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ORIGINAL ARTICLE

Rapid visual genotyping method for germline mutants with small genomic fragment deletion by allele-specific PCR and lateral flow nucleic acid biosensor

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Abstract

Background Genome-editing techniques incorporating artificial nucleases develop rapidly and enable efficient and precise modification of genomic DNA of numerous organisms. The present research aimed to establish a rapid, sensitive and visual method for genotyping of germline genome-edited mutants with small genomic fragment deletion.

Methods and Results The genome-edited pigs with 2-bp deletion and 11-bp deletion of Myostatin (*MSTN*) gene generated by TALENs system were used as test materials to check the proposed allele-specific PCR (AS-PCR) and lateral flow nucleic acid biosensor (LFNAB) cascade method. AS-PCR can produce products with different tags to distinguish genome-edited alleles and wild-type alleles. A LFNAB was applied to do visual detection of AS-PCR products without using additional instruments. Furthermore, we demonstrated that AS-PCR and LFNAB cascade could accurately and visually distinguish genome-edited pigs with small genomic fragment deletion of Myostatin (*MSTN*) gene and wild-type pigs with limit of detection (LOD) of 0.1 ng.

Conclusion The proposed AS-PCR and LFNAB cascade can do rapid and visual genotyping of genome-edited mutants with small genomic fragment deletion, serving as a platform for genome-edited animal genotyping.

Keywords AS-PCR · LFNAB · Genome-editing · Genotyping · Pigs

Introduction

In recent years, genome-editing techniques incorporating artificial nucleases such as zinc finger nucleases (ZFN) [1], transcription activator-like effector nucleases (TALEN) [2], clustered regularly interspaced short palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) systems [3] develop rapidly and enable efficient modification for genomic DNA in numerous organisms and successfully produce various germline mutations [4]. Genome-editing techniques have been widely used for knocking out genes or introducing desired mutations in animals to hold tremendous promise for improving various traits, such as meat production [5], meat quality [6], disease resistance [7], and animal welfare [8]. The genome-editing techniques can easily induce targeted DNA double-stranded breaks, which can be repaired by non-homologous end joining (NHEJ) and resulting in small genomic fragment deletion [9, 10]. Therefore, developing effective genotyping methods that differentiate heritable mutations from wild-type animals is important to select the identified mutants.

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To identify different alleles in a target loci, several methods have been developed to do genotyping of genome-edited mutation, such as the polymerase chain reaction (PCR)/restriction enzyme (RE) assay [11], T7 endonuclease 1 (T7E1) assay [12], Surveyor nuclease assay [13], high-resolution melting analysis (HRMA) assay [14, 15] and DNA sequencing [16]. PCR/RE assay is a simple and sensitive method that can screen genome-edited mutants, but PCR/RE assay is limited to restriction endonuclease restriction sites near the target loci [17]. T7E1 and Surveyor nuclease assays are widely used to perform genotyping of mutants induced by genome-editing techniques. However, the T7E1 and Surveyor nuclease assays have good ability of distinguishing double strand heteroduplex DNA with deletion substrates, but there is a limit to perfectly paired homoduplex DNA [18]. HRMA assay distinguishes genome-edited mutants based on different high-resolution melting curves caused by the nucleotide distribution difference, but HRMA assay requires special and expensive equipment [19]. DNA sequencing methods are the first choice and gold standard method for genotyping of genome-edited mutants. However, DNA sequencing methods are labor-intensive and time-consuming [20].

Recently, lateral flow nucleic acid biosensor (LFNAB) has great application for rapid visual detection due to easy-to-use, high sensitivity, less time-consuming and low-cost [21–23]. So far, researchers have successfully applied LFNAB for the detection of protein targets [24, 25], nucleic acids [26, 27], viruses [28, 29], and small molecules [30]. In addition, LFNAB is more suitable for on-site detection and analysis through portable cameras or smartphones [31, 32]. Furthermore, LFNAB has been successfully introduced for visual detection of genome-editing induced deletion and natural mutations in DNA [33, 34].

In this study, we aimed to establish a rapid, sensitive and visual method for genotyping of germline genome-editing mutants with small genomic fragment deletion. We use allele-specific PCR (AS-PCR) to identify genome-edited alleles and wild-type alleles in one reaction. AS-PCR is a powerful and simple system to detect point mutations or polymorphisms with a high level of selectivity and sensitivity [35–38]. It has be applied to rapid screen TALEN and CRISP/Cas9 edited point mutations or insertion and deletion (indel) mutations [39, 40]. To simplify the detection process of AS-PCR, we introduced the LFNAB in the test process to achieve fast data visualization without additional instruments. To prove this idea, the genome-edited pigs with 2-bp deletion and 11-bp deletion of Myostatin (*MSTN*) gene generated by TALENs system were used as test materials. The proposed AS-PCR and LFNAB cascade can do rapid and visual genotyping of genome-edited mutants with small genomic fragment deletion, serving as a platform for genome-edited animal genotyping.

Materials and methods

Samples

DNA samples of the genome-edited Meishan pigs with 2-bp deletion, Large White pigs with 11-bp deletion, Meishan pigs and Large White pigs were provided by the Institute of Animal Sciences of Chinese Academy of Agricultural Sciences. The genome-edited Meishan pigs (CN104059877B) have already been patented in China.

Materials

Sample pad (GL-b03), conjugate pad (MA0280), nitrocellulose membrane (NC), absorbent pad (H5072) and backing plate were purchased from Shanghai Jieyi Biotechnology Co., Ltd (Shanghai, China). Sucrose, chloroauric acid tetrahydrate, Tweenrisodium citrate dehydrate, boric acid, potassium carbonate, and polyethylene glycol 2000 (PEG2000) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). The sheep anti-FITC-UNLB was purchased from Southern Biotech (Birmingham, Alabama, USA). The rabbit anti-sheep IgG (H+L) antibody, IgG fraction monoclonal mouse anti-biotin antibody and IgG fraction monoclonal mouse anti-digoxin antibody were purchased from Jackson ImmunoResearch (West Grove, Pennsylvania, USA).

AS-PCR procedure

Primers were designed using Primer Premier 5.0 software. Primer design was applied to perform genotyping of genome-edited Meishan pigs. First, three specific primers for the target gene were designed including two forward primers and one common reverse primer. One forward primer that is used to detect the wild-type alleles, Fprimer-1: 5′-CCCTCTAACTGTGGATTTTGAAG-3′. To ensure that Fprimer-1 specifically hybridizes with the wild-type alleles, its 3′end exactly matches to the 3′end of cleavage position generated by genome-editing tools. The other forward primer is used to detect the small deletion alleles, Fprimer-2: 5′-CCCTCTAACTGTGGATTT TGACT-3′. To ensure that Fprimer-2 specifically hybridizes with the deletion alleles, its 3′end extends 2-bp across cleavage position to ensure specificity and sensitivity. The common reverse primer is Rprimer: 5′-CACCCACAG CGATCTACTACCA-3′. Biotin and digoxin were labeled at the 5′ end of the two forward primers, respectively, and FITC was added to the 5′ end of the reverse primer. The principle of primers design for genotyping of genome-edited Large White pigs is the same as above. All the

sequences of oligonucleotides used in this study are listed in Table S1.

The AS-PCR reaction system was performed in a 0.2 mL tube. The AS-PCR was conducted in a total reaction volume of 20 µL that contained 2 µL 10× PCR Buffer (25 mM $MgCl_2$), 1.6 µL dNTP Mix (2.5 mM), 0.1 µL Taq DNA Polymerase (5 U/µL), 0.6 µL of each forward primer (10 µM), 1.2 µL of reverse primer (10 µM), 1 µL template (50 ng/µL) and 12.9 µL ddH_2O. The components of AS-PCR were shown in Table S2. An initial denaturation was performed of 5 min at 95 °C, then 35 cycles were carried out at 95 °C for 30 s, 62 °C for 30 s, 72 °C for 30 s, and finally 72 °C for 5 min. The AS-PCR products were then stored at 4 °C.

LFNAB procedure

Briefly, 2 mL of 1% $HAuCl_4$ was added to 200 mL of boiled ddH_2O, and 3.6 mL of 1% trisodium citrate was slowly added. Then, the solution was boiled and stirred for 15 min. The heating was stopped when the solution showed a stable wine red and store at 4 °C for later use. The AuNPs and antibody coupling was carried out by adding 150 µL of 0.1 mol/L potassium carbonate and 60 µL of 10 mM FITC antibody to 10 mL of AuNPs solution, and then the coupling solution was incubated with constant stirring at room temperature for 30 min. After that, 1.2 mL of 10% BSA solution was added to the coupling solution and stirring for 5 min, following adding 292 µL of 2% PEG2000 and stirring for 30 min. Then, it was centrifuged at 12,000 rpm for 30 min at 4 °C, and the supernatant was removed and added a 0.002 mol/L borate buffer solution (pH 9.0) at 4 °C for 30 min. The AuNPs-antibody conjugate was finally concentrated to 2 mL with borate buffer solution. The conjugates were store at 4 °C for further use.

The LFNAB consists of five parts, including sample pad, conjugate pad, nitrocellulose membrane, absorbent pad, and backing plate. The sample pad was made of cellulose membrane, which quickly absorb the sample solution to flow laterally to the bonding pad by siphoning. The AuNP-antibody conjugates were coated on the conjugate pad with an amount of 50 µL per 1 cm^2, then placed in an oven at 60 °C for drying. The nitrocellulose membrane was made of cellulose membrane and contained a control line and two test lines. The control line and two test lines were sequentially assembled by dispensed with rabbit anti-sheep IgG (H+L) antibody (2.3 mg/mL), IgG fraction monoclonal mouse anti-biotin antibody (1.3 mg/mL), and IgG fraction monoclonal mouse anti-digoxin antibody (1.2 mg/mL) at different zones on one LFNAB with the amount of 1.0 µL/cm. The average distance between the three lines is approximately 0.5 cm. The sample pad and absorbent paper were located at the bottom and top of the LFNAB, respectively. Conjugate pad was placed between nitrocellulose membrane and sample pad. Nitrocellulose membrane was placed between conjugate pad and absorbent pad. Sample pad, conjugate pad, nitrocellulose membrane and absorbent pad were assembled to overlap approximately 2 mm, and they were fixed to the support plate to form the LFNAB. The whole assembly was cut to a size of 6.0 × 0.4 cm. The assembled LFNAB was stored under dry conditions at 4 °C for further testing.

Two µL of the AS-PCR amplified product was mixed with 48 µL of running buffer, and the mixture was applied onto the sample pad of the LFNAB. The visible red lines were formed of the LFNAB due to the accumulation of gold nanoparticles after 5 to10 min. The result was judged by visually observing whether the presence of two test lines and color changes of the test lines by the naked eye. Picture results were taken by a smartphone. The quantitative detection process was performed by measuring the grayscale value of each test line by ImageJ software (http://rsb.info.nih.gov/ij).

Results

Principle

The main working principle for rapid and visual genotyping of germline genome-edited mutations with small fragment deletion is illustrated in Fig. 1. The well-characterized genome-edited Meishan pigs with a 2-bp deletion on Myostatin (*MSTN*) exon 3 and wild-type pigs were used as test targets to check the performance of AS-PCR and LFNAB cascade strategy (Fig. 1a). To produce different PCR products for genome-edited alleles and wild-type alleles, the PCR primers were designed to hybridize with different alleles specifically. The primer set contains three primers, including two forward primers and one common reverse primer. One forward primer (Fprimer-1) is used to amplify wild-type alleles with its 3′end exactly matches the 3′end of cleavage position. The other forward primer (Fprimer-2) is used to amplify deletion alleles with its 3′end extends 2-bp across cleavage position to ensure specificity and sensitivity. In the AS-PCR reaction, the DNA template is first denatured at a high temperature, and then the annealing primers specifically hybridize with different DNA templates. In this step, if the wild-type alleles are present in the AS-PCR reaction, the Fprimer-1 and Rprimer hybridize with the target DNA templates. On the other hand, if the deletion alleles are present in the AS-PCR reaction, the Fprimer-2 and Rprimer hybridize with the target DNA templates. In addition, three primers are separately labeled with different tags for further LFNAB. A pair of biotin-Fprimer-1 and FITC-Rprimer for wild-type alleles, while a pair of digoxin-Fprimer-2 and FITC-Rprimer for deletion alleles. After AS-PCR reaction with three tag labeled primers, dual labeled

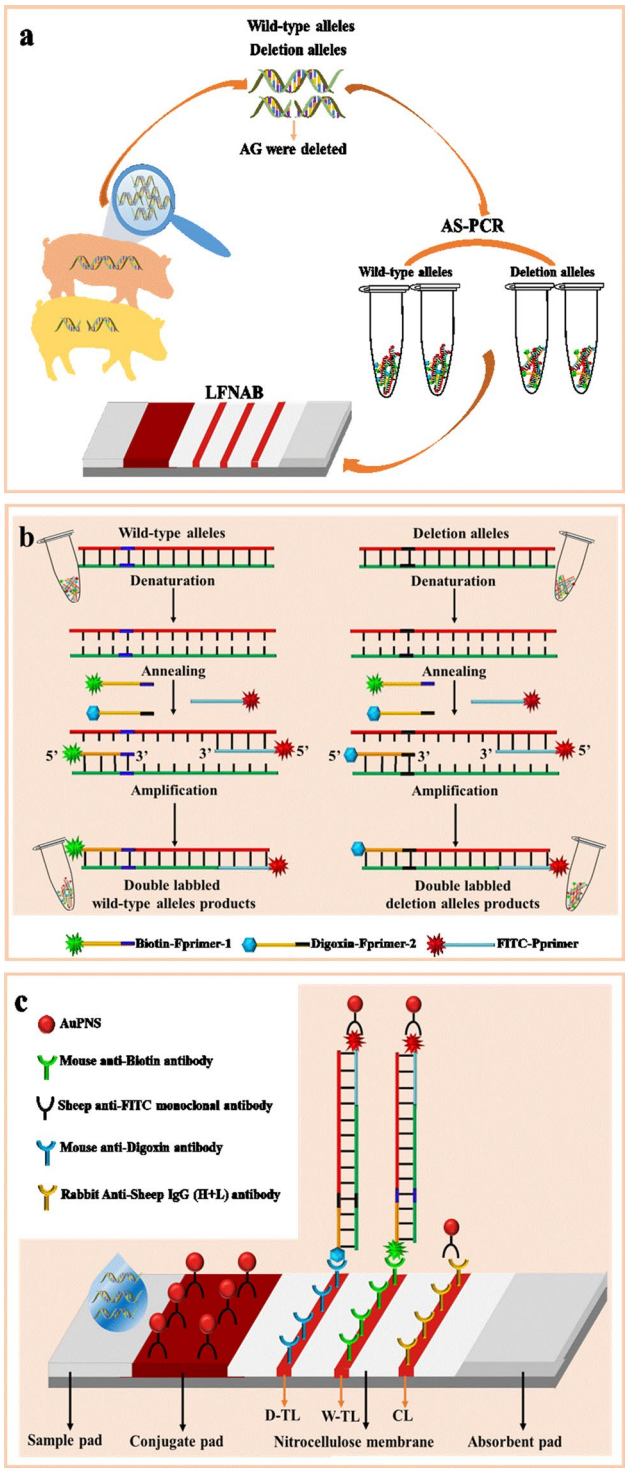

Fig. 1 Schematic illustration of AS-PCR and LFNAB cascade for genotyping of genome-edited pigs. **a** A simple overview; **b** AS-PCR procedure; **c** LFNAB procedure

PCR products with different tags were generated according to the target DNA templates (Fig. 1b).

LFNAB is employed to show the outcome for reading of AS-PCR results, which significantly shortens the testing time. Furthermore, LFNAB is no longer limited to specific instruments. The AS-PCR products are mixed with running buffer and loaded on the sample pad to run the reaction. Then, anti-FITC antibody modified AuNPs conjugates are captured by the FITC tag of AS-PCR products to form the complex when the mixture is passing through the conjugate pad. The anti-biotin antibody and anti-digoxin antibody on the nitrocellulose membrane will bind the complex to form two test lines for wild-type alleles (W-TL) and deletion alleles (D-TL), respectively. The rabbit anti-sheep IgG (H+L) antibody on the nitrocellulose membrane will capture anti-FITC antibody modified AuNPs conjugates to form a control line (CL) (Fig. 1c). Therefore, if wild-type alleles or deletion alleles are only present in the sample, the corresponded one visible red test line can be observed on the LFNAB (Fig. S1a and S1b). If the heterozygous alleles are present in the sample, two red test lines can be observed (Fig. S1c). The control line only can form in the blank sample (Fig. S1d).

Feasibility of AS-PCR

The feasibility of the AS-PCR was performed using the genomic DNA of the heterozygous genome-edited Meishan pig at different annealing temperatures and primer concentrations (Table S3) and different reaction systems (Table S4). To efficiently detect different PCR products caused by few base pair deletions through agarose gel electrophoresis, we loaded a 20-bp nucleic acid adaptor (GCTTATCTATAG CATTGAAG) at the 5′ end of the Fprimer-1 to enlarge the difference of fragment size of PCR products. The nucleic acid adaptor on the Fprimer-1 was aligned with BLAST (http://www.ncbi.nlm.nih.gov/BLAST) against the Meishan pig genome sequence. Nucleic acid adaptor did not find significant homology in the Meishan pig genome. After AS-PCR, two amplified PCR fragments, including one 290-bp DNA fragment and the other 268-bp DNA fragment, were observed in electrophoresis (Fig. S2). The results provided clear evidence that the primers of AS-PCR can effectively and correctly hybridize with different alleles and generate corresponding PCR products.

To further confirm the feasibility and accuracy of AS-PCR, fourteen pigs including three wild-type pigs, three homozygous genome-edited Meishan pigs, and nine heterozygous genome-edited Meishan pigs were used to perform AS-PCR reactions. Each sample was amplified twice to avoid technique error, and PCR products from the same individual were marked as a group in electrophoresis. As shown in Fig. 2, Group 2, 3, and 11 were identified as wild-type pigs that only 290-bp DNA fragments were amplified in AS-PCR. Group 4, 5, and 12 were identified as homozygous genome-edited Meishan pigs because only 268-bp DNA fragments were observed. Group 6, 7, 8, 9, 13, 14, 15,

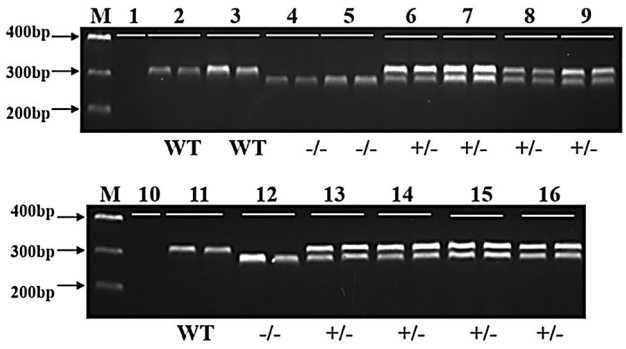

Fig. 2 AS-PCR test. M represents DNA Marker I. Group 1 and 10 represent blank control. Group 2, 3, and 11 represent wild-type pigs. Group 4, 5, and12 represent homozygous genome-edited pigs. Group 6, 7, 8, 9, 13, 14, 15, and 16 represent heterozygous genome-edited pigs

and 16 were considered as the heterozygous genome-edited Meishan pigs that two DNA fragments were observed in electrophoresis. Furthermore, the PCR results were consistent with DNA sequencing results. The results demonstrated that the AS-PCR had been successfully conducted in accordance with the design in the scheme.

Sensitivity test of AS-PCR and LFNAB cascade

Heterozygous genome-edited Meishan pigs can simultaneously show amplification of two alleles, so the genomic DNA of the heterozygous genome-edited Meishan pigs were used as template for sensitivity detection. The limit of detection (LOD) of AS-PCR and LFNAB cascade was assessed based on five genomic DNA content gradients of 50 ng, 10 ng, 0.1 ng, 0.01 ng, and 0 ng. As shown in Fig. 3, 0.1 ng and above content gradients appeared the three lines on the LFNAB including one control line and two test lines (W-TL and D-TL). The red color intensity of test lines on the LFNAB was correlated with the amount of the DNA template. For quantitative detection, the sensitivity of detection of the wild-type and deletion alleles was measured by each test line's peak intensity. The result showed an excellent linear relationship between the peak intensity of test lines and content of DNA template, with correlation coefficients of 0.97 and 0.99, respectively.

Real samples detection of AS-PCR and LFNAB cascade

To further confirm the accuracy and practicality of AS-PCR and LFNAB cascade for performing rapid genotyping, nine individuals including three wild-type Meishan pigs, three homozygous genome-edited Meishan pigs and three heterozygous genome-edited Meishan pigs were tested. As shown in Fig. 4a, the visualized results of three genotypes were clearly observed. The LFNAB 2, 3, and 4 were identified as wild-type Meishan pigs because W-TLs on the test zone were observed. The LFNAB 5, 6, and 7 were considered as homozygous genome-edited Meishan pigs due to the observation of D-TLs on the test zone. The LFNAB 8, 9, and 10 were identified as the heterozygous genome-edited Meishan pigs due to the observation of W-TLs and D-TLs on the test zone. Furthermore, the homozygous genome-edited Large White pigs with 11-bp deletion were used to verify the generality of the method (Fig. 4b). Therefore, AS-PCR and LFNAB cascade can achieve the purpose of rapid genotyping for genome-edited mutants.

Discussion

In this study, we presented a rapid and visual system for genotyping of genome-edited mutants. Compared to previously reported methods, this method is highly rapid and sensitive for genotyping of genome-editing induced small genomic fragments (Table 1) [19, 42]. Furthermore, this method is not limited to restriction endonuclease

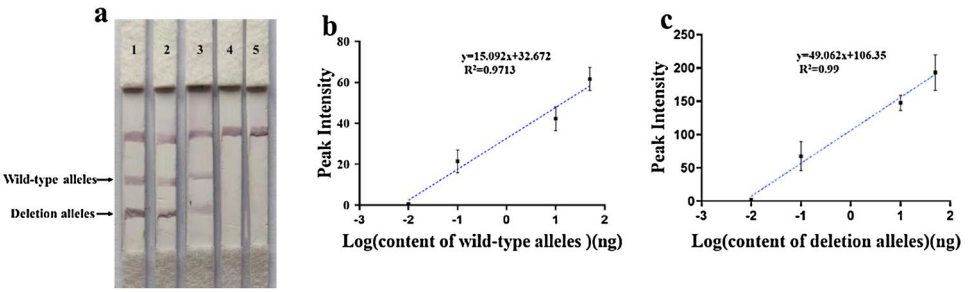

Fig. 3 Sensitivity of AS-PCR and LFNAB cascade for genotyping of heterozygous genome-edited Meishan pigs. **a** Sensitivity test of heterozygous genome-edited Meishan pigs. Strip 1, 2, 3, 4, and 5 represent genomic DNA content of 50 ng, 10 ng, 0.1 ng, 0.01 ng, and 0 ng, respectively; **b** Calibration curves of peak intensity and genomic DNA content of wild-type alleles; **c** Calibration curves of peak intensity and genomic DNA content of deletion alleles. Error bars indicate the standard deviation of three biological repetitions

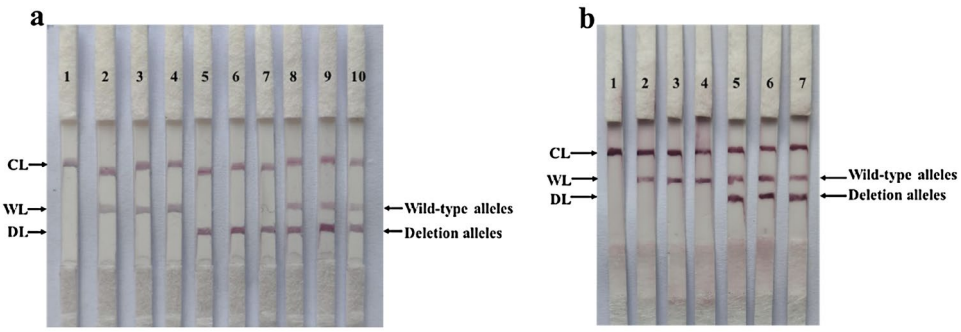

Fig. 4 Application of AS-PCR and LFNAB cascade for rapid genotyping of the real samples. **a** Genotyping results of Meishan pigs with 2-bp deletion. Strip1 represents blank control; Strip 2, 3, and 4 represent wild-type Meishan pigs; Strip 5, 6, and 7 represent homozygous genome-edited Meishan pigs; Strip 8, 9, and 10 represent heterozygous genome-edited Meishan pigs; **b** Genotyping results of Large White pigs with 11-bp deletion. Strip1 represents blank control; Strip 2, 3, and 4 represent wild-type Large White pigs; Strip 5, 6, and 7 represent heterozygous genome-edited Large White pigs

Table 1 Comparison of AS-PCR and LFNAB cascade with other methods for genotyping of genome-edited mutations

Strategy	Time[a]	Accuracy	Cost	References
DNA sequencing	1 days	High	High	[41]
PCR/RE assay	> 4 h	High	Moderate	[42]
T7E1 assay	> 4 h	Moderate	Moderate	[42]
HRM analysis	2–3 h	Moderate	High	[17]
PAGE-based method	> 4 h	Moderate	Moderate	[17]
Digital PCR assay	> 3 h	High	High	[43]
AS-PCR and LFNAB cascade	< 2 h	High	Low	(This work)

[a]It does not include DNA extraction time

restriction sites near the target loci. Moreover, this method can accurately distinguish different genotypes in one reaction. The AS-PCR and LFNAB cascade method does not require any expensive equipment, making this method cheap and time-saving.

The primer design is the critical factor to successfully perform AS-PCR and LFNAB cascade. The primer design of AS-PCR is more skillful than the design of traditional PCR [35]. Accordingly, the primary consideration of primer design is that non-specific application frequently occurs due to few genomic DNA differences between wild-type and genome-edited alleles. The primer pair of wild-type and genome-edited alleles share the same reverse primer, but the forward primers differ at their 3′end to guarantee allele-specific amplification. In addition, we introduced the LFNAB into the process to reduce analysis time, cost and implement visualization of the results directly. Furthermore, the method developed in this study can be extended to perform genotyping of different genome-edited organisms with a small deletion in the future.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-021-06734-x.

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Author contribution QS, XZ and BL conceived of the AS-PCR and LFNAB cascade method. QS performed the experiments. TW and KL contributed materials. QS and XZ wrote the manuscript. XZ, LB, WX, ZL, and PS edited the manuscript.

Declarations

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval Not applicable.

Consent to participate Not applicable.

Consent to publication Not applicable.

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