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Single universal primer recombinase polymerase amplification-based lateral flow biosensor (SUP-RPA-LFB) for multiplex detection of genetically modified maize

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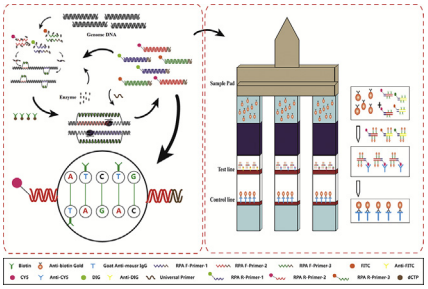
HIGHLIGHTS

- Construction of a single universal primer multiplex RPA-based lateral flow biosensor (SUP-RPA-LFB) for point-of-care detection.
- Development of a multiplex detection strategy based on RPA isothermal amplification.
- Single universal primer maintains the consistency of efficiency in multiple-target situations.
- Temperature-driven dimer subtraction technique improves specificity.
- SUP-RPA-LFB shows high sensitivity and selectivity for DNA detection.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, an isothermal paper biosensor, combining single universal primer recombinase polymerase amplification (SUP-RPA) and the lateral flow technique was developed for the multiplex detection of genetically modified maize (GMM). In pre-amplification stage, the event-specific primers contain a universal sequence at the 5' end, with a biotin-labeled deoxycytidine triphosphate (dCTP) deoxynucleotide providing additional amplification, which improves their amplification ability and ensures consistent multiplex amplification efficiency. In the signal recognition strategy, the SUP-RPA products are identified visually using the lateral flow biosensor (LFB) through dual hybridization. The accumulation of gold nanoparticles (AuNPs) produces a characteristic red band. Through this biosensor, a limit of detection of at least 50 copies was achieved, which is sensitive enough to detect MON810, MON863 and MON89034 simultaneously. The entire process of analysis was completed within 30 min and without any large-scale instrumentation. This biosensor, therefore, provides a novel rapid and portable multiple detection method for point-of-care applications, especially genetically modified organism (GMO) event-specific detection.

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1. Introduction

The advent of modern analytical technologies has provided researchers and laboratories involved in nucleic acid detection with new opportunities for the simultaneous detection of multiple DNA targets [1–3]. Multiple nucleic acid detection assays provide more information in a short time, which is particularly valuable in point-of-care (POC) testing [4–7]. Many applications have been developed for clinical diagnoses [8–12] and GMO identification [13–16]. Recombinase polymerase amplification (RPA) is a recent isothermal amplification method that simulates the progress of DNA replication in living cells and can amplify nucleic acid externally within 20 min in temperature conditions of 37–42 °C [17–19]. Based on RPA assays, researchers have established numerous new methods to achieve nucleic acid amplification and detection [20–25]. RPA assays can be used in the field to achieve target detection using portable testing instruments [26–30]. However, despite the progress made with RPA amplification protocols, multiplex RPA detection technologies remain relatively unexplored. Multiplex amplification reactions are affected by numerous factors, including length of amplicons, template concentration, annealing temperature, among others. However, the essence of all multiple amplification is the primer. The presence of multiple primers increases the complexity of the reaction system and amplification efficiency is different between primers. The emergence of universal primers effectively solved such challenges. Universal primers ensure uniform amplification efficiency with every target. Many detection methods based on universal primers have been used in the detection of multiple targets, including identification in four meat species (cattle, pig, horse and chicken) [31], discrimination of three kinds of bacteria in food samples [32,33] and event-specific detection in stacked genetically modified maize (Bt11 & GA21) [34].

A lateral flow biosensor (LFB) is used as an alternative method of assay to detect amplification products. LFB is one of the most popular and successful screening techniques in POC detection, because it is affordable, sensitive, specific, rapid and equipment free. In addition, LFB has been used in the detection of multiplex targets, including multiplex event-specific GM soybean detection [35], and multiplex detection of Aeruginosa and its toxin genes [36]. However, in the above assays the LFB was fabricated through nucleic acid hybridization, which is not stable in preservation.

In this paper, we developed a single universal primer-multiplex RPA-based lateral flow biosensor (SUP-RPA-LFB) for the event-specific detection of GM maize, based on the unique integration sequences between the maize genome and the exogenous gene. Single universal primers improved the amplification ability, as well as ensuring that the multiplex amplification efficiency was consistent. In addition, antibody hybridization was used instead of nucleic acid hybridization as the LFB signal identification method and antibodies provided additional stability and precision. Moreover, biotin-labeled deoxycytidine triphosphate (dCTP) was used as expansion material which raised the combination number of AuNPs on target DNA. The maize events of MON89034, MON863 and MON810 were chosen as the target analytes in this assay and the simultaneous detection of three GMO events showed high sensitivity and good applicability. Moreover, this biosensor system is miniaturized, low-cost and easy-to-operate.

2. Materials and methods

2.1. Chemicals and materials

Chloroauric acid tetrahydrate ($HAuCl4 \cdot 4H_2O$), NaCl, hemin, hydrogen peroxide, trisodium citrate, Triton X-100, Tween-20, bovine serum albumin (BSA), phosphate buffered saline (PBS, pH 7.4, 0.01 M), sodium chloride–sodium citrate (SSC) buffer (20 concentrate, pH 7.0), Tris-HAc buffer (pH 7.4, 10 mM) and borate buffer (BB, pH 9.0, 0.1 M) were purchased from Sigma Chemical Company (St. Louis, MO, USA). The anti-biotin antibody, anti-FITC antibody, anti-digoxin antibody, anti-CY5 antibody and goat anti-mouse IgG were purchased from Abcam Inc. (Cambridge, MA, USA). Double distilled water (ddwater) was used in all experiments. Backing cards (HF000MC100), glass fiber sample pads (CFSP001700), conjugation pads (GFCP000800), nitrocellulose membranes (135s) and absorbent pads (CFSP001700) were purchased from Millipore (Bedford, MA, USA).

Genetically modified maize events (MON863, MON810, MON89034, Bt176, Bt11, GA21, MIR162, MIR604, NK603, and TC1507), GM soybean events (MON89788, MON87705, and MON87769), and non-GM maize events were bought from Institute of Reference Materials (IRMM) and Measurement American Oil Chemists Society (AOCS). Additionally, the GM rice events (TT51-1 and kefeng 6) had been previously stored in our laboratory.

2.2. Event-specific SUP-RPA procedure

2.2.1. Genomic DNA extraction and quantification

The DNA of the highly processed samples was extracted using the cetyltrimethylammonium ammonium bromide (CTAB) method as previously reported [37]. The quantity and quality of purified DNA samples were measured and evaluated using the Nanodrop 1000 UV/Vis Spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA). Additionally, the quality of the DNA was analyzed by 2% (w/v) agarose gel electrophoresis (data not shown). A total of 50 ng genomic DNA was used as a template in each reaction.

2.2.2. Design of event-specific SUP-RPA primers

All the SUP-RPA primers were design and used in first time in this paper. The forward primers contained event-specific sequences and the FITC (DIG or CY5) were labeled at the 5′ end. The reverse primers contained event-specific sequences and a universal sequence at the 5' end with about 25 nt. All the reserve primers were tested in Primer 5 after design to check the sequence without any dimers or special structure. The detailed locations of the event-specific RPA primers in their genome are shown in Fig. S1. All primer sequences used in this study and the amplification products sizes are listed in Table S1 and were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China).

2.2.3. DNA amplification by SUP-RPA

To demonstrate the effectiveness of RPA primers and optimize the system for RPA, the basic information for the reactions was improved. RPA amplification was carried out with the TwistAmp Basic kit (TwistDX, Cambridge, UK) in an Eppendorf ThermoMixer (Eppendorf, USA). Each reaction was performed in a 47.5 μL reaction mixture containing 29.5 μL of rehydration buffer, 2.4 μL of forward and reverse primers (10 μM each) and 2 μL of the DNA template (100 copies). The reaction mix was then added to the freeze-dried tube, which was flicked until the freeze-dried powder fully dissolved. To initiate the reaction, 2.5 μL of 280 mM magnesium acetate (MgAc) was added. Since the RPA reaction started as soon as the MgAc was added, the tubes were immediately incubated for 20 min at 42 °C in a thermal cycler. The amplified products were analyzed using 2% (w/v) agarose gel electrophoresis.

The SUP-RPA reaction system was performed in a 47.5 μL reaction mixture containing 29.5 μL of rehydration buffer, 1 μL universal primer and 1 μL FITC (DIG or CY5) labeled forward RPA primer, 1 μL reverse primers, 2 μL of the DNA template, 2.5 μL of 280 mM magnesium acetate, and ddH2O was added to bring the final volume to 50 μL. The process was the same as that of the RPA reaction.

2.3. Preparation of AuNPs

AuNPs were prepared using a previously established method, with some modifications [38]. Before the reaction, bottles used in this procedure were soaked overnight in acid and then washed with tap water, distilled water and ultra-clean three times successively.

A round-bottomed flask was used as a heating vessel, in which 2 mL $HAuCl_4$ solution (1 wt %) was added to 190 mL ultrapure water. The solution was heated to boiling, then 8 mL sodium citrate solution (1 wt %) was added. When the color changed to pale yellow, dark and red successively, the reaction mixture was refluxed continuously for about 10 min, after which it was cooled in water to room temperature. The final color of the product presented cherry red. It was stored at 4 °C for later use.

2.4. Preparation of Anti-AuNPs

The AuNPs were modified with antibody. The procedure was as follows: 1 mL of a 10-fold-concentrated AuNP solution (pH 8.0) was mixed with 1 μg anti-biotin antibody. After gentle mixing for 1 h, 20 μL of 10% BSA and 100 μL of 10% NaCl were added, then gently mixed for 2 h after which the solution was centrifuged for 20 min at 9000 rpm. The red precipitate of anti-biotin-Au-NP conjugates was stored at 4 °C for later use.

2.5. Lateral flow fabrication

Lateral flow biosensors were prepared using a previously established method with some modifications [16]. The LFB consisted of three test strips comprising five components each: a sample pad, conjugate pad, nitrocellulose membrane, absorbent pad and backing, as it shown in Fig. S2. The sample pads were made of glass fiber and saturated with a buffer (pH 8.0) containing 0.15 M NaCl, 0.1 M BB and 0.25% Triton X-100. The sample pads were then dried at 37 °C for 2 h and stored in a desiccator. The anti-FITC antibody (1.0 mg/mL) solution, anti-digoxin (1.0 mg/mL) antibody solution and anti-cy5 antibody (1.0 mg/mL) solution were sprayed on the nitrocellulose membranes in three different strips as test lines, while the goat anti-mouse IgG (1.0 mg/mL) solution was sprayed at different locations on the nitrocellulose membranes in three strips as control lines. The distance between these two lines was approximately 5 mm. This operation was performed using a BioDot BioJet BJQ 3000 dispenser (Irvine, CA). Thereafter, the nitrocellulose membranes were dried overnight at 37 °C and stored at 4 °C. The conjugate pads and absorbent pads were assembled on backing with an overlap between them of approximately 1–2 mm to ensure that the solution could migrate through the biosensor. The test strips were cut at widths of 3.5 mm using a Bio-Dot Paper Cutter module CM4000 (Irvine, CA). Finally, the three different test strips with different test lines were assembled together like a trident to become the LFBs, with the sample pads used as linkers, and either used immediately or sealed. The LFBs were stored under dry conditions at room temperature until used.

3. Results and discussion

3.1. Principle

As illustrated in Scheme 1, because fluorescent labels were attached at the 5′ end of the primers, the products of the event-specific SUP-RPA were numerous duplex DNAs with FITC, DIG and CY5 labeled at the 5' end, which proved the existence of GM maize MON810, MON863 and MON89034, with biotin attached at the middle through biotin-labeled dCTP to enhance signal amplification. The detection of the amplified duplex DNA complexes using LFB is illustrated in Scheme 1. The principle of the basic LFB measurement was based on the sandwich type, anti-biotin labeled AuNPs combined with biotin labeled duplex DNA on the control line, FITC (DIG and CY5) labeled target products combined with anti-FITC (anti-DIG and anti-CY5) on the test line. The biotin attached on dCTP, which acted as signal amplification, increased the number of AuNPs. The red band on the test line of the left strip indicated a “MON810 positive” result, while the absence of a red band indicated a “MON810 negative” result. Similarly, the middle strip indicated the MON810 result and the right strip showed the MON89034 result. One lateral flow biosensor simultaneously detected triple targets. The red band on the control line indicated that the biosensor was working properly.

3.2. Verification of the SUP-RPA primer

The SUP-RPA products of MON810, MON863 and MON89034 were verified respectively by 2% (w/v) agarose gel electrophoresis. Amplification sequences of MON810, MON863 and MON89034 were of similar sizes of 321 nt, 338 nt and 344 nt, respectively, which enabled consistent amplification efficiency in the multiplex reaction. The results shown in Fig. 1, therefore, confirmed that the amplification product performed as expected.

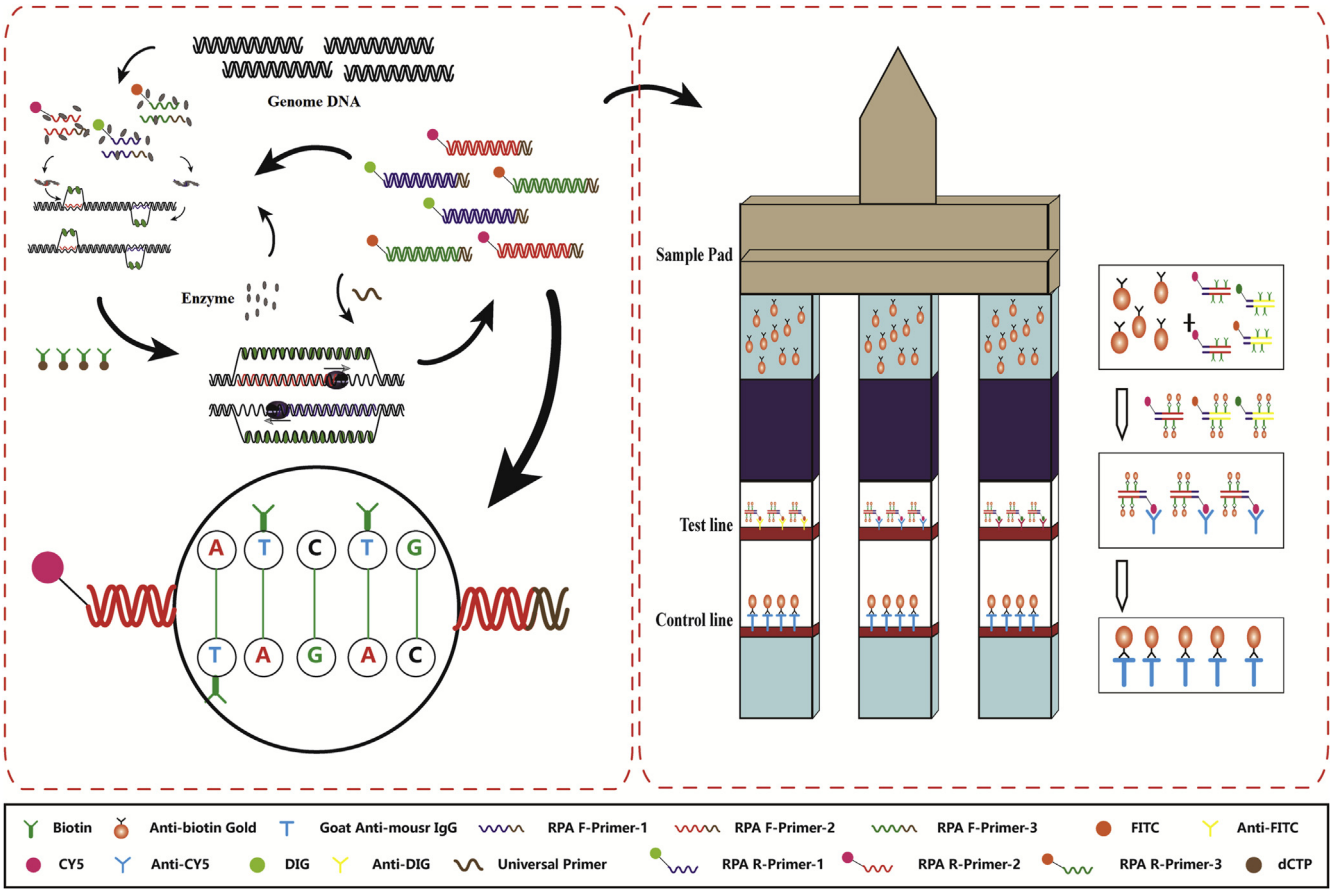
3.3. Characterization of AuNPs

The AuNPs were visualized by transmission electron microscopy (TEM) accurately measured to a mean diameter of approximately 20 nm (Fig. 2A). AuNPs-antibody conjugations were characterized by UV–vis analysis to determine the combination. The absorbance results showed that the maximum absorption peak of the AuNPs was at 520 nm. After modification with antibody, the absorption peak rose to 525 nm (Fig. 2B).

3.4. Optimization

3.4.1. Universal primer length

The RPA primer was 35–40 bp, according to the TwistAmp Basic kit manual, as excessive primers reduce the efficiency of amplification, especially in complex multiple reaction systems. Therefore, a single universal primer was used for the SUP-RPA instead of various high concentration primers to achieve multiple reactions. In addition, the length of the universal primer was a critical factor in amplification. Long primers cause the formation of dimers and short primers decrease the specificity of amplification and, therefore, different universal primers were designed, of between 10 nt and 25 nt, to test the optimum length. All the results were observed using 2% agarose gel electrophoresis. As shown Fig. 3A, the short universal primers (10 and 15 bp) kept amplifying non-specific product at approximately 100 bp (Fig. 3A, lanes 1 to 4). When the lengths were increased to 20 and 25 bp, this situation improved and the non-special band almost disappeared (Fig. 3A, lanes 5 to 8). Long universal primers enhanced the specificity of the RPA reaction. Thus, the universal primer length of 25 bp was considered



Scheme 1. Schematic of the design of the event-specific SUP-RPA and lateral flow biosensor.

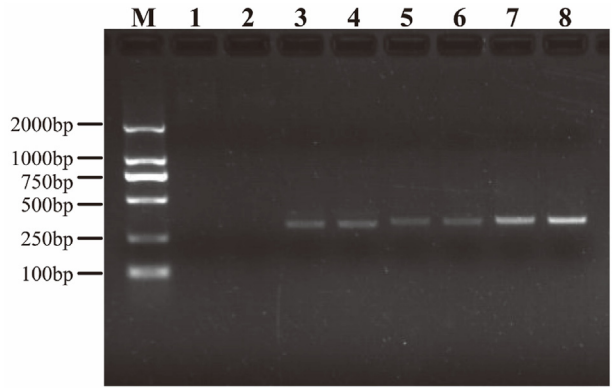


Fig. 1. Event-specific SUP-RPA agarose gel electrophoresis results: M, 100bp DNA ladder; lanes 1 and 2, two duplicates of negative control; lanes 3 and 4, two duplicates of MON810; lanes 5 and 6, two duplicates of MON863; lanes 7 and 8, two duplicates of MON89034.

optimal for detection in complicated multiple reaction systems.

3.4.2. Temperature of SUP-RPA reaction

The effect of temperature was tested on the SUP-RPA system. The optimum temperature for RPA proteins, according to the TwistAmp Basic kit instruction manual, is 37–42 °C, however, based on the results of our experiment with regular RPA progress, dimers are always accompanied with target sequence amplification in regular RPA progress. Ultimately, the gel electrophoresis was

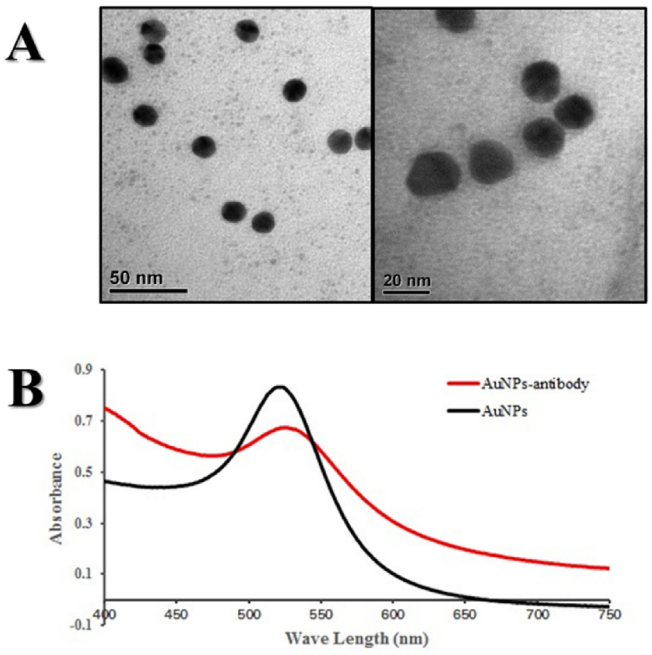


Fig. 2. Characterization of AuNPs. A, Transmission electron microscopy (TEM) images of AuNPs, the scale are 50 nm and 20 nm; B, Absorption spectrum of AuNPs (black) and AuNPs-antibody (red line). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

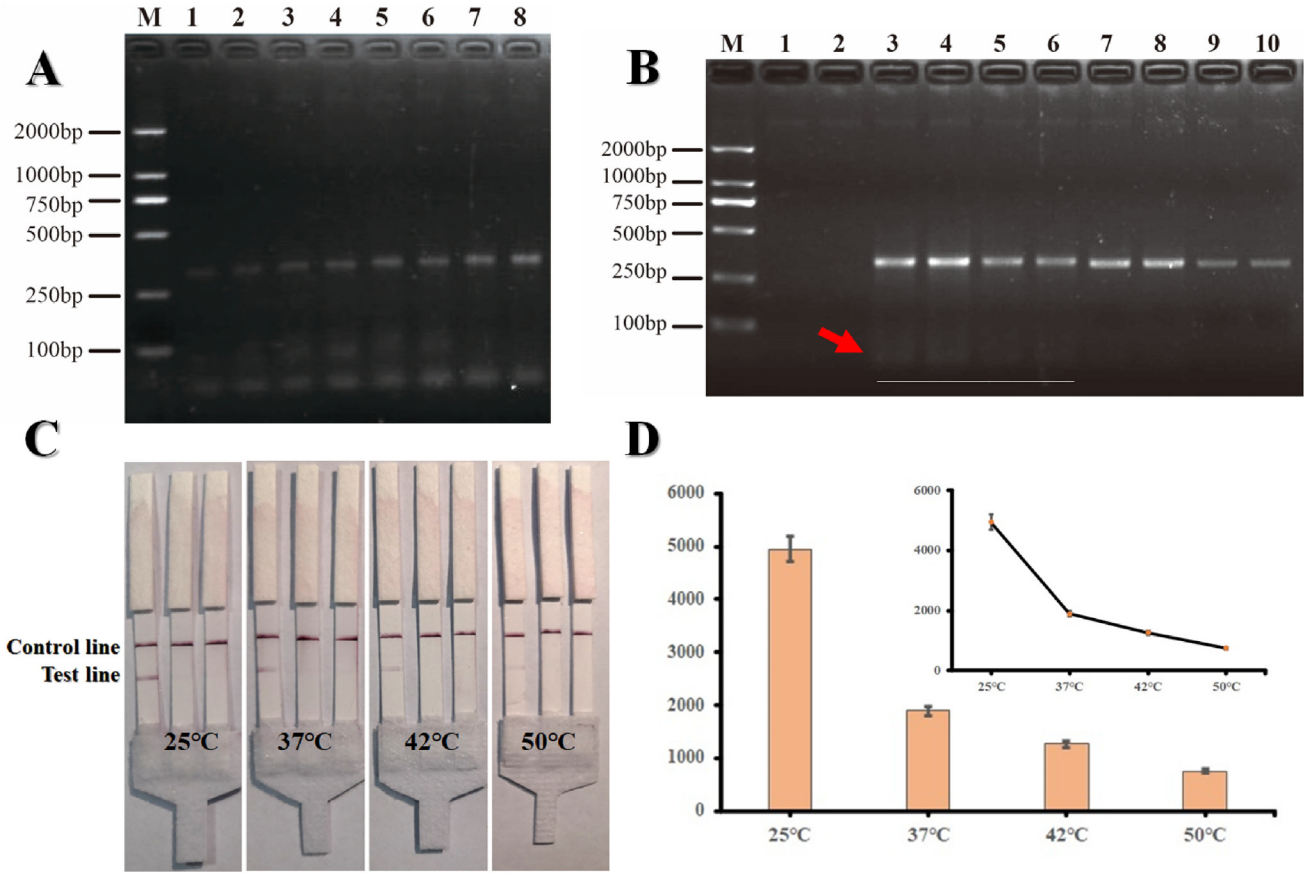


Fig. 3. Optimization of universal primers and temperature. **A**, The gel electrophoretogram for different universal primer lengths: M, D2000 DNA marker; lanes 1 and 2, two duplicates of 10 bp universal primer; lanes 3 and 4, two duplicates of 15 bp universal primer; lanes 5 and 6, two duplicates of 20 bp universal primer; lanes 7 and 8, two duplicates of 25 bp universal primer. **B**, The electrophoretogram for different reaction temperatures: M, D2000 DNA marker; lanes 1 and 2, two duplicates of negative control; lanes 3 and 4, two duplicates under 37 °C; lanes 5 and 6, two duplicates under 39 °C; lanes 7 and 8, two duplicates under 42 °C; lanes 9 and 10, two duplicates under 45 °C. **C**, temperature optimization of LFB reaction using MON810 sample at 25 °C,27 °C,42 °C,50 °C. **D**, grayscale measurement of LFB results at different temperature.

brighter at the bottom of the tunnel, which influenced the accuracy of the results. The formation of dimers was caused by the excessive length of the primers, but reducing the length decreased the specificity of the RPA reaction. Increasing the temperature also simultaneously reduced the dimers and increased the specificity. The performance of the SUP-RPA was tested by 2% gel electrophoresis in a temperature range of 37–45 °C. Between 37 and 39 °C, the SUP-RPA reaction still produced dimers, until the reaction temperature reached 42 °C. Because the high temperature decreased the contact probability of primers, the results showed a significant reduction in dimers (Fig. 3B). However, higher temperature (45 °C) would exceed the optimum temperature range (37–42 °C) and reduce the efficiency of the enzymes and, therefore, an optimal efficiency in vitro SUP-RPA reaction was selected of around 42 °C.

3.4.3. Temperature of LFB reaction

To further reduce the effects of primer dimers, the temperature of LFB reaction was optimized. SUP-RPA reaction with all primers only was performed and the product were incubated in thermostatic heater at different temperature range of 25–50 °C, then LFB was used to identify the results. At room temperature (25 °C), primer dimers caused the slight accumulation of AuNPs at test line. However, at higher temperature, this phenomenon was significantly reduced (Fig. 3C). Color degree was also measured using software ImageJ (Fig. 3D). But high temperature would reduce the binding efficiency between Antibodies, therefore the best temperature of LFB reaction was used 42 °C.

3.5. Performance of SUP-RPA-LFB method

The effective performance of the SUP-RPA-LFB system, including sensitivity and stability, was tested with different kinds of GMO events. The sensitivity test of SUP-RPA-LFB was performed using serial dilutions of each GMO genomic DNA (copy numbers arranged from 1000 to 10). As shown in Fig. 4, as the concentration of the template decreased, the number of amplification products with biotin-labeled dCTP were reduced until, eventually, the red band in the test line faded. MON810, MON89034 and MON863 genomic DNA were stably detected at 50 copies. When the number of copies had reduced to 10, a light red band appeared on the test line. Thus, the limit of detection (LOD) of MON810, MON89034 and MON863 was 50 copies (Fig. 4A, B, C). The stability of LFB was tested at different time periods of three days, one week, one month, six months and one year. The result showed that this LFB remained stable for at least one year (Fig. S3).

3.6. Verification of the feasibility of SUP-RPA-LFB in multiple detection

To exam the specificity of the SUP-RPA-LFB system, a series of templates with mixed DNA samples was used, including GM maize, rice, soybean and non-GMO crops. As shown in Fig. 5, the later fluid biosensor consisted of three pads representing MON810, MON863 and MON89034, respectively. With the presence of MON810, MON863 or MON89034 in the genome template in the solution, the

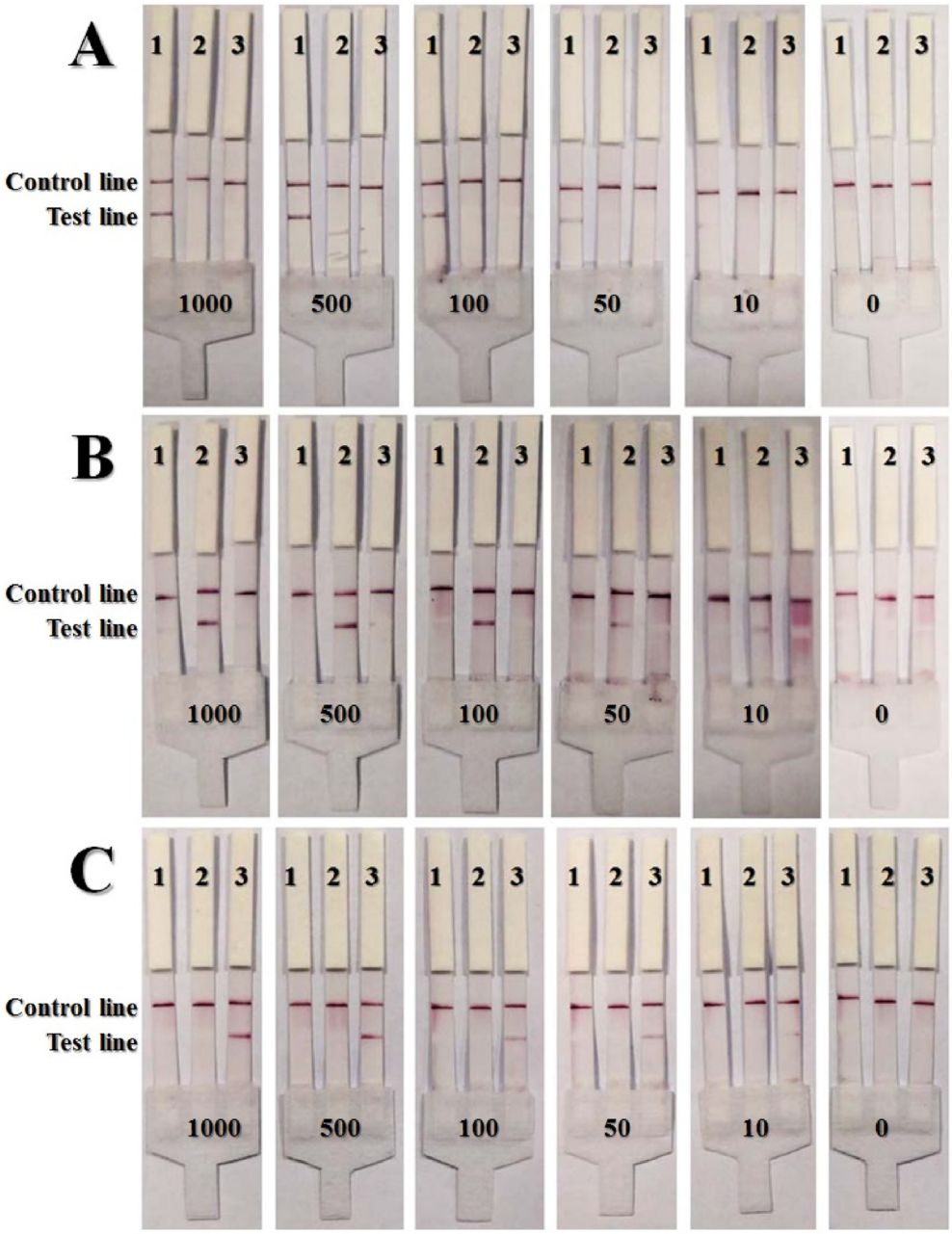


Fig. 4. Sensitivity of SUP-RPA-LFB was tested with serial copies of MON810, MON863 and MON89034: A, template of 1000, 500, 100, 50, 10 and 0 copies of MON810 genomic DNA; B, template of 1000, 500, 100, 50, 10 and 0 copies of MON8630 genomic DNA; C, template of 1000, 500, 100, 50, 10 and 0 copies of MON89034 genomic DNA.

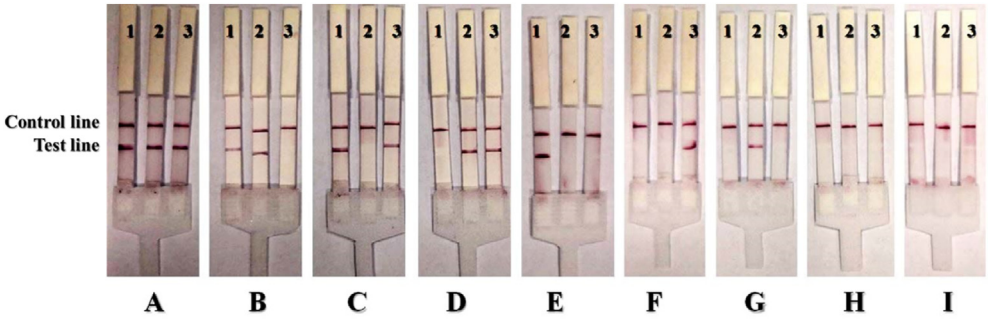


Fig. 5. Specificity of SUP-RPA-LFB was tested with different events: A, mix DNA of MON810, MON63 and MON89034; B, mix DNA of MON810, MON863 and Bt176; C, mix DNA of MON810, MON89034 and Bt11; D, mix DNA of MON863, MON89034 and TC1507; E, mix DNA of MON810, NK603 and GA21; F, mix DNA of MON89034, MIR162 and MIR604; G, mix DNA of MON863, GM rice TT51-1 and Kefeng 6; H, mix DNA of GM soybeans MON89788, MON87705 and MON87769; I, mix DNA of non-GM maize, rice and soybean.

LFB showed one positive signal result (Fig. 5E–G). Similarly, the presence of two targets showed two positive signals (Fig. 5B–D), and the presence of three targets were simultaneously detected too (Fig. 5A). GM soybeans, rice and non-GMO samples showed no color in the test line (Fig. 5H and I). The results demonstrated the high selectivity of this method for MON8106, MON863 and MON89034 detection against other GMO and non-GMO events.

4. Conclusions

The SUP-RPA-LFB assay developed herein provides an innovative multiplex GMO detection biosensor, combining the single universal primer recombinase polymerase amplification method with a lateral flow signal recognition technique. All reactions were performed in one system to achieve the amplification process and products were recognized simultaneously. The combination of RPA primer and single universal primer enabled the consistency of amplification efficiency and improved the level of sensitivity and simplicity. At the same time, the SUP-RPA primer changed the complex condition in the reaction system, providing an equal possibility of multiple targets. Through the modification of different kinds of groups (FITC, DIG and CY5) at 5' of the reverse primers, the SUP-RPA could be tested using an AuNP-based lateral flow strip. In the amplification process, a biotin labeled dCTP was used instead of normal dCTP as the target amplification material. As a result, amplification target sequences were labeled with rich biotin groups, which achieved the second signal increase which combined with the lateral flow strip. As expected, the different kinds of SUP-RPA amplification products contained FITC, DIG or CY5 at the end and biotin groups in the middle. AuNPs then accumulated by capillary action, providing a colorimetric signal.

The neoteric SUP-RPA-LF biosensor was shown to respond linearly over the GMO concentration range of 50–1000 copies and can be widely used to rapidly detect GMO. In conclusion, the SUP-RPA-LF biosensor developed herein provides the significant advantages of being highly sensitive, highly specific, cost-effective and time-saving, which makes it a promising and more practical technique for the multiple detection of GMO in POC environments.

CRediT authorship contribution statement

Kai Li: Methodology, Investigation, Writing - original draft, Visualization. **Yunbo Luo:** Resources, Supervision, Formal analysis. **Kunlun Huang:** Project administration, Resources, Visualization. **Zhansen Yang:** Investigation, Validation. **Yusong Wan:** Resources, Conceptualization, Writing - review & editing. **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.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2020.06.001.

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