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A 'turn-on' ultra-sensitive multiplex real-time fluorescent quantitative biosensor mediated by a universal primer and probe for the detection of genetically modified organisms

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ABSTRACT

Among the existing multiplex genetically modified organism (GMO) detection methods, significant problems are highlighted, including amplification asymmetry of different targets, and the low detection throughput, which limits their capacity to meet the requirements of high-throughput analysis. To mitigate these challenges, a 'turn-on' ultra-sensitive multiplex real-time fluorescent quantitative biosensor is developed. In this system, the multiplex ligation-dependent amplification (MLPA), universal primer and universal probe are innovatively combined, which can enhanced the amplification specificity, overcome asymmetric amplification and guarantee the homogeneity of amplification efficiency simultaneously. Furthermore, both single and multiplex detection results can be output by the fluorescent group labeled on universal TaqMan probes for different targets in real-time. After optimization, the quantitative detection limit was 5 pg. In conclusion, this strategy could serve as an important tool for GMO detection in processed and commercially available products, even in the fields that require reliable and sensitive detection of DNA targets.

1. Introduction

As the large-scale cultivation of genetically modified (GM) crops increases, so, too, does global attention to issues of food safety (Ahmed, 2002). Many countries have adopted mandatory genetically modified organism (GMO) labeling practices to protect consumers (Isaac & Kerr, 2003; Strauss, 2006). The European Union (EU), for example, provides regulations to ensure that the proportion of GMOs is never higher than 0.9% of the food ingredients (Dennis, 2018). However, some countries require product labels that disclose the GM ingredients regardless of their proportionate content. Nevertheless, the threshold of qualitative polymerase chain reaction (PCR) method is approximately 0.1–0.5% (Wurz, Bluth, Zeltz, Pfeifer, & Willmund, 1999). Event-specific GMO detection, therefore, faces ever-increasing challenges, due to the sheer number of GMOs entering the marketplace (Lu et al., 2009; Tian et al., 2019; Xu et al., 2012; Zhu, Fu, Wang, Du, Huang, Zhu, et al., 2016; Zhu, Wang, Huang, Luo, & Xu, 2016).

The quantitative technology for detecting GMOs has developed (Cheng et al., 2017; Cheng et al., 2018) and become widely available, especially the multiplex PCR technology (Gao et al., 2019; Shang, Xu, Wang, Xu, & Huang, 2017; Tian et al., 2018), which is a mature testing technology, and widely used to identify multiplex sequences (Niu et al., 2018). The amplification procedures for different targets can proceed simultaneously, thereby significantly enhancing amplification efficiency. However, the problems that presented in the existed quantitative detection methods, such as: 1) costly, labor- and time-consuming in traditional single detection; 2) the amplification asymmetry caused by different primers and probes, limited number of targets that are detected in multiplex detection, which limits their capacity to satisfy the needs of high throughput analysis. In an effort to mitigate these challenges, the universal primer multiplex PCR technology were developed (Wang, Feng, Ye, Huang, & Zhang, 2018; Xu et al., 2012). The key to

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universal primer multiplex PCR technology is, indeed, the universal primer, which can mediate the amplification of different targets with a single pair of universal primer in the middle and later stages, thus reduce the complexity of the reaction system and overcome the amplification asymmetry caused by the use of different primers.

Multiplex ligation-dependent probe amplification (MLPA) (Schouten et al., 2002; Shang et al., 2013) is a kind of qualitative and semi-quantitative universal primer multiplex technology for nucleic acid detection. It is widely applied in human genome, molecular cell biology, and cancer diagnostics (Bunyan et al., 2004; Kozlowski, Jasinska, & Kwiatkowski, 2008). The process of traditional MLPA includes the ligation-probes hybridization with target sequence, and ligation reaction under ligase, the universal primer-mediated PCR amplification the amplicons separation and analysis using capillary electrophoresis (Li, Zhang, Tian, & Xu, 2020). This MLPA technology, on one hand, can avoid non-specificity amplification due to the specific hybridization of the two ligation probes, moreover, the universal primer-mediated amplification can guarantee the amplification homogeneity among different targets (Guertler, Goerlich, & Busch, 2014). However, the capillary electrophoresis result analysis is tedious and time consuming (Holck, Signe, & Even, 2009; White, Vink, Kriek, Wuyts, & Dunnen, 2004).

TaqMan fluorescent probe technology has been previously used for the accurate quantification of nucleic acid molecules and has been found to improve the specificity and sensitivity of detection (Cai, 2003; Wang, Fu, Wang, & Hong, 2009). However, different lengths of TaqMan fluorescent probes can cause different annealing temperatures and further affect the amplification efficiency (Wang, Feng, Zhang, Wang, & Wang, 2012; Wang, Liu, Li, Zhang, & Yang, 2009).

Based on the advantages proffered by the MLPA and TaqMan fluorescent probe and overcoming their disadvantages (Niu et al., 2018), a 'turn-on' ultra-sensitive multiplex real-time fluorescent quantitative biosensor mediated by universal primer and probe was established, namely fluorescent-MLPA (F-MLPA) method. The GM maize MON810 and GM soybean GTS 40-3-2 were chosen as the research objects and sets of ligation probes were designed based on the genome sequences of the two grains (Yin et al., 2016; Yuan et al., 2016). In addition, the reaction conditions were optimized and the detection limit was explored. This study not only provided data support for the subsequent design of the ligation probe, but also proved to be a convenient technology for the detection of GMOs with high throughput and high sensitivity.

2. Material and methods

2.1. Materials

Six GM crops were used in this study, the seeds of GM maize MON810, GM soybean GTS40-3-2 and MON89788, and GM rapeseed GT73 were kindly supplied by Monsanto (MO, USA). GM maize BT11 and GA21 were kindly supplied by Syngenta (Basel, Switzerland). The non-GM maize and soybean were reserved by our laboratory.

The samples of MON 810 and GTS40-3-2 were used to identify the feasibility of this novel F-MLPA method, and BT11, GA21, MON89788 and GT73 were used to identify the specificity.

2.2. Samples preparation and primer design

Four sets of ligation probes were selected for the detection of GMO based on the gene sequences of GM corn and soybeans, including the endogenous reference gene of maize *ZSS* (Kim, Zhang, & Kim, 2014) and that of soybean *LEC* (You-wen, Xue-jun, Bang-ruo, Lu, & Zhen, 2012), and event-specific sequence of GM maize MON810 and GM soybean GTS 40-3-2 (Hernández et al., 2003; Holck, Vaïtilingom, Didierjean, & Rudi, 2002; Lau, Collins, Yiu, Xing, & Yu, 2004; Windels, Taverniers, Depicker, Van Bockstaele, & De Loose, 2001). All the primers were designed using Primer 5.0 software. The sequences of these ligation probes that hybridized with the DNA templates are listed in Table S1.

The universal primers should show less homologous with the DNA templates, which are required to be optimized, and the candidates were designed in this study and listed in Table S2. The different ligation probes for selecting the universal primer were designed according to the sequence of the specific primer, as listed in Table S3.

The universal primer pair CUP-F/R was chosen as the best for the subsequent study because of its superior amplification consequence. The sequence of TTTGGTCGTGGTGGTGG were connected at the 5′ end of ligation probe-F and GGGGAAGGAGGGAAGGG on the 3′ end of ligation probe-R. A sequence of TaqMan probe was inserted into the ligation probe-F, which is between the universal and specific sequences. The ligation probes of Mon810-2F and GTS-2F contained the same binding site of TaqMan probe GMO-FAM, which represented for the GM crops. The ligation probes of ZSS-2F and LEC-2F contained the same binding site of TaqMan probe EDO-VIC, which represented for the endogenous reference genes. The detailed sequences of ligation probes and universal TaqMan probes are listed in Table S4.

2.3. Extraction of genome and verification

The seeds of samples were ground into powder using a blender (Retsch GmbH & Co, Haan, Germany), and the genomic DNA was extracted using 100 mg powdered sample with the Plant Genomic DNA Kit (Tiangen Biotech Co., Ltd. (Beijing, China) and the extraction was performed following the instructions. The genomic DNA of MON810 acts as the targets of Mon810-F/R and ZSS-F/R, and that of GTS-40-3-2 acts as the targets of GTS-F/R and LEC-F/R. The DNA concentration and purity was determined and calculated using NanoDrop N2000 (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Each sample was measured three times. For further studies, the extracted DNA samples were diluted to a final concentration of 25 ng/μL.

The verification assays were conducted in a total volume of 25 μL on CFX96 thermal cycler (Bio-Rad Applied Science, Mannheim, Germany). Each reaction mixture contained 1 × SuperReal PreMix (SYBR Green) (Tiangen Biotech Co., Ltd., Beijing, China), 0.3 μM of each primer and 2 μL of template. The amplification program includes a 10-min denaturation at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 58 °C, and 10 min at 72 °C.

2.4. The ligation reaction of the probes

The reactions of the probe ligations (PL) consisted of denaturation (PL-1), hybridization (LP-2) and ligation of DNA (LP-3). The process parameters are listed in Table S5.

The 5 μL of DNA sample was added into the 100 μL centrifuge tube, in which LP-1 was performed. After that, the tube was quickly placed in the icy water for 20 min once the process was complete.

In the process of LP-2, the hybridization master mix was prepared, which consisted of 1.4 μL MLPA buffer (yellow cap, MRC-Holland, Amsterdam, Netherlands) and 1.4 μL probe mix (10 μM). Three microliters of hybridization master mix were added to each sample, after which the process of LP-2 was performed.

In the process of LP-3, the Ligase-65 master mix was prepared, which consisted of 25 μL H_2O, 3 μL ligase buffer A (transparent cap, MRC-Holland, Amsterdam, Netherlands) and 3 μL ligase buffer B (white cap, MRC-Holland, Amsterdam, Netherlands). After mixing, the 1 μL of ligase was gently added. Until the temperature reached to 54 °C, the 32 μL of Ligase-65 master mix were added into each sample gently. The program of LP-3 was continued.

2.5. Universal primer selection

A verification experiment was performed to determine which

universal primers were more efficient for the amplification according to the principle of MLPA. The PCR assays were conducted in a total volume of 25 μL on an ABI SimpliAmp thermal cycler (Applied Biosystems, USA). Each reaction contained 1 × buffer, 0.2 mM of dNTP, 0.3 μM of each primer, 2.5 units of Taq DNA polymerase (TaKaRa Biotechnology Co. Ltd., China), and 2 μL of ligation product. The experiment program comprised a 10-min denaturation at 95 °C, 45 cycles of 30 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C, and 10 min at 72 °C.

2.6. Universal primer mediated Real-time F-MLPA assays

Two systems of real-time fluorescent dual ligation-dependent probe amplification were built for GM maize MON810 and GM soybean GTS 40–3-2 detection, respectively. The F-MLPA assays were conducted in a total volume of 20 μL on CFX96 thermal cycler. Each reaction contained 1 × SuperReal PreMix (Tiangen Biotech Co., Ltd., Beijing, China), 0.3 μM of CUP-F/R, 0.1 μM of each probe (FAM probe and VIC probe) and 2 μL of ligation product. The experiment program was the same with that in Section 2.3.

3. Results and discussion

3.1. Principle of the assays

F-MLPA was developed for GMO detection (Scheme 1). This method is an innovative combination of MLPA, universal prime and probe. A pair of ligation probes were designed. The ligation probe-F included a universal sequence (red) at 5′ end and a specific sequence (green) that was complementary to the target gene (green) at 3′ end. The ligation probe-R included a universal sequence (red) at 3′ end, a specific sequence (green) that was complementary to the target primer (green) at 5′ end and a stuffer sequence (blue) between the two parts above, which was complementary to the fluorescence probe.

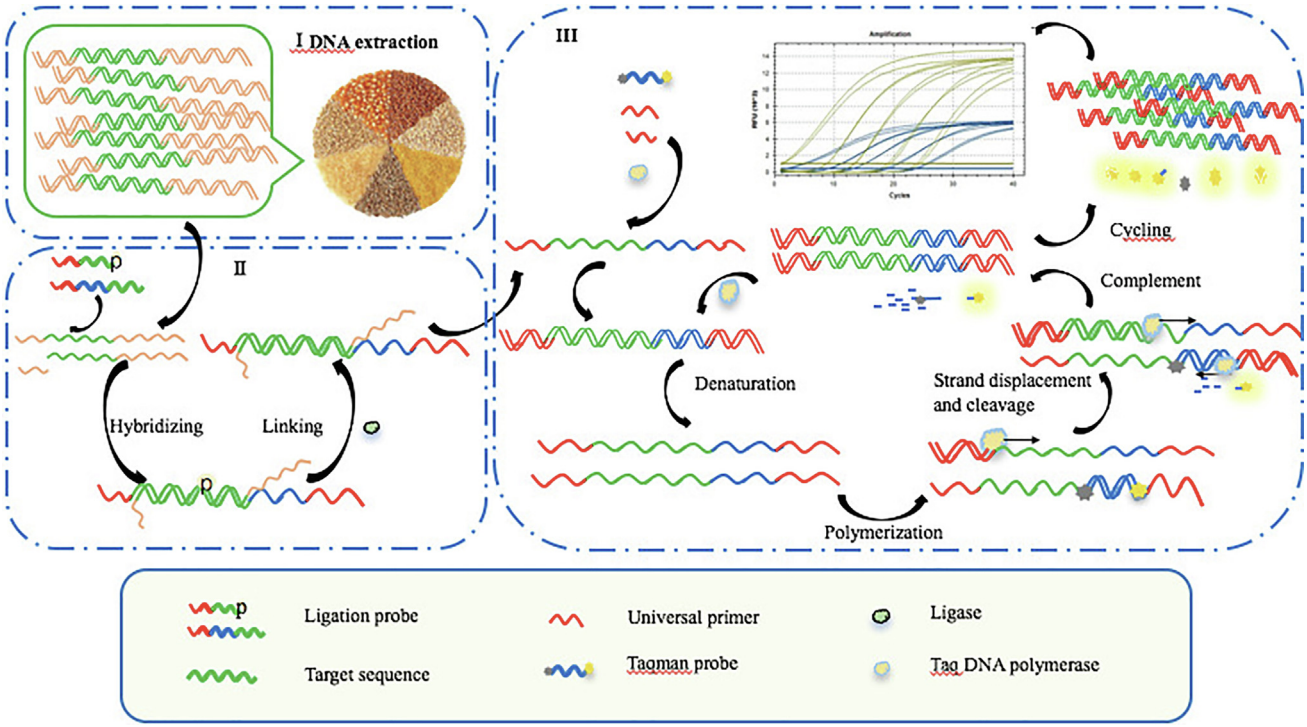
The F-MLPA reaction process comprised two steps. The first was the hybridization of ligation probes with the target sequence and the ligation of the ligation probes to a complete single strand catalyzed by ligase. The temperature of the hybridization was 60 °C, according to the sequence length of the ligation probe and target. The ligation reaction was conducted at 54 °C, according to the optimal activity of the ligase. The specificity of the detection system was improved by this step. The second step involved the universal primers mediated real-time fluorescent quantitative PCR reaction, with the single-strand acting as template in the amplification process. When the target was present, the TaqMan probes were hydrolyzed during the process of annealing (58 °C) and the fluorescence improved. The universal primer-mediated amplification was able to solve the imbalances of amplification efficiencies caused by traditional different primers. Significantly, one group of universal primers can mediate different amplifications for different targets, thus eliminating the need to design different primers for individual amplifications of different targets, which also reduces the cost of the experiment. Moreover, the use of Taqman probes was with easy operation and time-saving instead of capillary electrophoresis. A 'turn-on' fluorescent quantitative method was, thus, realized. When the target was absent, however, no single strand was available to be the template and, therefore, the TaqMan probe could not be hydrolyzed and the fluorescent signal remained low.

3.2. Extraction and verification of genome

Whether the sequence complementary to the ligation probe-F/R was integrated in the extracted genome DNA is very important, because it decides the process of hybridization and ligation. The DNA extraction results were verified and indicated in Fig. 1A, where it was evident that the curve of each sample was clear and smooth. The Ct values of Mon810-F/R and ZSS-F/R were approximately 28, and those of GTS-F/R and LEC-F/R were less than 28. The negative control group did not show the amplification and the extracted DNA could be utilized for further experiment and research.

3.3. Screening of the universal primers

According to the sequence shown in Table S2, probe ligation and



Scheme 1. Design Strategy of the 'turn-on' ultra-sensitive multiplex real-time fluorescent quantitative biosensor. (I) DNA extraction; (II) ligation-dependent probe amplification; (III) universal primer and TaqMan probe mediated RT-PCR.

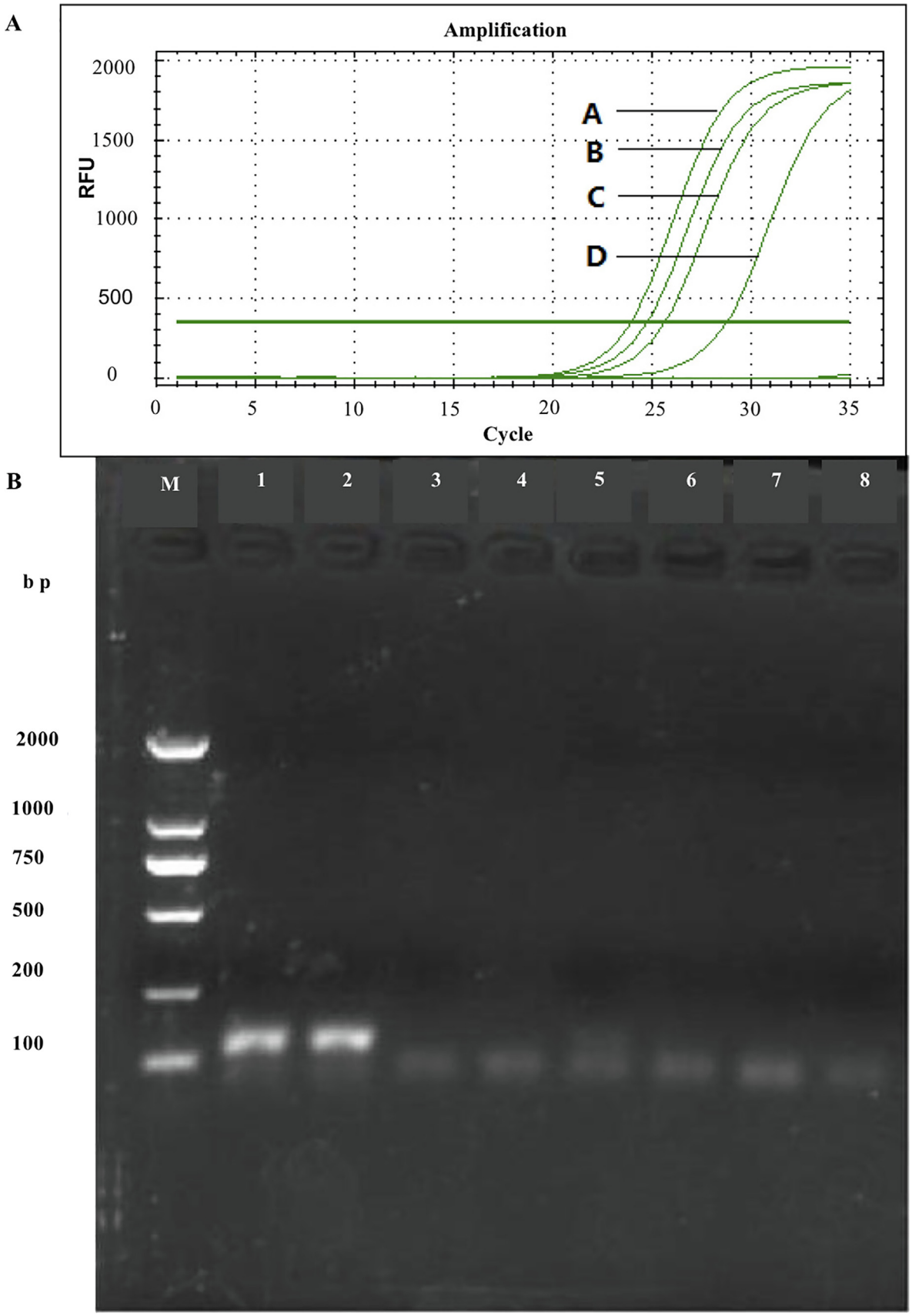


Fig. 1. The identification of GM soybean and GM maize. (A) A: LEC; B: ZSS; C: GTS 40-3-2; D: MON810. (B) Lanes 1–4 indicate the results of CUP-F/R: 1–2, MON810 (120 bp); 3–4, negative control using water in ligation step; Lane 5–8 are the results of SUP: 5–6, MON810 (120 bp); 7–8, negative control using water in ligation step; M: DNA Marker DL 2000.

universal primer amplification were performed and the genome DNA of MON810 was taken as the template. Next, the products were analyzed on 2% agarose of the gel electrophoresis (containing 0.3 μg/mL EB). The results were shown in Fig. 1B.

As indicated, the CUP-F/R achieved a higher amplification efficiency than the SUP, because the bands amplified by CUP-F/R were clearer than those of SUP. According to the design principle of the multiplex ligation probe, the sequence of SUP in Mon810-1F and Mon810-1R were complementarily reversed. However, the SUP primers were able to hybridize with each other more easily than the specific sequence hybridized with the template, which might explain why the ligase could not connect the ligation probes to a complete double strand, thus leading to a false negative result. The annealing temperature of SUP and the ligation probes were similar, so it was difficult to let the ligation probes hybridize with the template prior to the SUP by controlling the temperature. However, the universal primer CUP-F/R existed without any complementary element and did not disturb the ligation hybridization or the PCR process. Therefore, the ligation probes modified with CUP-F/R were applied in the follow-up experiment.

3.4. Optimization of F-MLPA system

The preparation operation of the single strand was optimized and the MON810 acted as the template, the result was tested on the 2% agarose of gel electrophoresis. As shown, the bands of ligation amplification products were clearer after optimization (Fig. 2A, lane 1–2).

The hybridization time decided the degree of hybridization and the high degree of hybridization was good to the catalytic activity of the ligase. A hybridizing time of 2 h was suggested in the instruction. Following the optimized single strand preparation, the hybridization time optimization was performed using RT-PCR and the MON810 acted as the template (Fig. 2B). As indicated in Fig. 2B, the Ct values were not markedly different, which suggested that the degree of hybridization had achieved the maximum and that extending the time would have no impact on the amplification. Hence, 0.5 h was selected for the further research.

Combined with the optimization results above, the annealing temperature during the PCR amplification was also discussed, the MON810 acted as the template and the products was analyzed on 2% agarose of gel electrophoresis. As indicated in Fig. 2C, when the temperature was lower than 58 °C, the non-specific products reduced with the temperature improved. However, when the temperature was higher than 58 °C, the efficiency of the amplification reduced because the temperature was higher than the Tm of the primer. Therefore, the temperature of 58 °C was chosen as the annealing temperature.

3.5. The feasibility of F-MLPA

The feasibility of F-MLPA was verified and the results were shown in Fig. 2D. The amplification products were the same as the theoretical value, while the negative control had no amplification products, which indicated that the four pairs of ligation probes could be ligated successfully, that the ligation products could be utilized in the universal probe-mediated PCR and, furthermore, that the specificity of the ligation primers and the universal primers were high.

Combined with RT-PCR, two fluorescence dual ligation-dependent probe amplification system were established for GM maize MON810 and GM soybean GTS 40-3-2 detections, respectively. The results were shown in Fig. S1. It indicated that all the ligation probes were ligated to form single strand successfully. Then, in the RT-PCR process, the

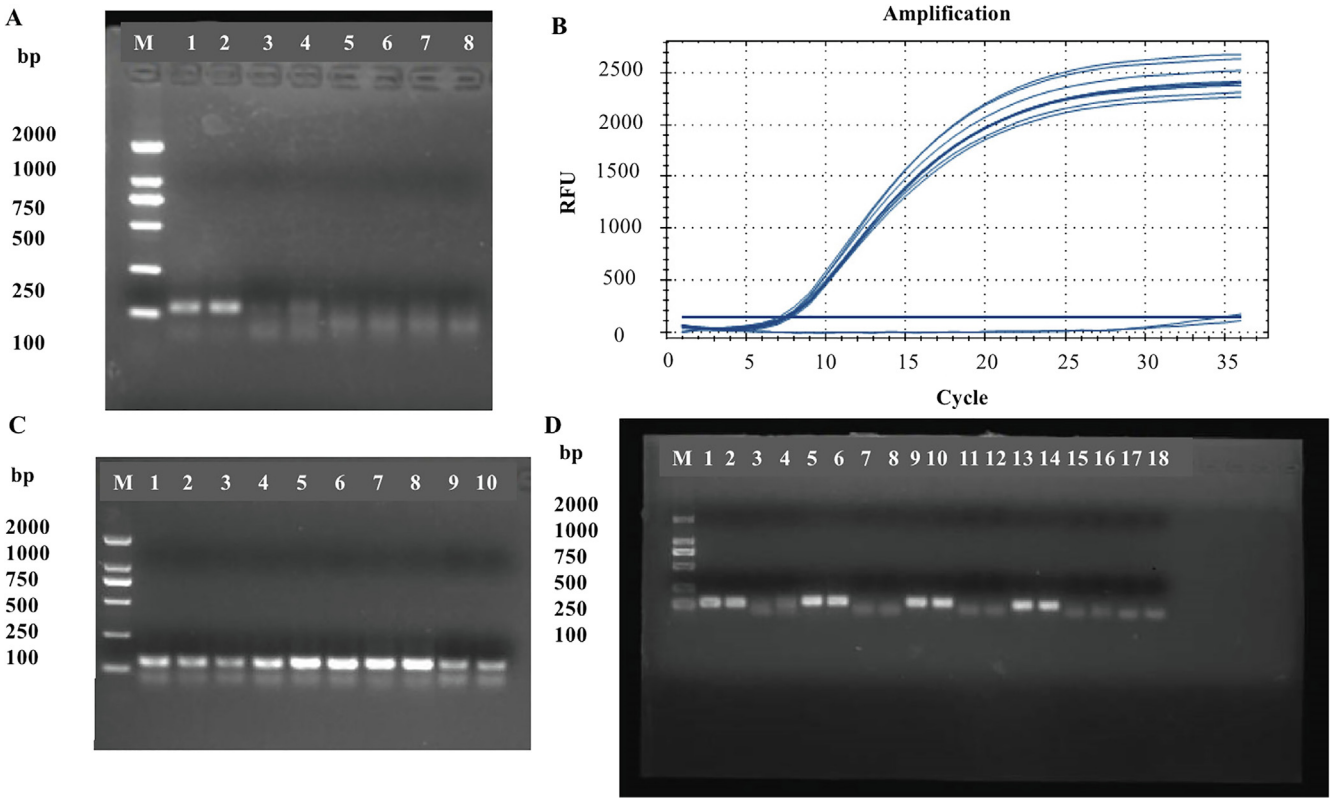


Fig. 2. Optimization and feasibility verification for the detection system. (A) Optimization for the process of obtaining single strand sequence. Lane 1–2: 95 °C 5 min, and then placed in ice water immediately; lane 5–6: the corresponding negative control; lane 3–4: 95 °C 5 min, and then cooled to 25 °C in PCR instrument; lane 7–8: the corresponding negative control; M: DNA marker DL2000. (B) Optimization for time of hybridization. From bottom to top: 0.5 h, 1 h, 1.5 h, 2 h, 3 h, and 4 h, respectively. (C) Optimization of annealing temperature in PCR. lane 1–2: 52 °C; lane 3–4: 54 °C; lane 5–6: 56 °C; lane 7–8: 58 °C; lane 9–10: 60 °C. (D) Feasibility verification results of 4 pairs ligation-dependent probes. 1–4: MON810 (3–4 as negative control); 5–8: ZSS (7–8 as negative control); 9–12: GTS 40-3-2 (11–12 negative control); 13–16: LEC (15–16 as negative control); NTC of 17–18: PCR process; M; DNA Marker DL 2000.

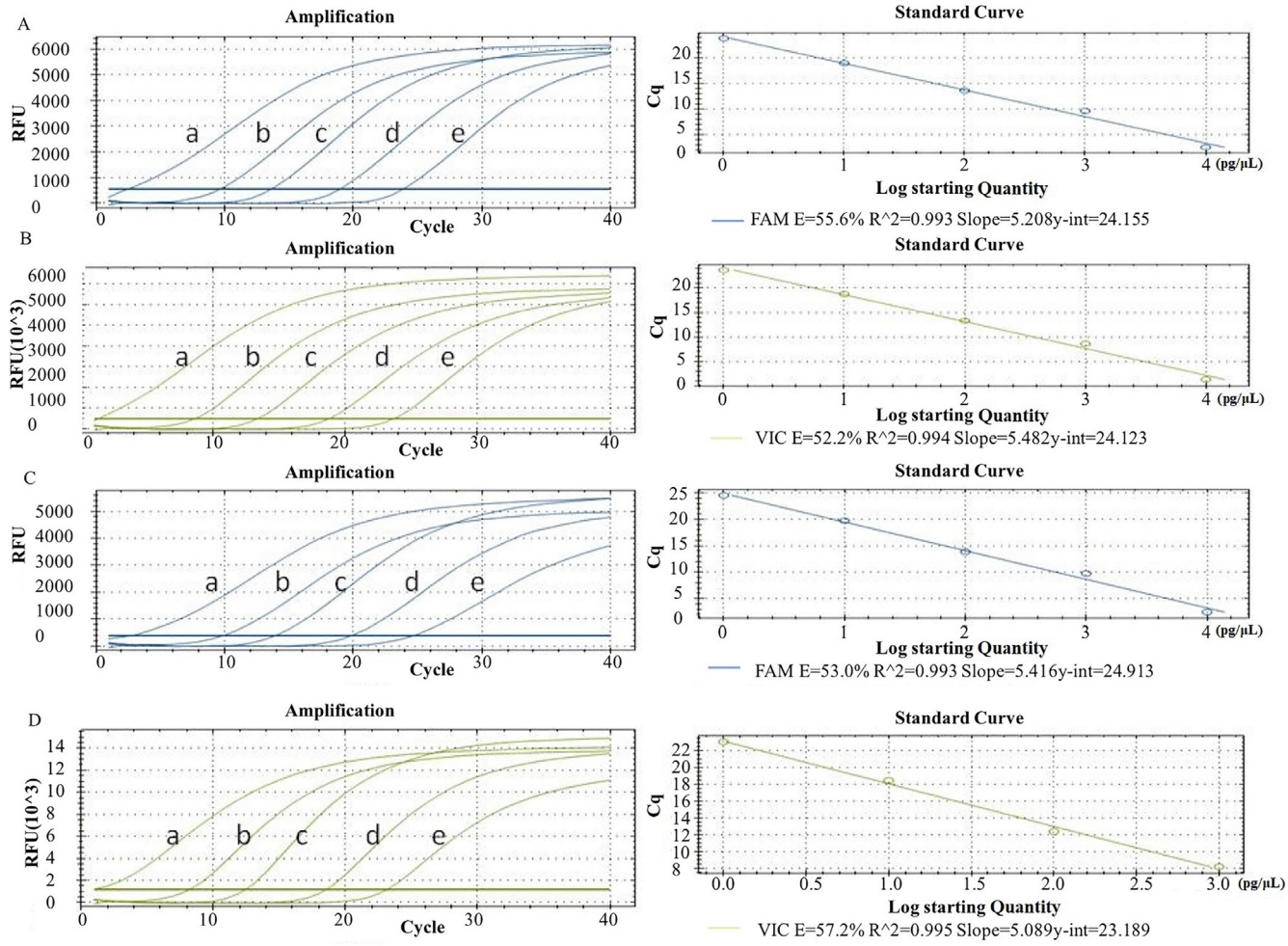


Fig. 3. Detection limit of F-MLPA in single-plex. A: Mon810-2F/2R; B: ZSS-2F/2R; C: GTS-2F/2R; D: LEC-2F/2R. Curve a: 10 μM; Curve b: 1 μM; Curve c: 0.1 μM; Curve d: 10 nM; Curve e: 1 nM.

fluorescence of the FAM and VIC were detected simultaneously, which presenting for the GM event MON810 and endogenous reference gene of maize, respectively (Fig. S1A). In Fig. S1B, the fluorescence of the FAM and VIC were also appeared simultaneously, which presenting for the GM event GTS 40-3-2 and endogenous reference gene of soybean, respectively.

3.6. Detection limit of F-MLPA

In order to verify the feasibility of F-MLPA, the single and dual detection processes were conducted separately. As shown in Fig. 3, the concentrations of genomic DNA were 10-fold diluted (10 ng/μL, 1 ng/μL, 100 pg/μL, 10 pg/μL, 1 pg/μL and 0.1 pg/μL). The amplification curve of the template with 0.1 pg/μL was no different to that of the negative control, so the standard curves were drawn according to the other five concentration samples. As indicated, when the template concentration was as low as 1 pg/μL, the standard curve showed good linearity. The standard deviations of the standard curves using primer pairs Mon810-2F/2R, ZSS-2F/2R, GTS-2F/2R and LEC-2F/2R were 0.993, 0.994, 0.993 and 0.995, respectively. Therefore, the detection limit of single ligation-dependent probe amplification was determined to be 1 pg/μL.

The universal primer and probe mediate F-MLPA containing the primer pairs of Mon810-2F/2R and ZSS-2F/2R, was performed using the serially diluted DNA of MON810. When the concentration was as low as 0.1 pg/μL, the feature of the amplification curve was not clear and the degree of fit of the standard curve deteriorated (standard deviations were 0.886 for FAM and 0.892 for VIC). When the template concentration was 1 pg/μL, the degree of fit for all the standard curves was good, and the standard deviation of the FAM curve was 0.996, while the standard deviation of the VIC curve was 0.996 (Fig. 4). Therefore, the detection limit of F-MLPA for GM maize MON810 was 1 pg/μL.

Similarly, the genomic DNA of GT 40–3-2 was taken as the template, as indicated in Fig. 5. When the concentration was as low as 0.1 pg/μL, the feature of the amplification curve was not clear and the degree of fit of the standard curve became bad (standard deviations were 0.872 for FAM and 0.887 for VIC). When the template concentration was 1 pg/μL, the degree of fit of all the standard curves was good, and the standard deviation of the FAM curve was 0.995, while the standard deviation of the VIC curve was 0.998. Therefore, the detection limit of F-MLPA for GM soybean was also 1 pg/μL.

The average weight of the maize genome is 2.595 pg per haploid, and that of soybean is 1.155 pg (Arumuganathan & Earle, 1991). In the initial detection, the 5 μL of sample was used, thus, the sensitivity of F-MLPA is an average of 2 copies of maize genomic DNA and 4 copies of soybean genomic DNA. In this quantitative detection system, the detection limit was much sensitive than that in quantitative LAMP and traditional multiplex real-time PCR assays (Huang et al., 2015; Samson, Gullí, & Marmiroli, 2013).

3.7. Specificity of F-MLPA

GM maize MON863, BT11 and GA21, GM soybean MON89788 and

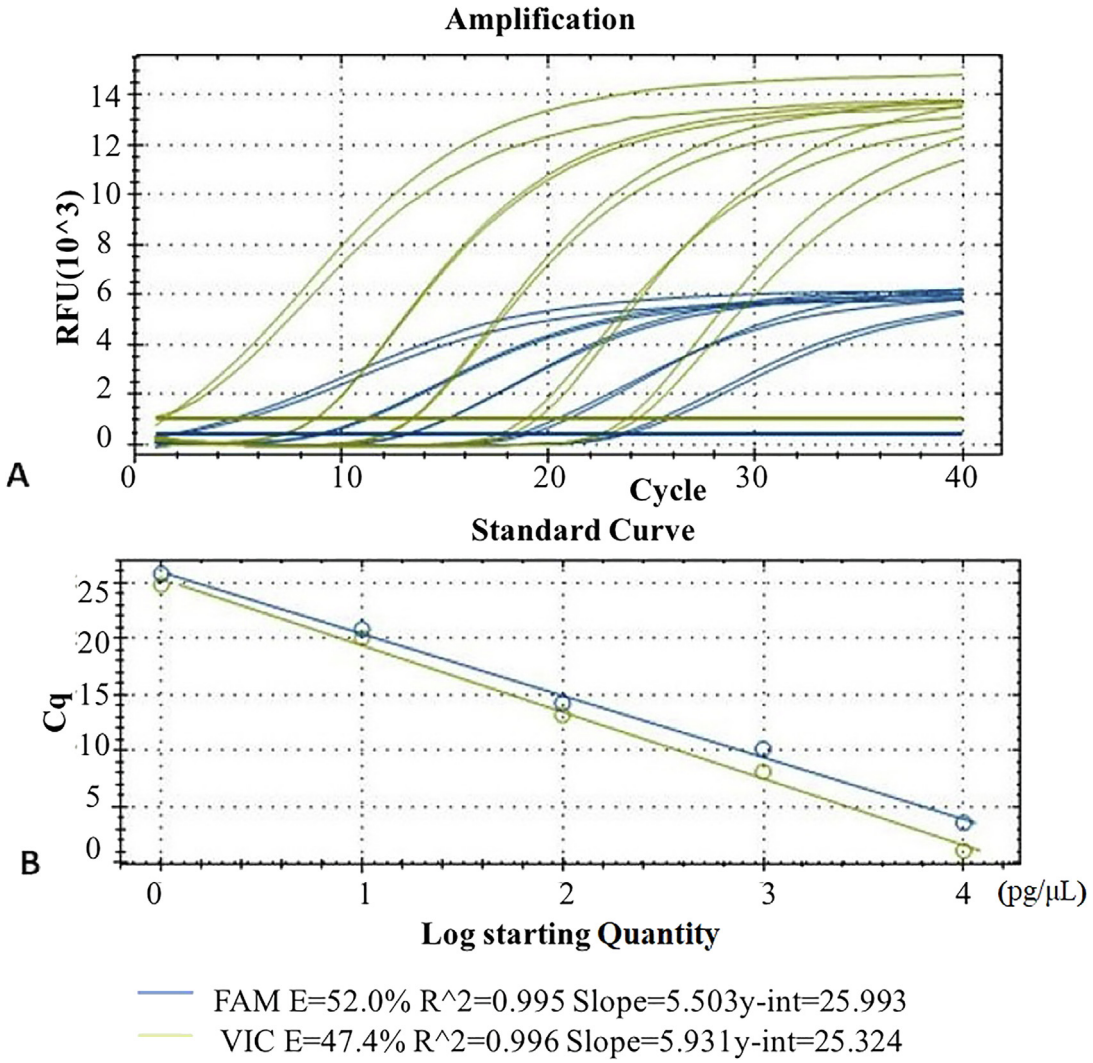


Fig. 4. Sensitivity of F-MLPA Maize. A: Amplification curve; B: Standard curve. Blue curve: FAM signal; Yellow curve: VIC signal.

GM rapeseed GT73 were used as templates to test the specificity of this method. The results of specificity towards different templates were shown in Table S6. As indicated, the signal of FAM could not be detected in non-GM maize and GM maize, with the exception of MON810 by the F-MLPA MON810 detection system. However, the signal of VIC could be detected only in the maize samples, which presented as the endogenous reference gene of maize. The detection system for GM soybean GTS 40–3-2 produced similar results. Hence, the specificity of the methods was determined to be good, with no false positives or false negatives resulting during the detection process.

4. Conclusions

In conclusion, an innovative accurate and quantitative biosensor has been developed for GM events detection, by integrating MLPA with delicately designed universal TaqMan probe. Employing a universal primer, universal TaqMan probe and ligation probe, the F-MLAP system was developed with excellent sensitivity and greater simplicity, compared with the traditional methods. The single and multiplex outputs of the signal were realized using the universal TaqMan probes for different targets. In addition, the cost of the experiment was reduced due to the design of the universal fluorescence probe. Under the optimal conditions of this study, the sequences in this system can reach the maximum degree of hybridization for only half 1 h and the detection limit of quantitation (LOQ) was as low as 1 pg/μL. The approach is a universal strategy, which can be applied to the detection of other highly broken genes in highly processed products. Because the connected probe is short, it can also be applied in related fields that require the reliable and sensitive detection of DNA targets.

Author contributions

All authors have given approval to the final version of the manuscript.

CRediT authorship contribution statement

Bing Xiao: Methodology, Investigation, Writing - original draft, Visualization. **Chenqi Niu:** Investigation, Validation, Writing - original draft. **Ying Shang:** Investigation, Writing - original draft, Visualization. **Yuancong Xu:** Formal analysis, Visualization. **Kunlun Huang:** Resources, Supervision, Project administration. **Xiujie Zhang:** Resources, Supervision. **Wentao Xu:** Conceptualization, Writing - review & editing, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

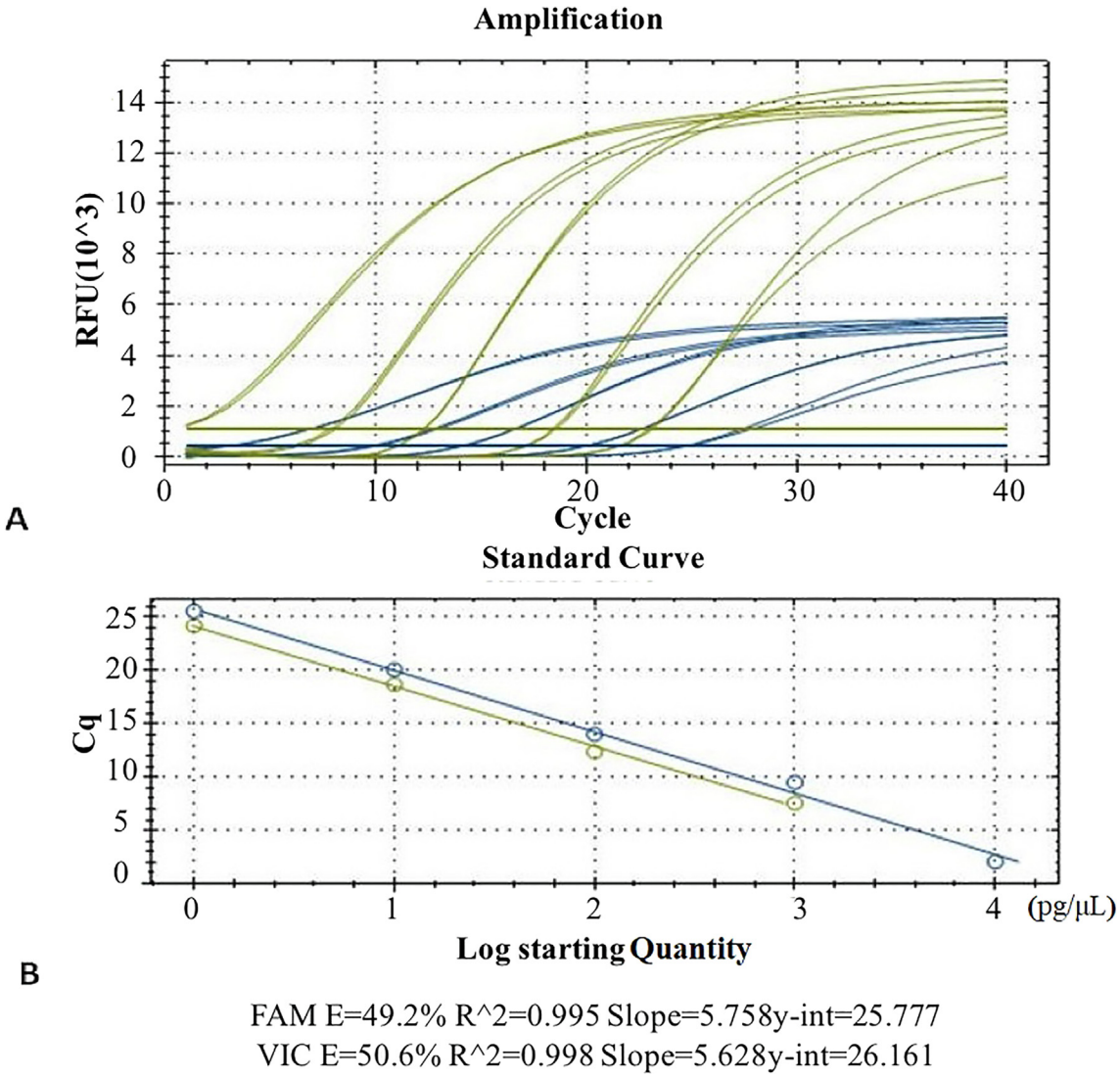


Fig. 5. Sensitivity of F-MLPA: Soybean. A: Amplification curve; B: Standard curve. Blue curve: FAM signal; Yellow curve: VIC signal.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.127247.

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