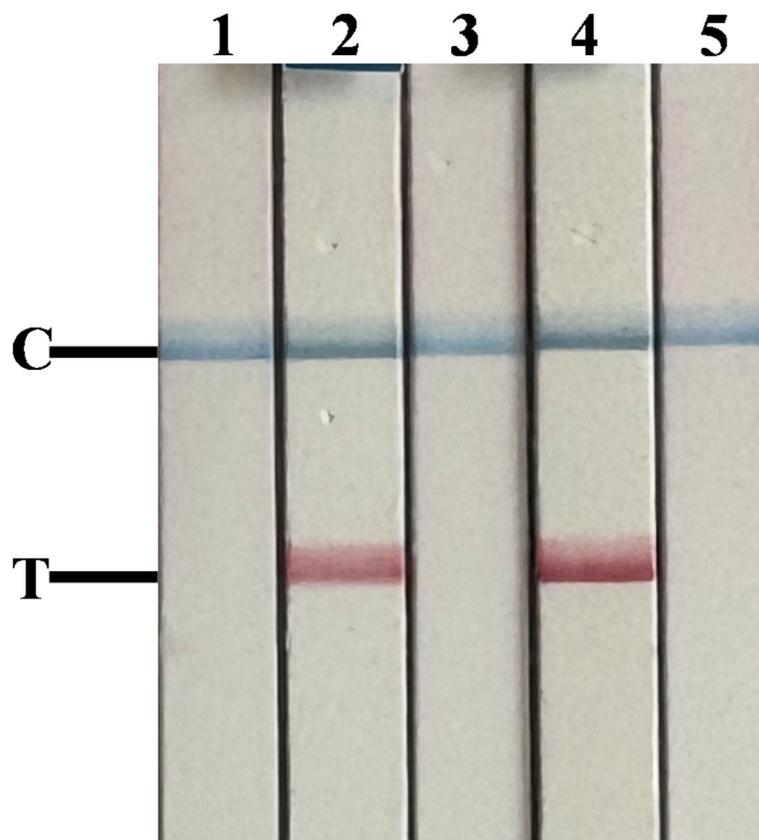


Fig. 4. SNP-locus PCR-LFD detection with the MC4R-732-F-G + MC4R-R primer. Templates 1–5 correspond to blood from homozygous CC individuals, blood from homozygous GG individuals, genomic DNA from homozygous CC individuals, genomic DNA from homozygous GG individuals, and double-distilled water as negative control, respectively.

In this study, primers consistently produced single, bright amplification bands at annealing temperatures of 55°C, 57°C, 59°C, 61°C, and 63°C. These bands became less distinct at 65°C, indicating suboptimal conditions. Nonetheless, 63°C was chosen because it met temperature requirements while minimizing primer dimer formation. If optimal results were not achieved by narrowing the temperature range or reducing temperature increments, strategies such as decreasing annealing time or reducing PCR cycle numbers could be employed. Repeating annealing temperature gradient PCR amplification may help identify the optimal temperature.

Two *MC4R* gene SNP detection primers, labeled with biotin and fluorescein isothiocyanate (FITC), were designed for this study. Both markers were incorporated into the double-stranded amplification products, enabling direct observation of reaction results using LFD without sequencing. The color development on the LFD followed the antigen-antibody binding principle²⁹. In detecting cyromazine and melamine in animal feed, LFD demonstrated detection limits of 0.22 ng/mL and 0.26 ng/mL, respectively, both lower than previously reported limits³⁰. LFD have been widely used for pesticide residue detection, including Sevin insecticide, oxyphosphomethyl, and chlorothalonil^{31,32}. They are also applied in hormone detection; for example, LFD identified testosterone in water with a detection limit of 5.00 ng/mL³², and estradiol (E2) in milk was detected using monoclonal antibodies against E2 combined with colloidal gold³³. Several studies employed LFD as a result-display terminal for detecting SARS-CoV-2 virus^{34,35}. This study developed a rapid SNP genotyping method using PCR-LFD, bypassing the complexity of traditional PCR sequencing and significantly improving SNP detection efficiency.

Streamlined sample processing: This method eliminated the need for genomic DNA extraction, allowing direct amplification from blood samples and reducing complex preprocessing steps such as DNA extraction.

User-friendly result presentation: Results were easily interpreted from the LFD after PCR, without requiring fluorescent detection equipment or specialized instruments. This simple method delivered rapid, intuitive results observable within minutes via color change, without further processing of PCR products.

Conclusion

This study developed a rapid and effective PCR-LFD detection system for distinguishing CC, CG, and GG genotypes at the *MC4R* gene g.732 C>G locus, demonstrating that PCR-LFD is a fast and simple method for SNP genotyping. PCR-LFD for SNP detection shows great potential in Hu sheep breeding. This method enables instant detection of target SNP, facilitating early screening of Hu sheep individuals that meet breeding requirements. It allows genotype identification at various stages of the Hu sheep's life cycle, enabling timely

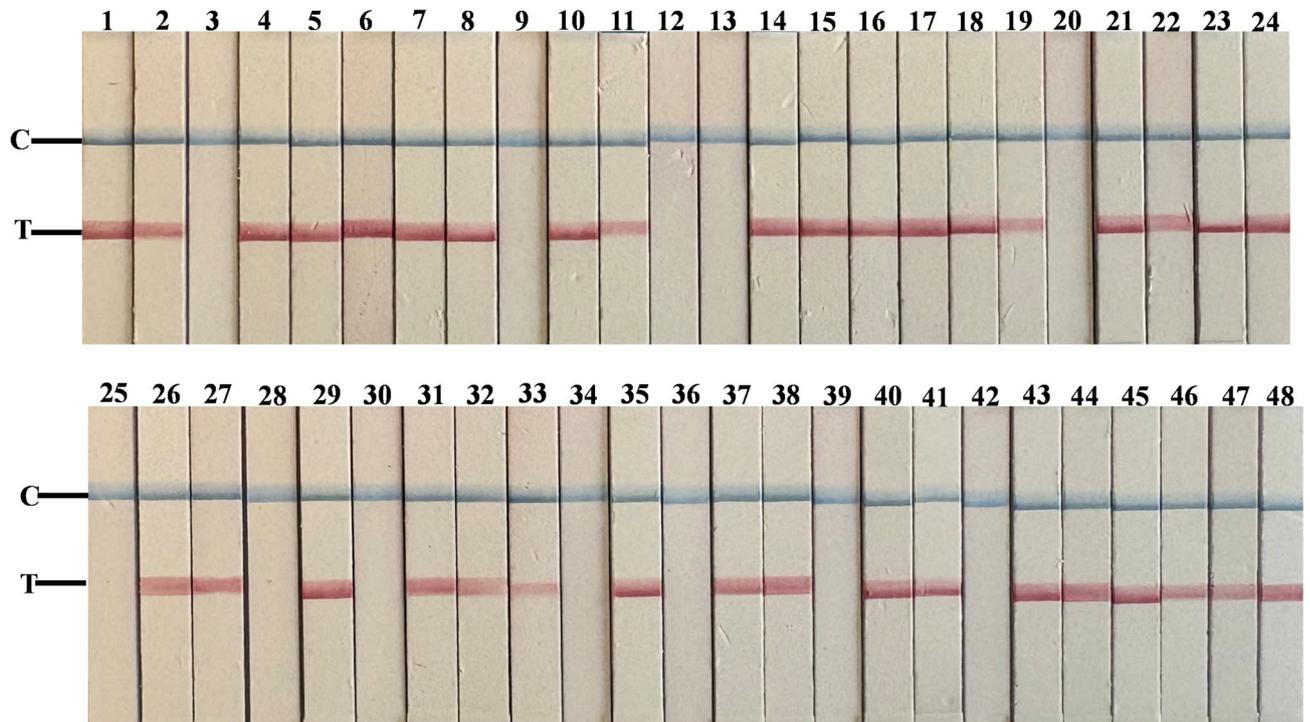


Fig. 5. SNP-locus PCR-LFD detection results of blood samples of Hu sheep. Primers used for the odd-numbered LFDs were MC4R-732-F-G, and primers for the even-numbered LFDs were MC4R-732-F-C. Sheep numbers 1–2: 6062; 3–4: 6341; 5–6: 6255; 7–8: 5546; 9–10: 5432; 11–12: 5378; 13–14: 5439; 15–16: 5497; 17–18: 6471; 19–20: 6944; 21–22: 6783; 23–24: 6369; 25–26: 6328; 27–28: 6346; 29–30: 5015; 31–32: 6303; 33–34: 5977; 35–36: 6451; 37–38: 5967; 39–40: 5271; 41–42: 6469; 43–44: 5572; 45–46: 5456; 47–48: 5902.

adjustments to breeding strategies. Particularly before mating, this method can rapidly screen suitable parent individuals, thereby improving breeding success rates.

Data availability

Data included in article/supp. material in article.

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Declarations

Competing interests

The authors declare no competing interests.

Additional information

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