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Optimization of a Multiplex PCR Assay for Detecting Transgenic Soybean Components in Feed Products

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Abstract A multiplex polymerase chain reaction (m-PCR) assay was developed for the simultaneous detection of multiple components of genetically modified (GM) soybean. It uses two sets of primers (I, lectin1/35S/CP4; II, lectin2/35S/CP4) specific for a soybean reference gene, the *35S* promoter, and an event-specific gene. Amplified fragments of 118, 414, 195, and 320 bp were easily detected by agarose gel electrophoresis and were positively confirmed by sequencing. Primer set concentrations and annealing temperatures in the m-PCR were optimized. The optimized m-PCR conditions were obtained for primer set I at a ratio of 1:2:3 and a 59.2 °C annealing temperature and set II at the same ratio and 58.6 °C, 60.3 °C, and 61.2 °C annealing temperatures. The sensitivities of the two m-PCR primer sets (I and II) were 0.25% and 0.5%, respectively. The results showed that this m-PCR assay provides rapid, reliable, and effective identification of multiple components of GM soybean in feed.

Keywords Genetically modified soybean · Feed · Multiplex PCR

Fang Tian and Xiumin Wang contributed equally to this paper.

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Abbreviations

GM	Genetically modified
RRS	Roundup ready soybean
CRMs	Certified reference materials
m-PCR	Multiplex PCR

Introduction

The cultivation area of genetically modified (GM) soybean is over 50% of the total world soybean area. The Roundup Ready soybean (RRS, Monsanto) is the most popular variety. Like non-GM soybean, the majority of GM soy was approved as beneficial and available for animal nutrition. To guarantee consumers' rights-to-know, regulations and labeling laws have been established in many countries. GM foods must be labeled at the threshold of 0.9% in EU [1], 3% in Korea [2], and 5% in Japan [3]. The methodology to enable the detection and labeling of genetically modified organisms (GMOs) is largely based on the use of polymerase chain reaction (PCR) to detect specific components of the inserted construct. It is possible to determine the components of GMOs in a wide range of feed matrices by the amplification of specific sequences found in transgenic cases.

PCR-based methods have been widely applied in the detection of GMOs in food [4, 5]. Multiplex PCR (m-PCR), a technique based on multiple targets being amplified simultaneously in the same reaction, is expected to save time and effort by decreasing the number of reactions required for GMO detection [6]. Several methods based on m-PCR have been developed for the detection of GM soybean and maize [7, 8]. However, there is still considerable room to improve the sensitivity and specificity of these m-PCRs by optimization of reaction conditions.

The objectives of the present study were to optimize the reaction conditions and establish an m-PCR-based system to rapidly identify and detect the housekeeping gene (*lectin*) and the inserted elements (*35S* promoter and *CP4-epsps* gene) of GM soybean in raw materials, soybean meal, and feed.

Materials and Methods

Materials

MON 810 maize, Bt cotton, and the certified reference material of RRS (ERM-BF 410a and 410e) produced by the Institute for Research Materials and Measurements (IRMM, Belgium) were used as controls.

Fifteen kinds of raw materials and diverse feeds, including four soybean samples (S1, S2, S3, and S4), four samples of the soybean meal (M1, M2, M3, and M4), one concentrated feed (C1), one mixed feed for porket (P1), three mixed feeds for broiler (B1, B2, B3), one mixed feed for layer (L1), and one mixed feed for fish (F1) were chosen as unknown samples.

DNA Extraction

DNA extraction was performed with the improved sodium dodecyl sulfate (SDS) method. First, 100 mg of each sample was mixed with 1 mL of 2% SDS buffer pre-heated at 65 °C

and 10 μL of RNase-A and incubated at 65 °C for 30 min, and then the mixture was centrifuged for 10 min at 12,000×*g*. A 0.6 volume of 5 M KAc (pH 4.8) was added to the supernatant and the mixture placed on an ice bath for 20 min. After centrifugation, the aqueous phase was mixed with two volumes of pre-cooled anhydrous ethyl alcohol and the mixture was placed at −20 °C for 30 min. After centrifugation at 12,000×*g* for 10 min, the liquid phase was discarded and the pellet washed with 70% ethanol. This step was repeated twice and then the precipitate was dried at room temperature, dissolved in 200 μL TE, and stored at −20 °C. The quality and quantity of the extracted DNA were evaluated using absorbance measurements at 260 and 280 nm and further checked by 2% agarose gel electrophoresis.

Primer Design

A total of five primer pairs specific for two soybean reference genes, the *35S* promoter, the *CP4-epsps* gene, and an event-specific gene were designed and checked for suitability for m-PCR using the Primer Premier 5.0 primer design software. The sequences of the oligonucleotide primers used in this study are listed in Table 1. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd. (China) and the annealing temperatures optimized using a Thermal Cycler (TECHNE) under single-target PCR conditions.

Single-Target PCR

For PCR with individual primer pairs, each reaction mixture contained 1 μL of genomic DNA template, 10×PCR buffer, 2.5 mM dNTP, 10 μM each primer, and 2.5 U *Taq* DNA polymerase in a total volume of 25 μL. PCR amplification conditions were 5 min of preincubation at 94 °C followed by 35 cycles of 94 °C for 30 s, 58.3 °C for 40 s, and 72 °C for 60 s and a final extension step at 72 °C for 6 min. Then, 5 μL of each PCR product was electrophoresed in 2.0% agarose gel with ethidium bromide (1 μg mL^{-1}) and 1×TAE buffer. DNA bands in gels were visualized on a Gel Doc system (Geliance 200 Imaging system, Perkin Elmer).

Multiplex PCR Conditions

The m-PCR reaction was performed using a final reaction volume of 25 μL containing 1× PCR buffer, 2.5 mM dNTP, 10 μM of each primer (*lectin1*/*lectin2*, *35S*, and *CP4-epsps*), 5 U of *Taq* DNA polymerase and genomic DNA. The thermal cycling procedure consisted of an initial denaturation step (94 °C, 5 min), 35 cycles consisting of denaturation at 94 °C for 30 s, annealing at 58–62 °C for 40 s, and elongation at 72 °C for 60 s, and a final elongation period at 72 °C for 6 min. The PCR products were analyzed by electrophoresis on 2% agarose gel.

Results

DNA Extraction

The results of the DNA isolation from all samples were shown in Fig. 1. The size of the genomic DNA was about 21 kb. The absorbance ratio of OD_{260}/OD_{280} ranged from 1.7 to 1.9 demonstrating high purity and yield of the DNA. The DNA yields from samples M1

Table 1 Primer sequences and amplification lengths for the PCR products

Target (GenBank accession no.)	Sequence (5′-3′)	Annealing temperature (°C)	Length (bp)	Reference
Lectin1 (K00821)	P1: GCCCTCTACTCCACCCCCATCC	67.53	118	GB/T19495.4-2004, China
	P2: GCCCATCTGCAAGCCTTTTTGTG	61.95		
Lectin2 (K00821)	P1: TGCCGAAGCAACCAAACATGATCCT	61.98	414	SN/T XXXX-2002, China
	P2: TGATGGATCTGATAGAATTGACGTT	57.06		
35S promoter (V00141)	P1: GCTCCTACAAATGCCATCA	55.41	195	GB/T19495.4-2004, China
	P2: GATAGTGGGATTGTGCGTCA	57.80		
CP4-epsps (AF464188.1)	P1: CTTCTGTGCTGTAGCCACTGATGC	63.68	320	Tan W et al., 2001
	P2: CCACTATCCTTCGCAAGACCCTTCC	65.26		
RRS event-specific gene (AJ308514.1)	P1: TCTTTGGGACCACTGTCGG	59.72	209	This work
	P2: TGCATGCTTTAATTTGTTTCTATC	53.43		

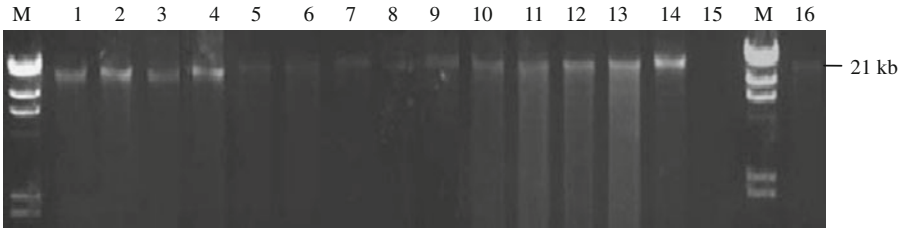

Fig. 1 Electrophoresis profile of soybean genome extracted from soya and deeply processed products with SDS. M, λ DNA/Hind III marker; *1* soybean (S1), *2* soybean (S2), *3* soybean (S3), *4* soybean (S4), *5* soybean meal (M1), *6* soybean meal (M2), *7* soybean meal (M3), *8* soybean meal (M4), *9* concentrated feed (C1), *10* mixed feed for porket (P1), *11* mixed feed for broiler (B1), *12* mixed feed for broiler (B2), *13* mixed feed for broiler (B3), *14* mixed feed for layer (L1), *15* blank control, *16* mixed feed for fish (F1)

through M4 were comparatively low but high quality. DNA isolated from the mixed feed for porket and broiler was observed to be slightly degraded. All DNA was extracted successfully from samples as proven by the amplification of endogenous DNA sequences of soybean.

Primer Design

For all primer sequences, homology searches were performed using the GenBank database and the BLAST program and the possibility of primer dimer formation was checked using the software (Table 1). The analyses indicated that all oligonucleotide primer pairs showed significant affinities only for their target genes. To facilitate PCR product detection, the primers were designed so that the predicted sizes of the amplification products of each target gene would be different to permit size discrimination by gel electrophoresis.

Single-Target PCR

Primers (*lectinl* and an RRS event-specific gene) were used in an attempt to amplify a conventional soybean sequence and a transgenic sequence from DNA samples extracted from the various samples. The applied single-target PCR system showed only specific amplicons of the expected size. The presence of the *lectin1* gene amplicons (118 bp) in all tested samples confirmed that the DNA extraction method used in this work was adequate for extraction of amplifiable soybean DNA from the 15 samples (Fig. 2a). The template-free negative control did not show the presence of any amplicons.

To identify the presence of GM soybean in the blind sample strains, a conventional PCR method was applied to detect the junction region between the inserted sequence of the *35S* promoter and the soybean genome in RRS. The 209-bp product was obtained from 13 of the 15 samples, except for S4 and M4 samples (Fig. 2b). Thus, these 13 feed products contained RRS ingredients.

Specificity of the triplex PCR assay was tested by amplifying each target gene from an equal volume of genomic DNA of GM maize and cotton seeds. The expected 195-bp amplicons of the *35S* promoter were observed and no specific amplification of the *lectin* and *CP4-epsps* genes were observed in either MON810 maize or Bt cotton using primer set II. At the same time, the above three target genes were successfully amplified from 1% to 2% RRS (Fig. 3; data of primer set I not shown). Therefore, the three primer pairs met the requirements for specific amplification of the target gene in m-PCR.

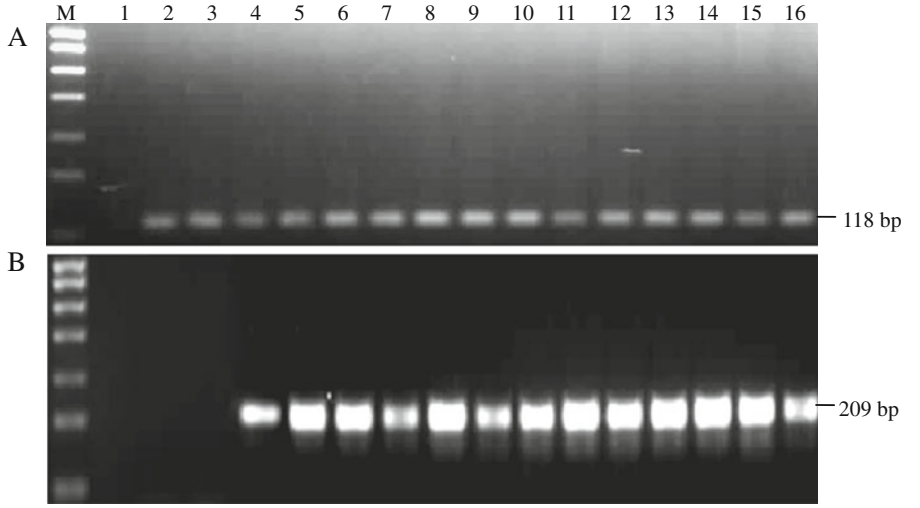

Fig. 2 PCR amplification of *lectin1* and event-specific genes of RRS. **a** PCR amplification of soybean *lectin1* gene. *M* DNA marker I, *1* blank control, *2* soybean (S4), *3* soybean meal (M4), *4* soybean (S1), *5* soybean (S2), *6* soybean (S3), *7* soybean meal (M1), *8* soybean meal (M2), *9* soybean meal (M3), *10* concentrated feed (C1), *11* mixed feed for porket (P1), *12* mixed feed for broiler (B1), *13* mixed feed for broiler (B2), *14* mixed feed for broiler (B3), *15* mixed feed for layer (L1), *16* mixed feed for fish (F1). **b** PCR amplification of the event-specific gene of RRS. *M* DNA marker I, *1* soybean meal (M4), *2* soybean (S4), *3* blank control, *4* mixed feed for porket (P1), *5* mixed feed for broiler (B1), *6* mixed feed for broiler (B2), *7* mixed feed for broiler (B3), *8* mixed feed for layer (L1), *9* mixed feed for fish (F1), *10* concentrated feed (C1), *11* soybean meal (M1), *12* soybean meal (M2), *13* soybean meal (M3), *14* soybean (S1), *15* soybean (S2), *16* soybean (S3)

The Optimization of m-PCR

Triplex PCR analysis was developed for screening an endogenous soybean gene, the *35S* promoter, and the *CP4* gene of GM soybean simultaneously. The specificity and sensitivity of m-PCR can be affected by many factors, such as the purity of the DNA, an effective concentration of the corresponding primers, annealing temperatures, primer design, and especially the ratio of primers (mol/mol). The annealing temperatures of the triplex PCR were between 52 °C and 62 °C, the ratio of primer set I (lectin1/35S/CP4) was 1:1:1 (mol/mol), and the amplicons of *lectin1*, *35S* and *CP4* were, respectively, observed at as bands of 118, 195, and 320 bp (Fig. 4a). However, the intensity of the nonspecific amplifications and

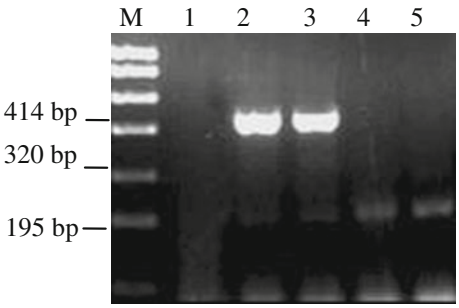

Fig. 3 Specificity of m-PCR using primer set II (lectin2/35S/CP4) *M* DNA marker I, *1* blank control, *2* 1% RRS, *3* 2% RRS, *4* Bt cotton, *5* MON810 maize

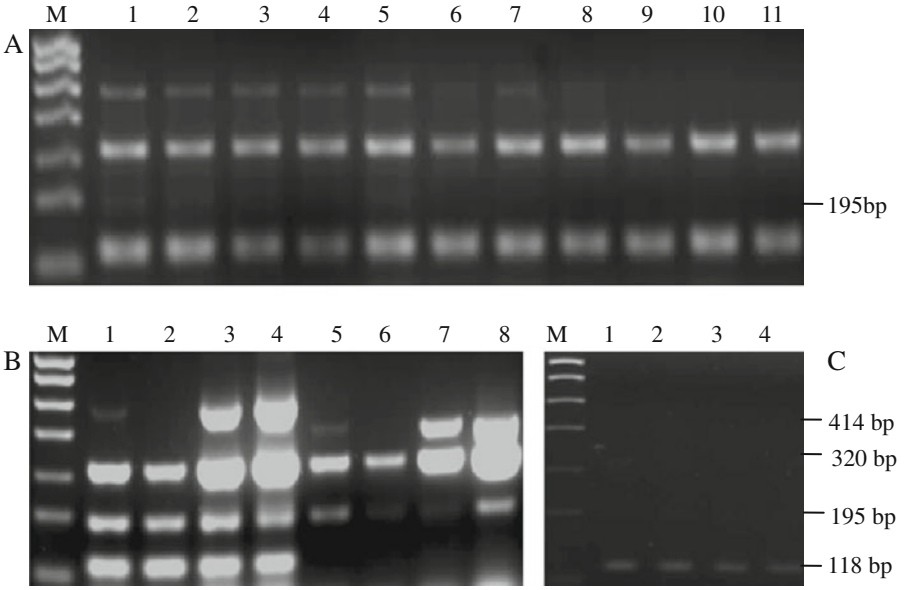

Fig. 4 Optimization of two different primer sets in the m-PCR. **a** Electrophoresis profile of m-PCR optimization in the ratio of 1:1:1 with the "lectin1/35S/CP4" primer set. *M* DNA marker I, *1* 52.8 °C, *2* 53.3 °C, *3* 54.1 °C, *4* 55.1 °C, *5* 55.8 °C, *6* 56.6 °C, *7* 57.2 °C, *8* 58.6 °C, *9* 59.2 °C, *10* 60.3 °C, *11* 61.2 °C. **b** Electrophoresis profile of triplex-PCR optimization in the ratio of 1:2:3 with two primer sets. *M* DNA marker I, *1–4* primer set I (lectin1/35S/CP4), *5–8* primer set II (lectin2/35S/CP4), *1* and *5* 58.6 °C, *2* and *6* 59.2 °C, *3* and *7* 60.3 °C, *4* and *8* 61.2 °C. **c** Electrophoresis profile of m-PCR optimization in the ratio of 1:1.5:2 with the "lectin1/35S/CP4" primer set. *M* DNA marker I, *1* 61.2 °C, *2* 60.3 °C, *3* 59.2 °C, *4* 58.6 °C

195-bp band weakened with increased annealing temperatures. When the proportion of the above primer sets was changed to 1:1.5:2 (mol/mol) and the annealing temperature scope was between 58 °C and 62 °C, only one PCR product corresponding to the *lectin1* gene locus (118 bp) was observed (Fig. 4c).

To determine whether a particular primer set was significantly better for the detection of GM soybean, two sets of primers (lectin1/35S/CP4, set I and lectin2/35S/CP4, set II) were examined at the same ratio of 1:2:3 (mol/mol) and four different annealing temperatures (58.6 °C, 59.2 °C, 60.3 °C, and 61.2 °C). The best PCR result from primer set I was obtained at a 59.2 °C annealing temperature and the best from primer set II at 58.6 °C, 60.3 °C, and 61.2 °C (Fig. 4b).

Application of the m-PCR System for the Analysis of Feed

The m-PCR system was then applied to detect the presence of GM soybean in 15 blind samples using primer sets I and II. Consistent m-PCR results were observed. Among the 15 blind samples tested, 13 were positive for the *CP4* gene and *35S* promoter. Samples S4 and M4 did not have these bands (Fig. 5a, b).

Sensitivity of the m-PCR System

To validate the sensitivity of the m-PCR system, three genomic DNA samples containing 0.5%, 0.25%, and 0.125% (*w*/*w*) RRS were prepared by serially diluting the genomic DNA

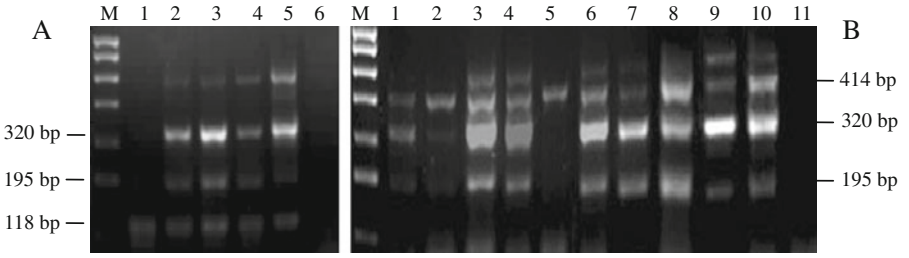

Fig. 5 Electrophoresis profile of triplex PCR products using two primer sets. **a** Electrophoresis profile of triplex PCR products using the "lectin1/35S/CP4" primer set. *M* Marker I, *1* soybean (S4), *2* concentrated feed (C1), *3* soybean (S1), *4* soybean (S2), *5* soybean (S3), and *6* blank control. **b** Electrophoresis profile of triplex PCR products using the "lectin2/35S/CP4" primer set. *M* DNA marker I, *1* mixed feed for porket (P1), *2* soybean meal (M3), *3* soybean meal (M2), *4* soybean meal (M1), *5* soybean meal (M4), *6* mixed feed for broiler (B1), *7* mixed feed for broiler (B2), *8* mixed feed for broiler (B3), *9* mixed feed for layer (L1), *10* mixed feed for fish (F1), and *11* blank control

of 0% and 2% RRS. The sensitivity of the m-PCR was 0.25% and 0.5% using primer sets I and II, respectively (Fig. 6a and b). This result indicates that the m-PCR established in this work can be used for the simultaneous detection of multiple target sequences in GM soybean in feed products in one PCR reaction.

Discussion

Several reports describing m-PCR techniques used to detect GM soybean have been published [9, 10]. As a cost- and time-saving measure, m-PCR can be used to detect several GM components in feed products simultaneously. GM components can be discriminated very quickly and reproducibly by m-PCR assay [11–13]. In this study, two primer sets of three primer pairs each were identified that reliably differentiated GM soybean from non-GM soybean by simultaneously amplifying DNA products of different lengths from the DNA of feed. Factors important in achieving successful multiplex amplification include DNA quality and extraction procedure, primer sequences and their ratios, annealing temperatures, and thermal cycling parameters [10, 14].

The template concentration, often overlooked, was shown to be a critical factor in some reactions. A simple SDS extraction procedure of genomic DNA from feed was developed in

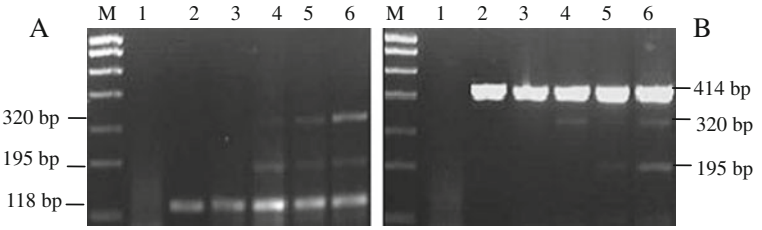

Fig. 6 Sensitivity analysis of triplex-PCR procedures for detecting RRS with two primer sets. **a** Sensitivity analysis of triplex-PCR procedures for detecting RRS with the "lectin1/35S/CP4" primer set. *M* DNA marker I, *1* blank control, *2* 0% RRS, *3* 0.125% RRS, *4* 0.25% RRS, *5* 0.5% RRS, *6* 2% RRS. **b** Sensitivity analysis of triplex-PCR procedures for detecting RRS with the "lectin2/35S/CP4" primer set. *M* DNA marker I, *1* blank control, *2* 0% RRS, *3* 0.125% RRS, *4* 0.25% RRS, *5* 0.5% RRS, and *6* 2% RRS

this work that avoids the phenol or chloroform used usually in cetyltrithyl ammonium bromide methods [15].

Primer design is the major challenge for a universal m-PCR system. We initially designed different primer sets solely based on published DNA sequences to obtain the specific amplicons for this triplex PCR system. Unfortunately, several samples could not be detected using the designed primer sets (data not shown). Then, four pairs of primers to detect *lectin1*, *lectin2*, the *35S* promoter and *CP4-epsps* were chosen from the published literature for this m-PCR and divided into two groups (set I, lectin1/35S/CP4; set II, lectin2/35S/CP4) according to the tested samples because of the variable degree of genomic DNA degradation during the deep processing [16, 17]. The effect of the primer ratio on the PCR products was as significant as that of the annealing temperature (Fig. 4a and c). In addition, we also found differences in the detection of GM soybean using primer sets I and II in the range of 58.6–61.2 °C annealing temperature. Nonspecific amplification was observed using m-PCR with primer set I (Fig. 4b). Therefore, use of an appropriate primer set is critical for a successful m-PCR with appropriate annealing temperatures within this range.

Two previous studies found the sensitivity of the m-PCR using the same target genes to be 0.1% and 0.5% [7, 18]. In this study, the sensitivity in triplex PCR with primer sets I and II was 0.25% and 0.5%, respectively, which may be due to different primer ratios, target genes, and DNA templates.

Taken together, the protocol presented here has a series of advantages over common PCR, including cost, efficiency, and sensitivity and could be suitable for rapid identification of GM components in raw materials, soybean meal, and feed.

Conclusions

In this study, m-PCR is optimized and established for the simultaneous detection of three transgenic elements of RR soybean in one reaction. Compared with regular PCR, this m-PCR shows many merits, including specificity, low cost, high efficiency, and reproducibility. Therefore, this method may be applied to qualitatively and quantitatively detect multiple inserted elements of RR soybean in various feed products.

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