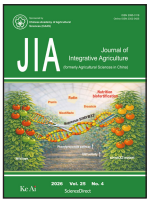
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RESEARCH ARTICLE

Rapid on-site genotyping of the ovine prolific *FecB*^{B} mutation using a CRISPR/Cas12a-based detection system

Tingjie Wu[1], Jiayuan Sun[1], Lijin Lu[1], Chen Wang[1], Shiwei Zhou[2, 3], Yulin Chen[1, 3, 4], Xinjie Wang[5#], Xiaolong Wang[1, 3, 4#]

[1] *International Joint Agriculture Research Center for Animal Bio-breeding, Ministry of Agriculture and Rural Affairs/College of Animal Science and Technology, Northwest A&F University, Yangling 712100, China*
[2] *College of Veterinary Medicine, Northwest A&F University, Yangling 712100, China*
[3] *Key Laboratory of Livestock Biology, Northwest A&F University, Yangling 712100, China*
[4] *School of Future Technology on Bio-breeding, Northwest A&F University, Yangling 712100, China*
[5] *Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen 518124, China*

Highlights

- A rapid, precise, on-site, portable, and naked-eye visualization method for genotyping the *FecB*^{B} mutation in sheep has been established.
- A genotyping strategy for important SNP site in livestock breeding programmes is provided, theoretically applicable to any SNP site.

Abstract

BMPR1B is a pivotal gene that influences reproductive performance in sheep. The sheep populations that carry the *FecB*^{B} mutation within this gene exhibit significantly higher lambing rates compared to wild-type populations. Therefore, screening for individuals carrying the *FecB*^{B} mutation is crucial for effective sheep breeding programs. This study aims to establish a rapid, precise, and visualised on-site detection method for genotyping the prolific *FecB*^{B} mutation in sheep. We combined the CRISPR/Cas12a system with the recombinase-polymerase amplification (RPA) technique. We introduced an additional nucleotide mismatch on the amplification primers to form a Cas12a-recognised protospacer adjacent motif (PAM) sequence. In addition, mismatches were introduced in CRISPR-derived RNA (crRNA) to enable naked-eye differentiation of the assay results. Subsequently, we validated the accuracy of the method by examining additional blood samples from 56 sheep representing four breeds. The results of using our developed system were highly consistent with the Sanger sequencing. Overall, the CRISPR/Cas12a-based detection provides a rapid and more versatitle method for *FecB*^{B} genotyping. It holds promise in enhancing efficiency in livestock breeding programmes for any single nucleotide mutations.

Keywords: CRISPR/Cas12a, isothermal amplification, SNP detection, *BMPR1B*, *FecB*^{B} mutation, sheep

1. Introduction

The *BMPR1B* gene is the first significant gene identified to be a strong controller for multiple births in sheep, with a highly potential missense mutation (known as *FecB*^{B}, c.A746G) located within the gene's 8th exon (Souza *et al.* 2001). In ewes with the *FecB*^{B} mutation, the response of granulosa cells to *BMP4*-stimulated *AMH* production was reduced in a dose-dependent manner, effectively increasing ovulation rates and lambing numbers (Mulsant *et al.* 2001; Wilson *et al.* 2001; Estienne *et al.* 2015). In modern sheep production practices, the economic benefits of producing double lambs are 1.6 times greater than those of producing single lambs (Notter 2012). Thus, identifying and screening the *FecB*^{B} mutation can provide firm technical support for selecting and breeding multiparous sheep and improving the sheep industry's economic benefits.

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Tingjie Wu, E-mail: tingjie.wu@nwafu.edu.cn; #Correspondence Xinjie Wang, E-mail: wangxinjie@caas.cn; Xiaolong Wang, Tel: +86-29-87091130, E-mail: xiaolongwang@nwafu.edu.cn

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Single nucleotide polymorphisms (SNPs) are the most common type of genetic variation. Currently, common SNP detection techniques include the gene chip method, restriction fragment length polymorphism (RFLP) technology, Sanger sequencing, TaqMan probe technology, and real-time qPCR. Among them, Sanger sequencing serves as the gold standard for SNP detection. Previous detection methods for the *FecB*^{B} mutation included polymerase chain reaction (PCR)-based Sanger sequencing, restriction fragment length polymorphism (RFLP) (Guan *et al.* 2007), and real-time qPCR (Zhou *et al.* 2022). However, these methods are not applicable to on-site testing due to expensive specialized equipment and high operational requirements, thus limiting their application to small- and medium-sized farming enterprises or individual farmers on remote pastures.

The clustered regularly interspersed short palindromic repeats (CRISPR) system has shown great potential for rapid and accurate nucleic acid detection (Myhrvold *et al.* 2018; Li *et al.* 2019; Teng *et al.* 2019; Wang *et al.* 2020b). One of the type V-A CRISPR effector proteins, Cas12a, which recognizes a T-rich protospacer adjacent motif (PAM) sequence (Zetsche *et al.* 2015), can specifically recognize and cleave the target double-stranded DNA (dsDNA) with the guidance of a CRISPR-derived RNA (crRNA) that is complementary to the target sequence at a single-base resolution (Li *et al.* 2018). At the same time, Cas12a also indiscriminately cleaves nearby single-stranded DNA (ssDNA, including fluorescent reporter probes), a property known as *trans*-cleavage (Chen *et al.* 2018). This *trans*-cleavage strategy is widely used for sensitive and specific target sequence detection (Ding *et al.* 2020; Wu *et al.* 2020; Ding *et al.* 2021; Zhu *et al.* 2022). CRISPR/Cas12-based nucleic acid detection methods are sensitive, specific, rapid, simple, and inexpensive. However, there have been no reports on detecting the desirable mutations in animals based on isothermal amplification technology and the CRISPR/Cas12a system.

In this study, we established a rapid and accurate method for the on-site genotyping for the sheep *FecB*^{B} mutation based on the combination of recombinase-polymerase amplification (RPA) and the CRISPR/Cas12a system. Taking into account the PAM sequence constraint, we inserted the PAM sequences at a suitable position by amplification with RPA. Next, we used a dual-crRNA strategy to genotype the *FecB*^{B} mutation and introduced mismatches into the crRNA sequence to visualize the assay results. Finally, we verified the accuracy and feasibility of this method by comparing the detection results of multiple sheep breeds using PCR-based Sanger sequencing. Together, this method provides a rapid and precise on-site strategy for *FecB*^{B} mutation genotyping, demonstrating its ability to be widely used in selective breeding of highly reproductive livestock species.

2. Materials and methods

2.1. Experimental sample collection and DNA extraction

All sheep blood samples were collected from the jugular vein using a blood collection vessel containing EDTA2K anticoagulant and stored at –20°C. Sheep genomic DNA (gDNA) was extracted using a blood genome extraction kit (CWBIO, China) and stored at –20°C. The 56 blood samples of the Chinese Tan sheep were collected from Tianyuan Sheep Breeding and Raising Co., Ltd., Hongsibao District, Ningxia Hui Autonomous Region, China. The blood samples of Dorset sheep, Hu sheep, and Dorset-Hu crossbreed sheep were collected from Gansu Qinghuan Meat Sheep Breeding Co., Ltd., Qingyang City, Gansu Province, China. The gDNA samples of Chinese Tan sheep with known *FecB*^{B} genotypes were previously stored by our laboratory.

2.2. Generation of *FecB*^{B} dsDNA targets

The PAM sequence recognized by LbCas12a (TTTV) was lacking near the 5′ end of the *FecB*^{B} mutation site. Thus, the Chinese Tan sheep gDNA with the three known *FecB*^{B} genotypes (AA, AG, and GG) was used as templates, and a single-base mismatched primer was designed to provide the required PAM sequences. Wild-type (WT), heterozygous, and mutant-type (MT) fragments were amplified using PCR. Then, fragments were purified and diluted to the same concentration (1×10^{10} copies μL^{-1}) for subsequent experiments. Three 161 bp DNA fragments covering the *FecB*^{B} site were PCR amplified with primers P1 and P2 from AA, GG and AG genotypes gDNA of sheep (Appendix A). The 25 μL PCR system included 2× Fast Pfu Master Mix (12.5 μL, Novoprotein, Suzhou, China), P1 (10 μmol L^{-1}, 0.5 μL, all primers were synthesized by Sangon, China), P2 (10 μmol L^{-1}, 0.5 μL), gDNA (1 μL), and ddH_2O (10.5 μL). PCR reaction conditions were 94°C for 1 min 30 s, 32 cycles of 94°C for 20 s, 53°C for 20 s and 72°C for 20 s, 72°C for 5 min.

2.3. Isothermal amplification of *FecB*^{B} target region

The isothermal amplification of the *FecB*^{B} target region was performed with a commercial GenDx ERA Kit (Suzhou GenDx Biotech, China). According to the manufacturer's instructions and with reference to the position of the single-base mismatch introduced by the PCR primers, we designed and synthesized 30–35 bp RPA primers (Appendix A). The size of the fragment containing the *FecB*^{B} site was 75–168 bp. The 25 μL RPA reaction system included reaction buffer (10 μL), ERA basic buffer (5 μL), forward primer (10 μmol L^{-1}, 1.25 μL), reverse primer (10 μmol L^{-1}, 1.25 μL), DNA template (3 μL), activator (1 μL), and supplementary RNase-free water. Then, the mixture was incubated at 39°C for 20 min. Three 214 bp DNA fragments covering the *FecB*^{B} site were PCR amplified as temples for the RPA reaction test with primers P3 and P4 from AA, GG and AG types gDNA of sheep (Appendix A). The 25 μL PCR system included 2× Fast Pfu Master Mix (12.5 μL, Novoprotein, Suzhou, China), P3 (10 μmol L^{-1}, 0.5 μL), P4 (10 μmol L^{-1}, 0.5 μL), gDNA (1 μL), and ddH_2O (10.5 μL). PCR reaction conditions were 94°C for 1 min 30 s, 32 cycles of 94°C for 20 s, 50°C for 20 s and 72°C for 20 s, 72°C for 5 min.

2.4. Cas12a fluorescent cleavage assay and analysis

The *FecB*^{B} mutation was identified by the incubating mixture reactions at 39°C for 1 h using a Biotek Microplate reader (Synergy H1, USA). Fluorescence kinetics was measured every minute, and the fluorescence results were recorded

using a smartphone camera or Thermo Fisher automated gel imaging analysis system (iBright 1500, USA). For screening crRNAs, we used the signal-to-noise ratio (the ratio of the fluorescence intensity of the positive signal to the noise signal) as a measure. For example, when we were screening MT-crRNAs, we selected the crRNA with the highest ratio of fluorescence intensity value between the GG genotype sample and the AA genotype sample at the same moment. The 20 μL reaction system included LbCas12a (1 μmol L^{-1}, 1 μL, NEB, USA), crRNA (1 μmol L^{-1}, 1 μL, synthesized by GenScript, China), 10× NEbuffer 2.1 (2 μL, NEB, USA), RNase inhibitor (40 U μL^{-1}, 0.1 μL, Solarbio, China), ssDNA-FQ reporter (25 μmol L^{-1}, 1 μL, Genewiz, USA), an appropriate amount of PCR product or 2 μL RPA product to test, and supplementary ddH_2O. After sufficient mixing on the vortex shaker, the mixture was incubated at 39°C for 20 min.

2.5. Preprocessing of sheep blood samples

Three nucleic acid releasing agents (Ras) were used to optimize blood samples preprocessing session, Ra1 (La, Suzhou GenDx Biotech, China), Ra2 (GD005, Suzhou GenDx Biotech, China), Ra3 (GZ011201, CYGZ, China), respectively. For genomic DNA extraction, 300 μL jugular vein blood samples were mixed and shaken thoroughly with 600 μL red cell lysing reagent (Biosharp, Hefei, China). Then, 1 min of minicentrifuge was used to separate lysed red blood cells (RBCs) and unlysed white blood cells (WBCs). Repeat the above steps once. Finally, the precipitated WBCs were split by a 100 μL nucleic acid releaser (Suzhou GenDx Biotech, China) at 95°C for 5 min to release genomic DNA. The 3 μL treated sample were used for the subsequent RPA.

2.6. *FecB*^{B} mutation genotyping

The *FecB*^{B} mutation genotyping was based on a dual-crRNA detection system, including on- and off-target crRNAs designed to complement MT or WT gDNA completely, respectively. The genotype was determined by testing the reaction of two crRNA tubes for each sample. The green fluorescence of the ssDNA-FQ reporter was visualized by the naked eye under a 485 nm blue lamp (Sangon, China) when there is a target DNA that is completely complementary with the crRNA. We determined the genotype of the sample based on whether on-target and off-target crRNAs produced fluorescence signals.

2.7. Statistical analysis

The experimental results are shown as mean±standard error of the mean (SEM) unless stated otherwise. Statistical analysis was performed using GraphPad Prism 9.0.

3. Results

3.1. CRISPR/Cas12a-based *FecB*^{B} mutation genotyping

Generally, the workflow of sheep *FecB*^{B} mutation genotyping based on the CRISPR/Cas12a system includes sample collection, gDNA release, target DNA amplification, Cas12a fluorescence detection, and result analysis (Fig. 1). Briefly, gDNA was rapidly released from the sheep blood samples under 95°C for 5 min, target DNA was amplified at 39°C for 20 min using RPA technology, and the amplified products and the fluorescence reaction system were incubated and detected at 39°C for 10 min. When the crRNA is specifically recognized, Cas12a stimulates *trans*-cleavage ssDNA activity, the ssDNA fluorescent reporter is cut to produce fluorescence, and finally the genotype of the sample is determined based on the fluorescence results. The whole process can be completed within 1 h.

3.2. Breaking PAM limitations through primer mismatch

PAM sequences are essential when detecting nucleic acids in the CRISPR/Cas12a system. However, not all target DNA contains PAM sequences, or PAM sequences are in the appropriate position. In this study, a PCR amplification system inserted PAM sequences into the 5′ direction of the target DNA mutation site. On the one hand, when designing the PCR primers, it was considered that the introduction of mismatched bases at the 3′ end of the primers would significantly reduce the amplification efficiency. On the other hand, the single nucleotide sensitivity would be significantly reduced when the PAM sequences are more than six bases away from the mutation site (Chen *et al.* 2018). Thus, we introduced only a single-base mismatch at the 3′ end of the primer to form the TTTV sequence (Fig. 2-A). The primer does not contain the mutation site and is theoretically applicable to the three genotypes. The gel electrophoresis results of the PCR-amplified products showed that single bands were obtained for AA, AG, and GG genotypes (Fig. 2-D). The PCR-based Sanger sequencing results confirmed that the PAM sequences had been successfully inserted into the expected position in the amplified products (Fig. 2-E).

3.3. Screening specific crRNAs for genotyping the *FecB*^{B} mutation

Modifying crRNAs is required in the Cas12a reaction to avoid generating noisy signals that interfere with genotype determination. This study used a dual-crRNA system, including WT-crRNA and MT-crRNA, for detecting *FecB*^{B} WT and MT, respectively. The PCR products (AA, AG, and GG) with inserted PAM sequences were used as templates for the CRISPR/Cas12a detection system (Fig. 3-A). These PCR products were used to test the potential of the dual-crRNA system to discriminate between homozygous and heterozygous genotypes. The eight crRNAs were firstly designed for *FecB*^{B} mutant sequences, in which crRNA-g matched perfectly with the *FecB*^{B} mutant sequences, while crRNA-g1 to crRNA-g7 introduced a single-base mismatch at different positions at the 3′ end of the mutation site (Fig. 2-B; Appendix B). crRNA-g1 has a strong inhibitory effect on fluorescence signaling of WT. We inferred that the vicinity of the position where the mismatched base was introduced in this sequence is a sensitive region for the specific recognition of the single-base mismatch in Cas12a (Fig. 3-B and E). Therefore, we designed eight additional engineered crRNAs (crRNA-g8 to crRNA-g15) with substitutions of A, T, C, and G from the –1 to +1 position around the site of introducing the mismatch of crRNA-g1 (Fig. 2-B; Appendix B). crRNA-g11 has the best suppression of the WT noisy signals, but with

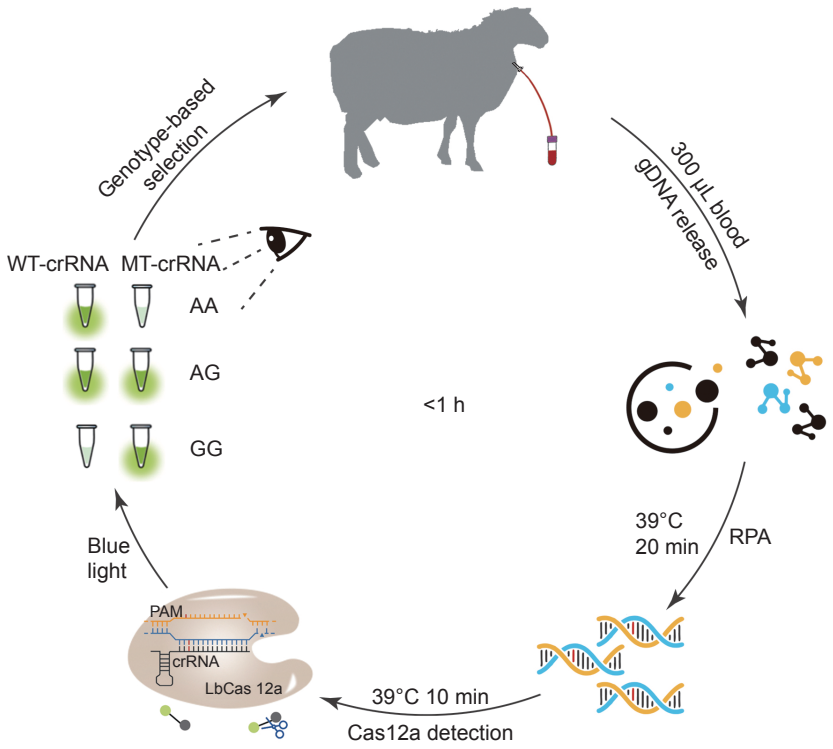


Fig. 1 Schematic diagram of the rapid *FecB*^{B} detection using the Cas12a system. The workflow includes sample preparation, target DNA amplification, Cas12a fluorescence detection, and the analysis of the result. RPA, recombinase-polymerase amplification.

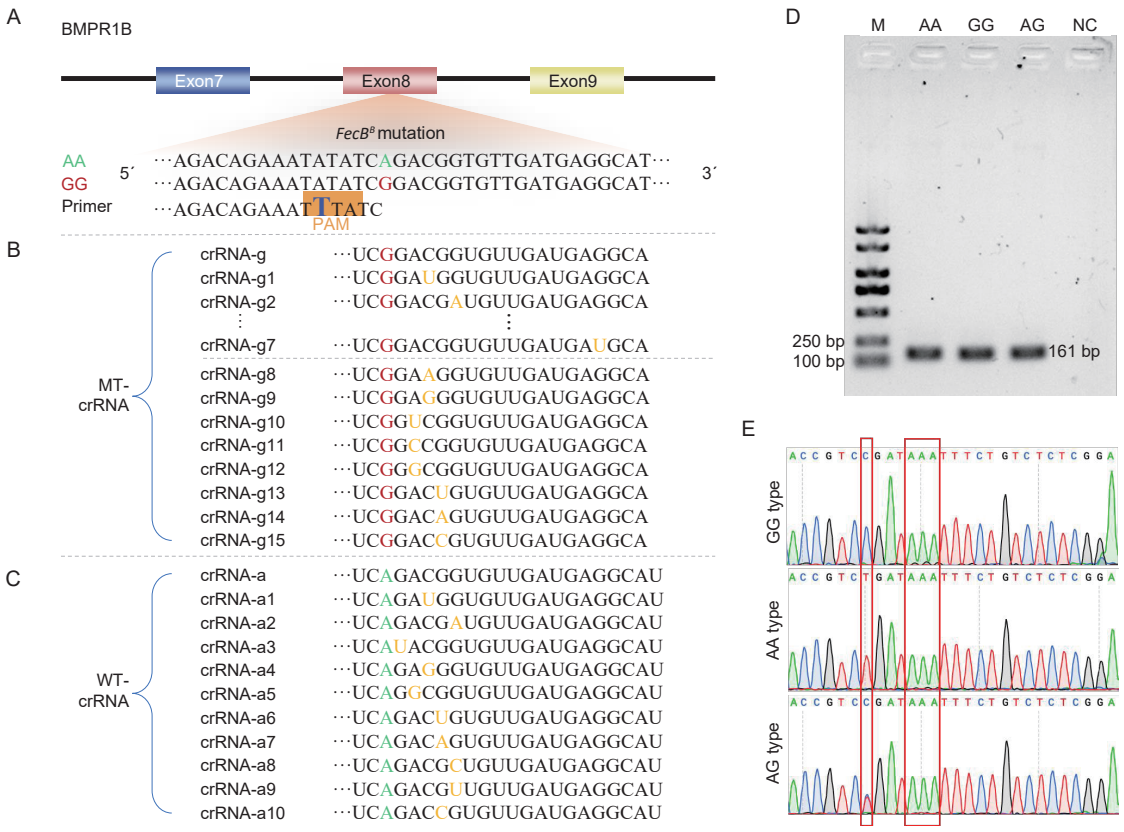


Fig. 2 Strategy of design and optimization amplification primers and specific crRNAs for *FecB*^{B} site. A, sequence information of the forward primer, and AA and GG target DNA. A mismatched base is indicated by the letter in larger font. B and C, sequence information of the crRNA-g to crRNA-g15 and crRNA-a to crRNA-a10. Mismatch positions are shown in orange. D, the AA, GG, and AG gDNA were amplified as single target products through agarose gel. E, the reverse Sanger sequencing results of AA, GG, and AG genotypes indicated that the PAM sequences had successfully been inserted into the AA, GG, and AG amplification products.

a greater loss of positive signals (Fig. 3-C and F). For the *FecB*^{B} WT sequence, we designed 10 crRNAs (crRNA-a1 to crRNA-a10) near the above Cas12a recognition-sensitive region (Fig. 2-C; Appendix B). crRNA-a2 exhibited an extremely high signal-to-noise ratio (Fig. 3-D,G and H). In a comprehensive comparison, the combination of crRNA-a2 and crRNA-g11 was the most effective for subsequent experiments.

3.4. Optimization of the RPA primer

A suitable pair of RPA primers would greatly improve the amplification efficiency and sensitivity of the assay. For this reason, we tested 16 pairs of RPA primers to amplify the *FecB*^{B} fragment to optimize RPA-mediated preamplification,

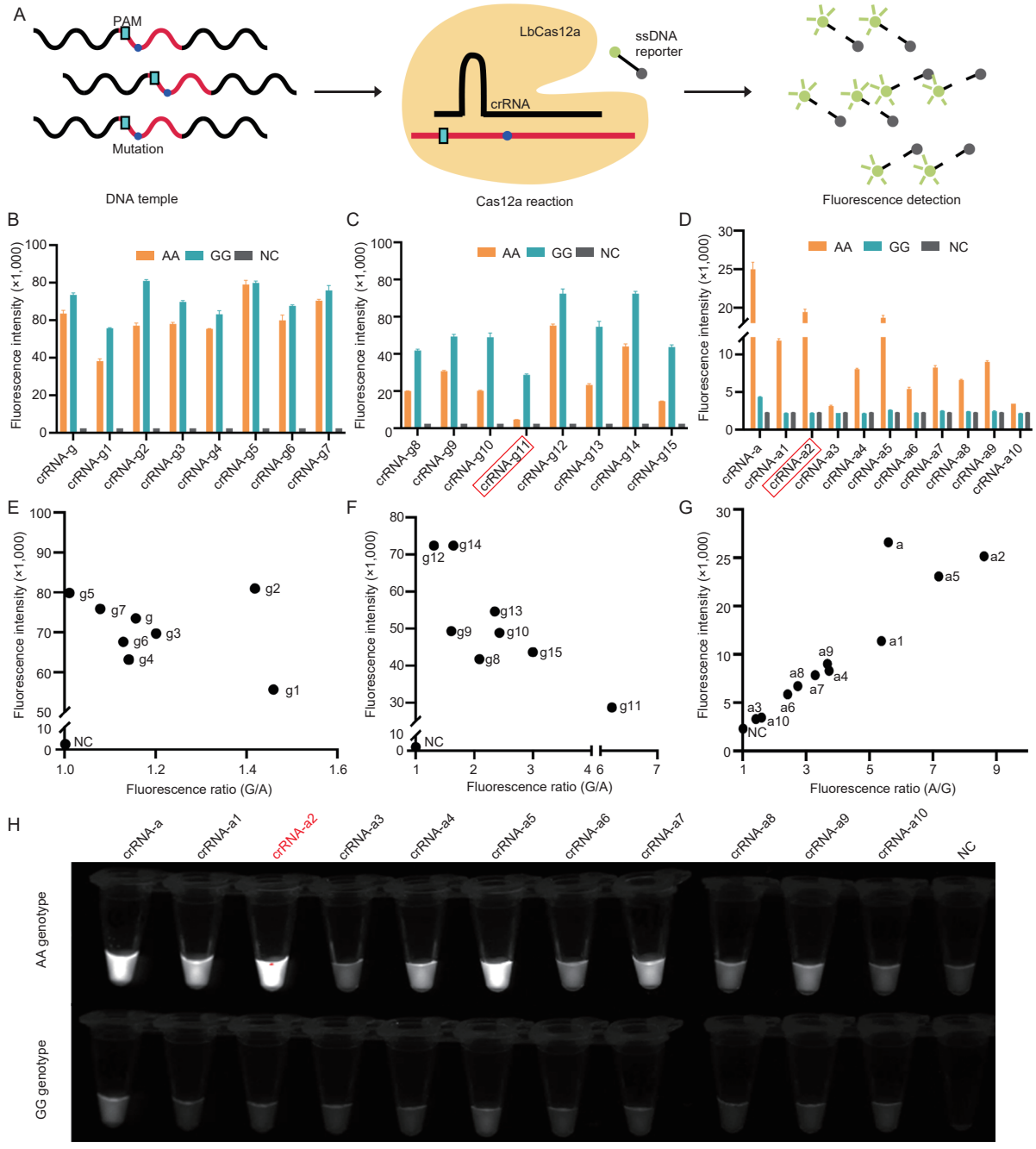


Fig. 3 Specificity of the crRNA combination for CRISPR/Cas12a-based *FecB*^{B} detection. A, schematic of screening specific crRNA combination for *FecB*^{B} site. B and C, specificity assay of crRNA-g to crRNA-g15 using 1×10^{10} copies of AA and GG genotype fragments, including the fluorescence intensity after 2 h of Cas12a reaction (*n*=3). D, specificity assay of crRNA-a to crRNA-a10 using 1×10^{10} copies of AA and GG genotype fragments, including the fluorescence intensity after 30 min of the CRISPR/Cas12a reaction (*n*=3). E and F, screening of crRNA-g to crRNA-g15 targeting the *FecB*^{B} mutant sequence. Specificity was determined as the ratio of the on-target DNA-induced fluorescence intensity over the off-target DNA-induced fluorescence intensity. The fluorescence intensity was obtained after 2 h of the CRISPR/Cas12a reaction. G, screening of crRNA-a to crRNA-a10 targeting the *FecB*^{B} WT sequence. H, imaging results under UV light at 30 min of CRISPR/Cas12a reaction. NC, negative control.

using the PCR products containing the *FecB*^{B} site as the template. The primer pair, F3 and R4, had the highest fluorescence intensity during CRISPR/Cas12a detection, demonstrating the highest amplification efficiency (Fig. 4-A–C). This pair of primers was used in subsequent analyses.

3.5. Isothermal amplification coupled with Cas12a-mediated efficient nucleic acid detection

After confirming the crRNA combination and RPA primer pair, we next used the PCR products of the three *FecB*^{B} genotypes as templates and used the screened RPA primer pair and crRNA-a2 to validate the detection effect of the combination of the RPA technology and the CRISPR/Cas12a system. It was shown that the AA and AG genotypes could be effectively detected, while the noisy signal of the GG genotype is suppressed more strongly, proving that CRISPR/Cas12a system and RPA can be effectively combined and detected (Fig. 4-D).

Immediately after that, we tried the initial genotyping of the *FecB*^{B} mutation at the genomic level. crRNA-a2 showed a strong signal-to-noise ratio as usual (Fig. 4-E). Although crRNA-g11 could suppress the noisy signal of the WT samples to a very low level, the positive signal was also suppressed to some extent, resulting in a limited increase. The sample genotypes were simply discriminated by naked eye based on the reaction tube luminescence between the dual-crRNAs, though the difference in the positive signals generated is obviously large.

3.6. Optimization of the detection system

Then, we optimized the sample processing for a more convenient and simplier DNA release. We tested the DNA release efficiencies of three commercial nucleic acid releasers (Ra1, Ra2 and Ra3) on the gDNA from sheep WBCs. Ra1 produced the highest release efficiency after incubation at 95°C for 5 min (Fig. 5-A). It was shown that the high gDNA release increases the intensity of the positive signal of the MT-crRNA system. The required temperature to activate Ra1 to release DNA is 95°C, which is an easy-to-achieve temperature under normal field conditions. Thus, we

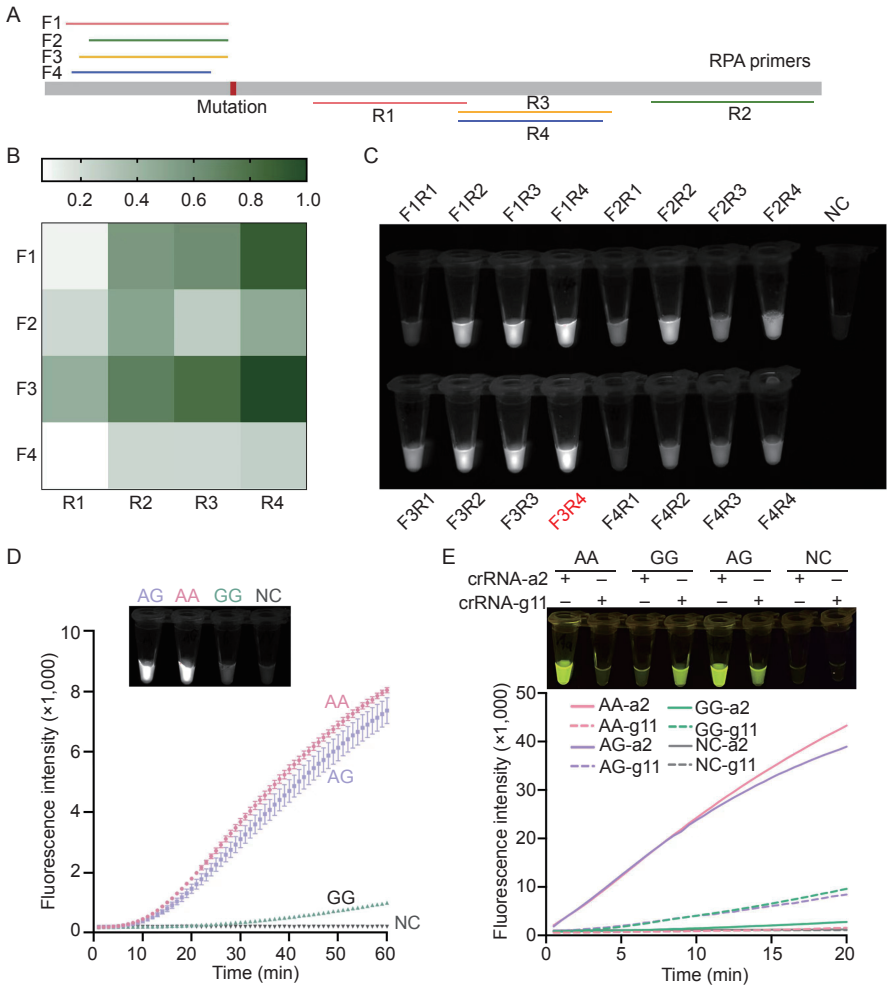


Fig. 4 RPA-combined CRISPR/Cas12a-based SNP detection. A, schematic of the position of RPA primers sequence. B, optimization of efficient RPA primers for the amplification of the *FecB*^{B} mutation site. The amplification efficiency of 16 pairs of RPA primers was evaluated, and quantification of normalized fluorescent intensity of the CRISPR/Cas12a detection signal was performed at 30 min. C, imaging results under UV light at 20 min of the CRISPR/Cas12a reaction. D, time-course of fluorescence intensity and imaging results of the PCR products of three *FecB*^{B} genotypes after 60 min of Cas12a reaction (n=3). E, time-course of fluorescence intensity and naked-eye observation of the gDNA samples of three *FecB*^{B} genotypes after 20 min of Cas12a reaction.

selected Ra1 based on its efficiency and simple requirements to be used as a DNA releaser in subsequent experiments.

In order to solve the problem of a large difference in positive fluorescence signal during genotyping, we made different adjustments to the WT-crRNA and MT-crRNA systems (Table 1). It was shown that increasing the dose of Cas12a and crRNA-g11 in the mutant-type processing group 1 (MT-PG1) significantly increased the rising speed of positive signals in the MT-crRNA system, and the noisy signals are slightly elevated over time (Fig. 5-B). However, by controlling the assay time, we could get fluorescence results that can be readout by the naked eye. For the WT-crRNA system, we gradiently reduced the dose of Cas12a and crRNA-a2, and the positive signals were weakened sequentially (Fig. 5-C). The positive signals of the MT-PG1 and WT-PG2 groups were relatively similar at 10 min of detection. Considering the accuracy of the fluorescence results observed by naked-eye discrimination, we determined that a total of 2 μL Cas12a and 2 μL crRNA-g11 in the MT-crRNA system and a total of 0.25 μL Cas12a and 0.25 μL crRNA-a2 in the WT-crRNA system are the optimal doses. Additionally, the assay time was determined to be 10 min and it was used for the subsequent experiments. Finally, we successfully achieved efficient genotyping of samples with the three *FecB*^{B} mutation types using the optimized system (Fig. 5-D).

3.7. Sheep blood sample validation

Finally, we validated the accuracy of the WT-crRNA/MT-crRNA system for genotyping the *FecB*^{B} mutation in sheep. The present method and PCR-Sanger sequencing were applied simultaneously to test 56 sheep blood samples from four breeds (Tan sheep, Dorset sheep, Hu sheep, and Dorset-Hu sheep crossbreed), and the sample fluorescence results showed that 10 samples were MT, 25 were WT, and 21 were heterozygous, which showed consistency with the results of PCR-based Sanger sequencing (Table 2; Appendix C).

Table 1 Optimization of MT-crRNA and WT-crRNA systems

Group[1)]	crRNA type	crRNA dosage (μL)	LbCas12a dosage (μL)	Temple input (μL)
MT-PG1	crRNA-g11	2	2	2
MT-PG2	crRNA-g11	1	1	5
MT-PG3	crRNA-g10 +crRNA-g11	0.5+0.5	1	2
MT-Con	crRNA-g11	1	1	2
WT-Con	crRNA-a2	1	1	2
WT-PG1	crRNA-a2	0.5	0.5	2
WT-PG2	crRNA-a2	0.25	0.25	2

[1)] PG, processing group. Con, control group. MT-PG1 and WT-PG2 were the optimal groups.

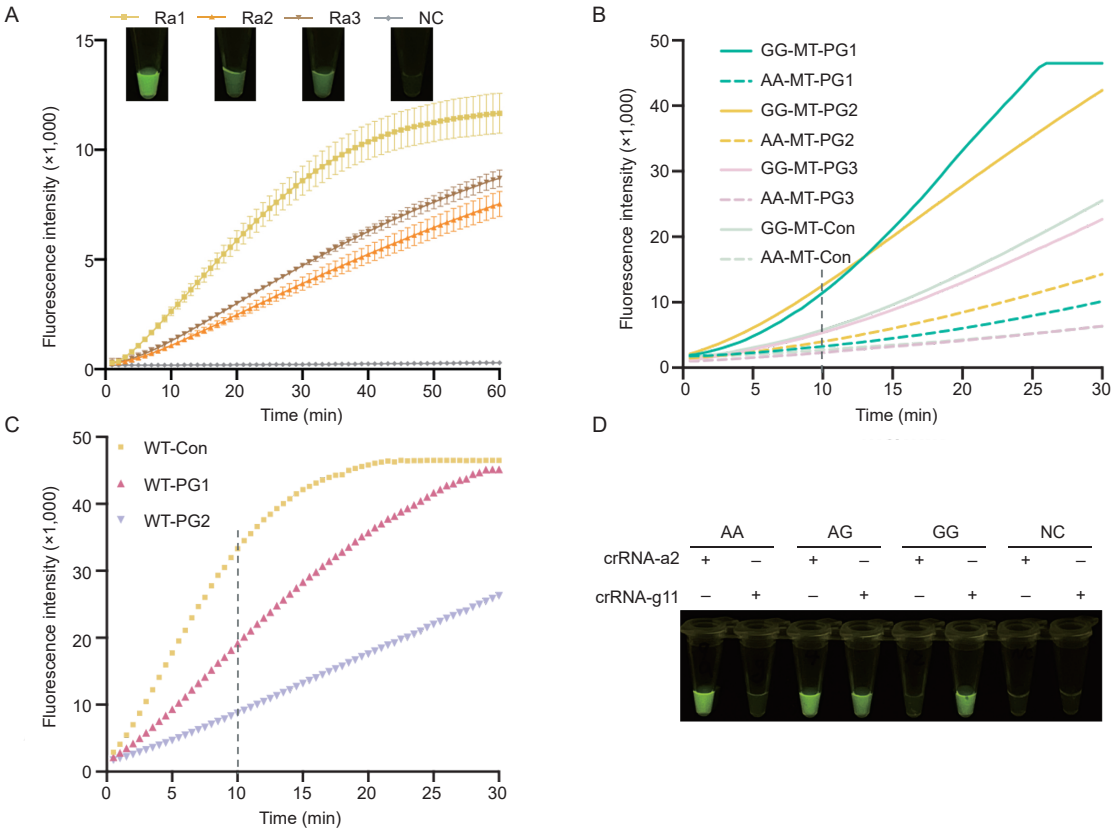


Fig. 5 Optimization of the CRISPR/Cas12a detection system. A, the time-course of fluorescence intensity and visual observation after 60 min of the Cas12a reaction with the addition of three nucleic acid releasers (Ra1, Ra2, and Ra3; *n*=3). B and C, the time-course of fluorescence intensity for optimizing MT-crRNA (B) and WT-crRNA (C) systems after 30 min of the Cas12a reaction. D, naked-eye fluorescence observations of different genotypes after optimization (at 10 min).

4. Discussion

Previous studies have found that the *FecB*^{B} mutation is more widely distributed in Asian sheep breeds. The presence of the *FecB*^{B} mutation was found in Indonesian Javenese sheep (Kumar *et al.* 2008), Iranian Kalehkoohi sheep (Mahdavi *et al.* 2014), Chinese Small-tailed Han sheep (Chu *et al.* 2007), Hu sheep (Guan *et al.* 2007), among other breeds. Previous studies by our research team demonstrated that lambing rates in Chinese Tan sheep populations with the *FecB*^{B} mutation were significantly higher than in WT Tan sheep (Zhou *et al.* 2022). Therefore, it is highly expected to establish a method for immediate and precise on-farm detection of the prolific *FecB*^{B} variant for a more efficient selection of prolific sheep.

Currently, the genotyping of the *FecB*^{B} mutation is still in the laboratory stage. PCR-based Sanger sequencing is considered the gold standard test for SNP genotyping. However, PCR requires sophisticated temperature control equipment, takes a long time, and requires high purity of samples, and Sanger sequencing relies heavily on the professionalism of the staff. These factors limit the use of this strategy for on-site testing. Recently, real-time PCR has been widely used in nucleic acid testing due to its high sensitivity and specificity (Batten *et al.* 2015). However, real-time PCR requires expensive and specialised equipment. Currently, there is no on-site, visualized detection method for the ovine *FecB*^{B} mutation.

The urgent need for rapid diagnosis highlights the need to improve effective diagnostic methods or develop novel methods. To overcome these shortcomings, we combined the CRISPR/Cas12a system and the RPA technology to develop a new *FecB*^{B} mutation genotyping method that can be applied on-site in a short period of time (less than 1 h). In order to achieve on-site, rapid, and portable detection, we optimized each step. First, in order to simplify the form of result determination, we (i) adopted a dual-crRNA strategy, (ii) designed a total of 25 crRNAs with the introduction of single-base mismatches for different base types at the *FecB*^{B} site, and (iii) screened out a pair of crRNAs with the highest signal-to-noise ratio. Then, we adjusted each system's Cas12a and crRNA dosages to make the positive fluorescence signals of the two systems, WT-crRNA and MT-crRNA, relatively similar. Finally, we used a nucleic acid releaser compatible with the RPA kit to release the gDNA from sheep WBCs under five-minute incubation, eliminating DNA extraction, greatly saving sample processing time. Although the results of the Cas12a-based detection were consistent with those obtained via the PCR-based Sanger sequencing, the time used for this method was much shorter than that of the PCR-based Sanger sequencing. In addition, this method does not require complex and expensive equipment, only a microcentrifuge, a small vortex shaker, pipettes, tips, a heat blocker, and a blue light, and the cost of the assay is about US$1.5.

Table 2 Comparison of the optimized CRISPR/Cas12a-based detection method and the results of PCR-Sanger sequencing

Genotype	Number of samples	Test results	
		Sanger sequencing	CRISPR-Cas12a method
AA	25	25 (100%)	25 (100%)
AG	21	21 (100%)	21 (100%)
GG	10	10 (100%)	10 (100%)

The CRISPR/Cas12 system and the isothermal amplification technology were widely used in molecular diagnostic studies of major infectious diseases affecting human public health security, high incidence cancers and diseases, and major infectious diseases of livestock and poultry. Such as the COVID-19 (Wang *et al.* 2020c), HPV (Liu *et al.* 2021), *Mycoplasma* (Wang *et al.* 2019), African swine fever (ASF) (Wang *et al.* 2020a). For pathogen detection, it is only necessary to design the crRNA with high recognition and binding efficiency at the target sequence, resulting in a yes or no result. However, for SNP identification, we need to minimize the noise signal due to the inclusion of single mismatched base by the CRISPR/Cas12a system on the basis of the former, in order to achieve the naked-eye determinable results. At present, this is undoubtedly a challenge.

Although our study could achieve efficient genotyping of the *FecB*^{B} mutation, it still has some limitations. First, the method could only be applicable in nucleic acid assays where the sequence is known due to the need for crRNA involvement. Second, RPA and CRISPR/Cas12a detection are accomplished in two steps, which increases detection time and operation difficulty. In future research, optimization of the reaction system and the use of the one-pot method for genotyping to meet the demand for high-throughput assays are expected. There is also potential for further improvement in this study. Recently, a strategy of switching on Cas12 activity using PAM-engineered toehold switch DNA was developed, which may be beneficial for the detection of nucleic acid sequences that lack PAM sequence or PAM sequence that is not in the proper position (Bagheri *et al.* 2024). When complexed with engineered modified non-canonical crRNAs, some Cas12a showed superior trans-cleavage activity than when complexed with wild-type crRNAs, which provides a new idea for screening crRNAs in SNP detection studies (Nguyen *et al.* 2024). Taken together, this study provides technical support for the selection and molecular breeding of high-prolificacy sheep.

5. Conclusion

In this study, we established a rapid, on-site, precise, portable, and naked-eye visualization method for genotyping the *FecB*^{B} mutation based on the CRISPR/Cas12a system and the RPA technology. This approach is expected to be widely applied for the detection of variants linked to high reproductive performance in livestock species. The selection of superior sheep individuals with highly desirable variants has the potential to improve the overall efficiency of production performance.

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Declaration of competing interest

The authors declare that they have no conflict of interest.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use AI in the preparation and writing of this manuscript.

Ethical statements

This research did not involve animal ethics experiments.

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