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A highly integrated system with rapid DNA extraction, recombinase polymerase amplification, and lateral flow biosensor for on-site detection of genetically modified crops

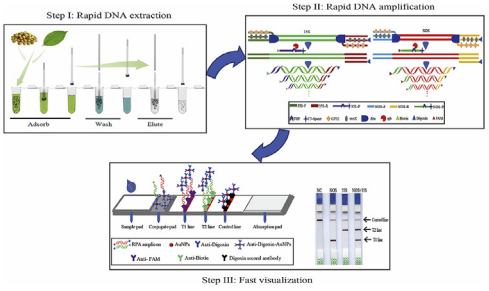
Xiaofu Wang [a, b], Yu Chen [a, d], Xiaoyun Chen [a], Cheng Peng [a, b], Liu Wang [a], Xiaoli Xu [a], Jian Wu [c], Wei Wei [a, b], Junfeng Xu [a, *]

[a] State Key Laboratory for Quality and Safety of Agro-products, Zhejiang Academy of Agricultural Sciences, Hangzhou, 310021, China
[b] Key Laboratory of Information Traceability for Agricultural Products, Ministry of Agriculture of China, Hangzhou, 310021, China
[c] College of Biosystems Engineering and Food Science, Zhejiang University, Hangzhou, 310058, China
[d] College of Chemistry and Life Science, Shenyang Normal University, Shenyang, 110034, China

HIGHLIGHTS

- A highly integrated system for on-site detection of GM crops was proposed.
- The system exhibited high selectivity and sensitivity.
- The entire detection process was accomplished in approximately 20–30 min.
- The system was used to detect field samples demonstrated the practicability for on-site detection.

GRAPHICAL ABSTRACT



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ABSTRACT

With the large-scale planting of genetically modified (GM) crops, the development of a rapid and convenient method for on-site monitoring GM crops is needed. In this study, a duplex recombinase polymerase amplification (DRPA)-based, quick and simple detection system is presented for on-site detection of GM crops. In this system, a rapid DNA extraction method, a DRPA, and a lateral flow biosensor (LFB) are integrated to allow for rapid DNA extraction and amplification, and fast visualization of the detection results. Using the rapid DNA extraction method, high-quality DNA suitable for RPA and polymerase chain reaction (PCR) was quickly isolated from various crops within 5 min. Utilizing the optimal DRPA assay, the universal screening sequences (*Cauliflower mosaic virus* 35S promoter [35S] and *Agrobacterium tumefaciens* NOS terminator [NOS]) were rapidly and simultaneously amplified with high selectivity and sensitivity. The sensitivity threshold of the DRPA assay was ~10 copies for GM soybean genomic DNA and 100 ng DNA of 0.1% GM soybean. In combination with the LFB in an enclosed cassette, the entire detection process was performed in approximately 20–30 min and eliminated the carryover contamination. No special or expensive equipment was needed for the detection process. The system was successfully applied and validated for on-site detection of GM rice, demonstrating its suitability for on-site testing of GM crops and high potential for application to other fields, especially in low-resource regions that require rapid detection of DNA targets.

* Corresponding author.
E-mail address: njjfxu@163.com (J. Xu).

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1. Introduction

In the past two decades, the cultivation area of genetically modified (GM) plants has rapidly expanded [1]. However, although many countries and regions have strengthened the management of GM and adopted mandatory GM labeling regulations [2], unauthorized GM plant releases have been reported frequently. Many detection methods, including polymerase chain reaction (PCR), DNA or protein chips, Western blotting, and enzyme-linked immunosorbent assay (ELISA), have been developed and applied for GM crops detection [3–7]. Most of these detection methods require special instruments or complicated and time-consuming procedures, making them unsuitable for on-site detection of GM crops.

The protein strip test (PST) is suitable for on-site GM crop detection [8]. This method is simple and fast, requiring only simple protein extraction and then placing the protein strip in the protein extract, and provides specific and sensitive results on the basis of immunolabeling antigens with antibodies. Protein-based detection methods, however, require specific antibodies and the process to develop new antibodies is complicated and time-consuming [3]. In addition, protein-based detection methods have been created to detect exogenous proteins expressed by GM crops, and the targeted exogenous proteins are limited [9]. At present, only a few protein test strips are available, e.g., CP4-EPSPS, Cry1Ac, Cry1Ab, NPTII, and Hpt. Furthermore, the methods based on protein detection cannot detect DNA elements, such as the *Cauliflower mosaic virus* 35S promoter (35S), *Agrobacterium tumefaciens* nopaline oxidase synthase terminator (NOS), and event-specific sequences.

DNA, with its high stability and well-established manipulation methods, is a popular candidate target for GM detection [10,11]. While PCR is the primary method for detecting specific DNA in GM crops [12,13], it is not suitable for on-site detection due to the two melting steps, and the requirement for large and expensive equipment. To meet the requirement of on-site detection based on DNA, the method should have a fast, sensitive, and specific DNA identification process. Usually, the identification process consists of DNA amplification and detection of the amplified products. The emergence of isothermal nucleic acid amplification (INAA) methods has high potential applicability for on-site detection. Various INAA methods have been reported, such as loop-meditated isothermal amplification (LAMP) [14], strand displacement amplification [15], cross-priming amplification [16], rolling circle amplification [17], and recombinase polymerase amplification (RPA) [18]. These methods do not require large-scale equipment for their reactions, which are performed at a constant temperature and overcome the limitations of PCR thermo cyclers. This feature endows INAA methods with a wide range of possible applications, including testing in resource-limited settings, on-site testing, and testing of environmental samples [19].

Among these INAA methods, RPA is a novel, rapid, and highly specific isothermal system that simulates DNA replication in vivo [18]. Initial denaturation is not required for the template DNA and exponential amplification can be accomplished within a short time under a wide range of temperatures. RPA has great potential for on-site detection, but some limitations remain. First, identification of the products amplified by RPA is traditionally performed using agarose gel electrophoresis, which is time-consuming and has high risk of carryover contamination. Some new DNA detection methods have been presented, such as fluorescent labeling [20,21], novel materials [22], and micro-fluidic devices [23], but these methods require special or expensive instruments. On the other hand, although some INAA methods for detection of GM crops have been reported [24,25], it is difficult to omit the DNA extraction and purification steps for on-site detection. RPA and other INAA methods are more tolerant to some inhibitors of conventional PCR amplification [26], crude cell lysates can be used as the template to be amplified by RPA or other INAA methods [21,27]. Although these methods are simple and fast, they are not stable.

In the present study, we developed a simple and rapid DNA-based system for on-site detection of GM crops. 35S and NOS were the most common elements found in the molecular composition of GM crops [28]. According to a previous report the two elements could be contained in 88.3% of GM crops [29]. The universal screening sequences of 35S and NOS were selected as the detection targets. For rapid and simultaneous amplification of the two targets, duplex RPA (DRPA) of 35S and NOS was established. For rapid identification of the amplified products, we developed a novel nucleic acid lateral flow biosensor (LFB). This system is an innovative combination of DRPA and LFB (DRPA-LFB). To shorten the DNA extraction time, a novel and fast DNA extraction method was also designed. The three major steps of this system are shown in Scheme 1. Taking advantage of these technologies, the proposed DRPA-LFB system allows for fast and simple on-site detection with high sensitivity and selectivity. This system requires no specific instruments, and here we report the applicability of this system for on-site detection of GM crops.

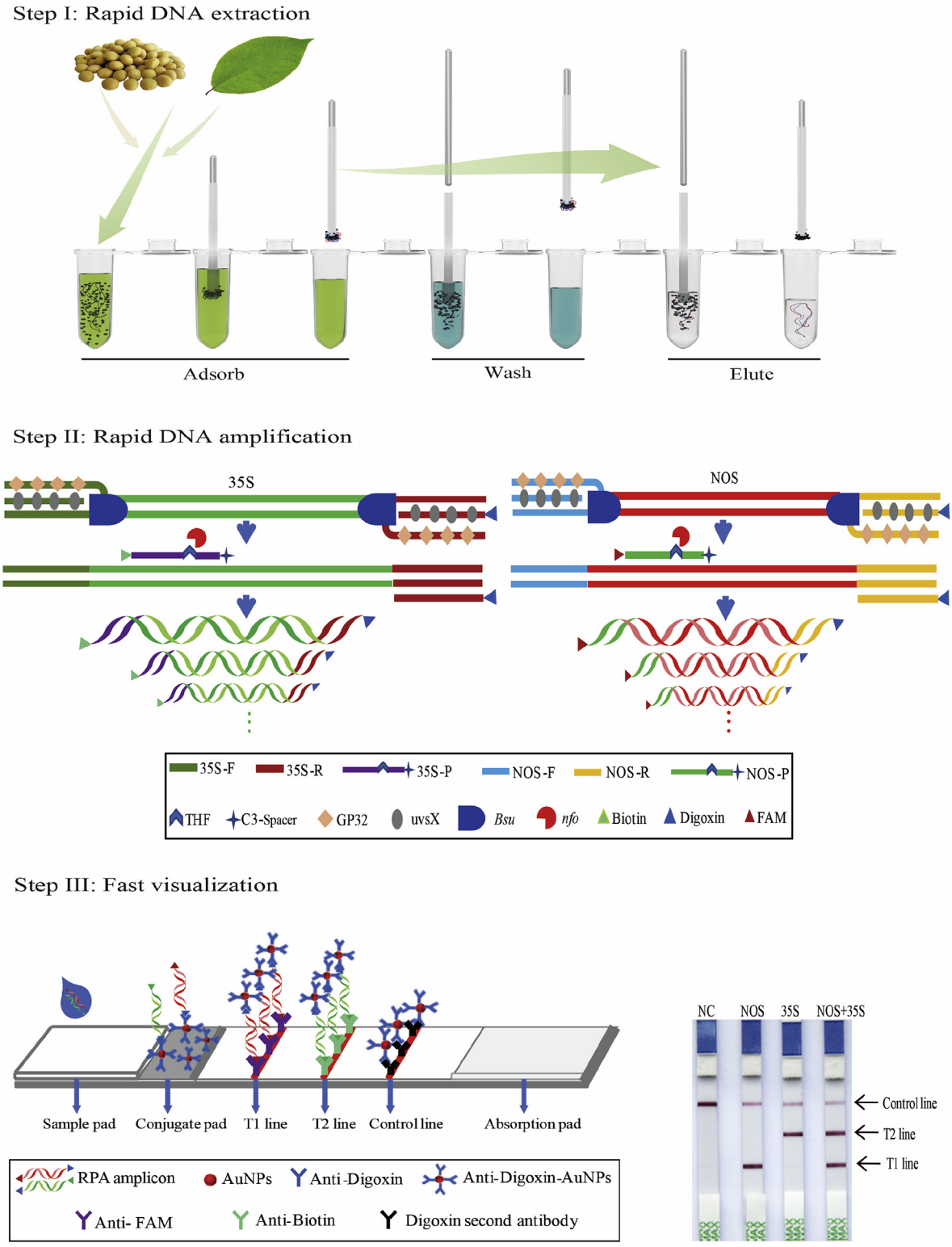
2. Materials and methods

2.1. Plant materials

Non-transgenic seeds and fresh leaves of maize, soybean, canola, rice, and cotton were collected and the absence of transgenic elements was confirmed by our lab. GM soybean, GTS-40-3-2, containing both 35S and NOS elements, was purchased from Shenzhen Excellent Biotech Co., Ltd. (Shenzhen, China). Seeds of GM rice TT51-1 and KMD1 were provided by Zhejiang University (Hangzhou, China), and the two GM rice strains were grown in the greenhouse as samples to verify the feasibility of DRPA-LFB for on-site testing.

2.2. DNA extraction using a novel magnetic bead method

The novel magnetic bead method was based on the Wizard Magnetic DNA Purification System for Food (WDPS, Promega, Madison, WI, USA). To quickly collect and separate the magnetic beads from the extraction buffer or wash solution, a simple and small tool was devised consisting of a bar magnet and a plastic cover. The bar magnet in the plastic cover adsorbs the magnetic beads, and the magnetic beads are released from the plastic cover when the bar magnet is removed. The outline of the modified DNA extraction method based on the WDPS is shown in Scheme 1 step I and Fig. 1A. For on-site detection, approximately 100 mg of fresh leaves from test plants were placed into a 1.5-mL tube. Then, 500 μL of lysis buffer A and 5 μL of RNase A was added to the tube and the leaves were homogenized using a disposable plastic pestle. After standing at room temperature for 1 min, the supernatant was directly transferred to a new 2-mL tube through a layer of gauze (pore size, ~1 mm). Next, 250 μL of lysis buffer B was added and the mixture was shaken for 10–15 s until completely mixed. Precipitation Solution (750 μL) was added and the mixture shaken vigorously for 10–15 s. The magnetic bead solution (50 μL) provided by the WDPS kit was added to the tube, and the tube was shaken vigorously by hand for 5 s. Isopropanol (800 μL) was added to the tube, and the mixture was inverted 10–15 times. The bar magnet/plastic cover tool was used to adsorb and transfer the magnetic beads to another tube containing 250 μL lysis buffer B. The magnetic beads were released from the plastic cover then the bar magnet was pulled away from the plastic cover. The tube was then gently shaken for 5 s to wash the magnetic



Scheme 1. Schematic illustration of the three major steps in the integrated system. Step I: Rapid DNA extraction using a novel magnetic bead method. Step II: Rapid DNA amplification based on DRPA using primers and probes labeled with biotin, digoxin, and FAM. Step III: Fast visualization of the DRPA products using LFB.

beads. The magnetic bead washing procedure was repeated three times with 1 mL of 70% ethanol. Finally, the magnetic beads were washed with 100 μL TE buffer to remove the DNA, collected, and removed using the bar magnet/plastic cover tool, and the genomic DNA was suspended in the TE buffer. For seed samples, the seeds were ground to a powder and approximately 100 mg seed powder was transferred to a 2-mL tube. The seed powder was then processed as described above. Simultaneously, the WDPS was used to extract duplicate samples to compare the performance of the novel DNA extraction method with that of the WDPS.

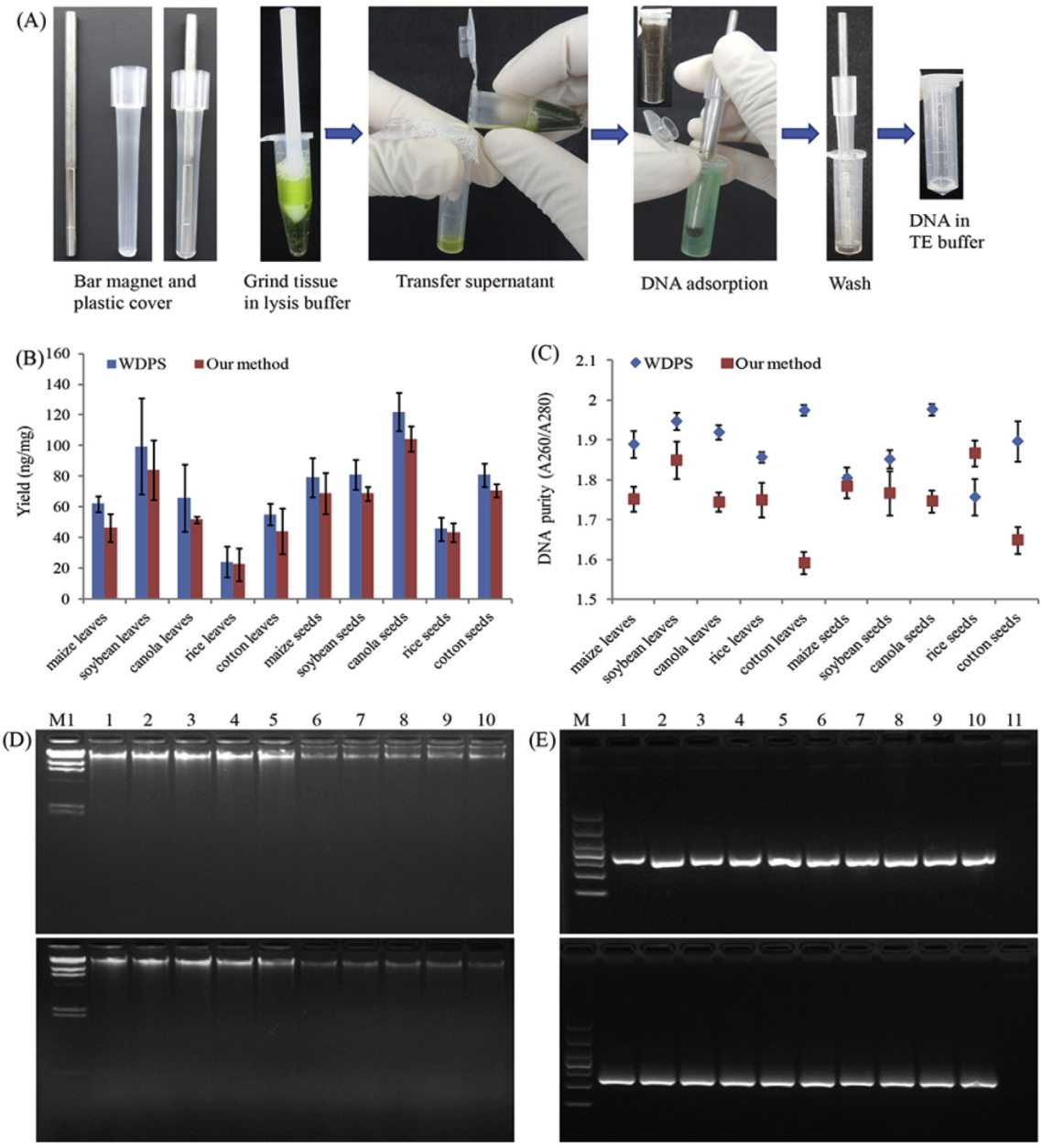


Fig. 1. DNA extraction and evaluation. (A) An outline of our DNA extraction method using a bar magnet and plastic cover. (B) Comparison of DNA yields of the WDPS method and our method. (C) Comparison of DNA purity of the WDPS method and our method. (D) Agarose gel electrophoresis of extracted genomic DNA from the leaves and seeds of five crops using the WDPS method and our method. Top panel shows the result of the WDPS method. Bottom panel shows the result of our method. (E) Conventional PCR and basic RPA amplification of 18S rRNA gene from all extracts by our method. Top panel is agarose gel showing the results of conventional PCR. Bottom panel is agarose gel showing the results of basic RPA. Lane M1:λ DNA/HindIII + EcoRI marker. Lane M: DL1000 DNA marker. Lane 1–10: maize leaf, soybean leaf, canola leaf, rice leaf, cotton leaf, maize seed, soybean seed, canola seed, rice seed, cotton seed. Lane 11: No template control.

The concentration and purification of all extracts were evaluated using a NanoDrop™ 2000c Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). DNA for the sensitivity detection was quantified using the Qubit dsDNA BR Assay Kit (Invitrogen, Shanghai China). The quality and integrity of the extracted DNA was analyzed by gel agarose electrophoresis.

2.3. Primers and probes

For the RPA assay of 35S and NOS, RPA primers and probes were designed according to the sequence information from the European Union Reference Laboratory. Conventional PCR primers for 35S and NOS were synthesized according to previous reports [30,31]. Conventional PCR primers and RPA primers for the 18S ribosomal RNA gene (18S rRNA) were designed according to the homologous sequence of 18S rRNA of maize (NO.AF168884.1), soybean (No. X02623.1), canola (No.KT225363.1), rice (No.LC086814.1), and cotton (No.L24145.1). The homologous sequences and location of the primers for PCR and RPA are shown in Fig. S1. All primers and probes were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). Detailed information on all primers and probes is provided in Table S1.

2.4. Conventional PCR reactions

Conventional PCR (25 μL volume) was performed with 10 × PCR buffer, 0.4 μM of each primer, 0.2 mM of each dNTP, 1.25 U Taq DNA polymerase (TaKaRa Biotechnology Co., Ltd, Dalian, China), and 2 μL DNA template. The PCR program included an initial denaturation step (95 °C for 5 min); followed by 35 cycles at 95 °C for 30 s, 58 °C for 30 s, and 72 °C for 30 s; with a final elongation step of 72 °C for 7 min. The results of the conventional PCR were analyzed through agarose gel electrophoresis.

2.5. Preparation of the lateral flow biosensor

The LFB contained three reaction lines: one control and two targets. The details of the LFB, comprising a sample pad, conjugation pad, three test lines, and an adsorption pad, is shown in Scheme 1 step III. Gold nanoparticles (AuNPs) coated with anti-digoxin antibody were sprayed onto the conjugation pad. Anti-FAM and anti-biotin antibodies were immobilized to test lines 1 and 2, respectively. The control line contained immobilized digoxin secondary antibody. For final visual detection, 20 μL of the DRPA amplification products and 80 μL of running buffer (10 mM PBS, pH 7.5 with 1% Tween 20) were mixed and directly added to the sample pad.

2.6. Establishment of DRPA-LFB

RPA reactions were performed according to the manufacturer's instructions (TwistAmp nfo kit/basic kit, TwistDx, Cambridge, UK). Briefly, all reactions contained 29.5 μL rehydration buffer, 2 μL DNA template, 2.5 μL magnesium acetate, 1 lyophilized enzyme pellet, and primers with or without probe depending on the different experiments, and up to 50 μL nuclease-free water. Reactions were performed at 39 °C for 20 min.

To achieve the best results, the concentrations of the primers and probes in the DRPA system were optimized. We performed the DRPA for 35S and NOS with various concentrations of primers and probes at 39 °C, the recommended temperature. Six different combinations of concentrations (I–VI) of primers and probes were evaluated, and detailed information is provided in Fig. 2A. To select the optimal temperature for the DRPA, the DRPA with the optimal concentration of primers and probes was performed at different temperatures (30, 32, 34, 36, 38, 40, 44, 48, and 50 °C). For system optimization experiments, the genomic DNA of soybean GST40-3-2 (500 copies) was used as a template, and the amplification results were detected using the LFB, agarose gel electrophoresis, or fluorescent signal to assess the feasibility of the optimized system.

2.7. Sensitivity and selectivity of DRPA-LFB

Soybean GTS40-3-2, containing both 35S and NOS, was used to determine the limits of detection (LOD) of the DRPA-LFB system. A dilution series of genomic DNAs of GTS40-3-2 was prepared at concentrations of 10, 50, 100, 200, and 500 copies. Another series of various proportions (1%, 0.5%, and 0.1%) of GTS 40-3-2 samples was also prepared.

Thirty-five samples (12 GM maize samples, 5 GM soybean samples, 7 GM canola samples, 8 GM cotton samples, 1 non-GMO-maize sample, 1 non-GMO soybean sample, and 1 non-GMO canola sample) were used to test the selectivity of the DRPA-LFB. In addition, all the samples were also tested by conventional PCR using 35S and NOS primers. The DNA of the Thirty-five samples and the rice samples for on-site testing was extracted only by the novel magnetic bead method, the extracted results of DNA yields and purity of these samples were provided in Table S3.

3. Results and discussion

3.1. Evaluation of the novel magnetic bead method for DNA extraction

The magnetic bead method of extracting DNA is quick and convenient, and widely used in many areas. This method works by coating magnetic beads with a substance having high affinity for DNA. DNA in solution is mixed with the magnetic beads, then a magnetic field is applied to the tube to immobilize the beads and the other contaminating factors are washed away. Changing the salt concentration releases the DNA from the magnetic beads to complete the purification process. The Wizard Magnetic DNA Purification System for Food (WDPS, Promega, Madison, WI, USA) is commonly used for extracting DNA from plant materials and food. The extraction system requires a centrifuge and a special magnetic device to absorb the magnetic beads, but the entire extraction process takes up to 1 h according to the manufacturer's protocol, making it unsuitable for on-site testing. To overcome these limitations, we modified the magnetic bead DNA extraction method to meet the requirement for on-site DNA extraction.

To evaluate the feasibility of this novel method for rapid DNA extraction, leaves and seeds from five crops (maize, soybean, canola, rice, and cotton) were used for DNA extraction by this method and the WDPS. The results for each method are provided in Table S2. The mean DNA yield of all the samples ranged between 22 and 104 ng/mg using our method, and between 24 and 122 ng/mg using the WDPS (Fig. 1B). The OD A260/280 ratios of DNA extracted from leaves and seeds of the five crops ranged from 1.59 to 1.87 using our method, and from 1.75 to 1.98 using the WDPS method (Fig. 1C). Both the yield and purity of the DNA extracted by our method were generally lower than those extracted using the WDPS method, indicating that the rapid DNA extraction sacrifices DNA yield and purity. This decrease is likely due to the omission of the centrifugation, vortexing, incubation and long washing steps that are included in the WDPS method. The vortexing and incubation steps are highly related to the extracted DNA yield, because they increase the interaction between the magnetic beads and DNA and result in more DNA being absorbed. The centrifugation and washing steps were used to remove other metabolites and inhibitors, such as protein, polysaccharides, phenol, and salts, to obtain high purity DNA. The quality of the DNA was checked by agarose gel electrophoresis with 50 ng of extracted DNA and was similar between the two methods (Fig. 1D). Further, to verify whether the DNA extracted by our method was applicable for DNA amplification, an endogenous plant gene, the 18S rRNA gene, was amplified by conventional PCR and RPA amplification using DNA extracted by our method as a template. DNA amplicons of the expected size of the 18S rRNA gene were observed for both the PCR and RPA assays (Fig. 1E). These results indicated that, although the yield and purity of the DNA extracted using our method was not as high as when using the WDPS method, they were sufficient for DNA amplification using RPA.

For on-site detection of GM crops using DNA-based methods, DNA extraction is the first hurdle. Traditional and common methods for extracting DNA from plants, such as the cetyltrimethylammonium bromide (CTAB) method, the sodium dodecyl sulfate (SDS) method, and commercial kits, are complicated and time-consuming and therefore unsuitable for on-site detection. Some investigators tried to rapidly extract DNA by treating the plant tissue with a simple cell lysate treatment [21,27]. However, because they contain more secondary metabolites, such as polysaccharides and phenol compounds, that are not eliminated and affect the DNA amplification [13,32]. In this study, we took advantage of the characteristics of magnetic beads for DNA

(A)

Combination	Concentration (nM)			
	35S primers	35S probe	NOS primers	NOS probe
I	200	60	200	60
II	300	80	300	80
III	400	100	400	100
IV	500	120	500	120
V	600	140	600	140
VI	700	160	700	160

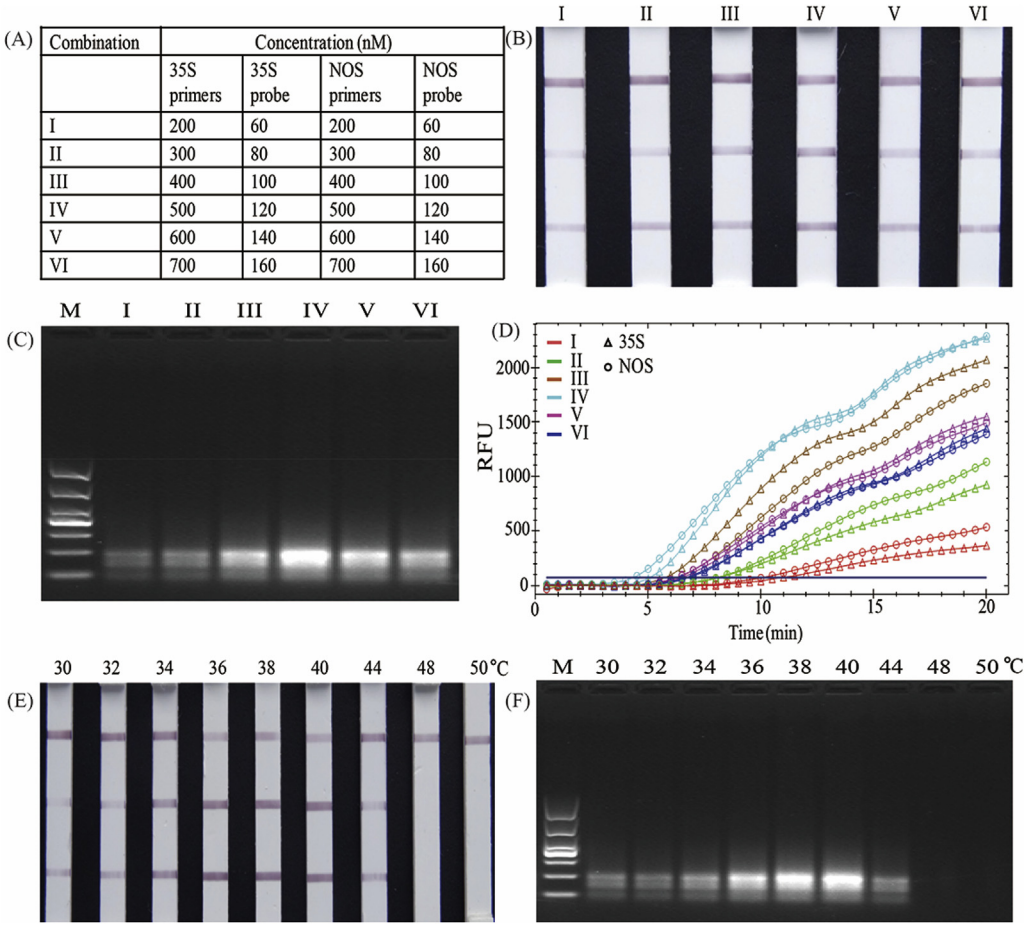


Fig. 2. Optimization of the DRPA-LFB system. (A) Various combinations of primer and probe concentrations (I-VI) for DRPA-LFB. (B) The LFB detection results of the DRPA with combinations I-VI. (C) Amplification results of DRPA with combinations I-VI on an agarose gel. (D) The fluorescence signal of DRPA with combinations I-VI. (E) The LFB results of DRPA at various temperatures. (F) The DRPA amplification products on an agarose gel at various temperatures.

extraction, using only a bar magnet and a plastic cover to accomplish the entire DNA extraction process within 5 min. Despite the decreased DNA yield and purity compared with that obtained using a commercial kit, the amplification of the DNA in PCR and RPA was not affected. The proposed method is simple, rapid, and economical, showing good applicability for rapid on-site DNA extraction, especially for on-site detection of robust materials such as plant samples. In addition, the bar magnet and plastic cover used in this method are commonly available, making it easy to apply this method for on-site detection, especially in low-resource regions.

3.2. Design of the RPA-LFB detection system

In RPA, T4 *Uvs*X recombinase and primers form a complex that scans double-stranded DNA, and strand exchange is facilitated at cognate sites. The resulting structures are stabilized by GP 32, a single-stranded DNA-binding protein. The primer is then extended along another single-stranded DNA by *Bsu* polymerase. This process is repeated until exponential amplification is accomplished. The schematic of RPA amplification is presented in Fig. S2A. In our DRPA system, to be compatible with the following LFB detection, digoxin was labeled at the 5′ end of the reverse primer for the 35S and NOS targets. To obtain highly specific amplification products, a special probe (Scheme 1 step II and Fig. S2B) was employed for each target. The probe comprised an antigenic label at the 5′ end, biotin for 35S, and FAM for NOS; a nucleotide was replaced with tetrahydrofuran (THF); and a C3-spacer at the 3′ end. The THF residue was cleaved with endonuclease *nfo* only when the probe was bound to its target. With the cutting step, the probe was transformed into a new primer for polymerase extension. Along with the amplification of DRPA, the amplicons produced by the processed probe and the 5′-labeled reverse primer effectively incorporated two antigenic residues into the amplified RPA. For the 35S target, biotin and digoxin were at each end of the RPA amplicons; for the NOS target, FAM and digoxin were at each end of the RPA amplicons (Scheme 1 step II). Thus, the 35S and NOS amplicons containing specific antigenic residues could be detected by the LFB.

The principle for the visualization of the DRPA products in the LFB is presented in Scheme 1 step III. The double-labeled DRPA amplicons first combined with AuNPs that were coated with anti-digoxin. With the migration of the reagents, the AuNPs carrying the NOS amplicons are specifically captured by the anti-FAM antibody immobilized in test line 1 (T1), and the AuNPs carrying 35S amplicons are specifically captured by the anti-biotin antibody immobilized in test line 2 (T2). Excess AuNP conjugates are captured by the digoxin secondary antibody immobilized in the control line. The AuNPs accumulate in each line through the interaction of each antigen and antibody, resulting in a red line at the corresponding analysis line. The four possible test results are shown in Scheme 1 step III. Three red lines appearing in each of the analysis lines, T1, T2, and control line, indicate the tested sample contained both 35S and NOS. Two red lines appearing in the T2 line

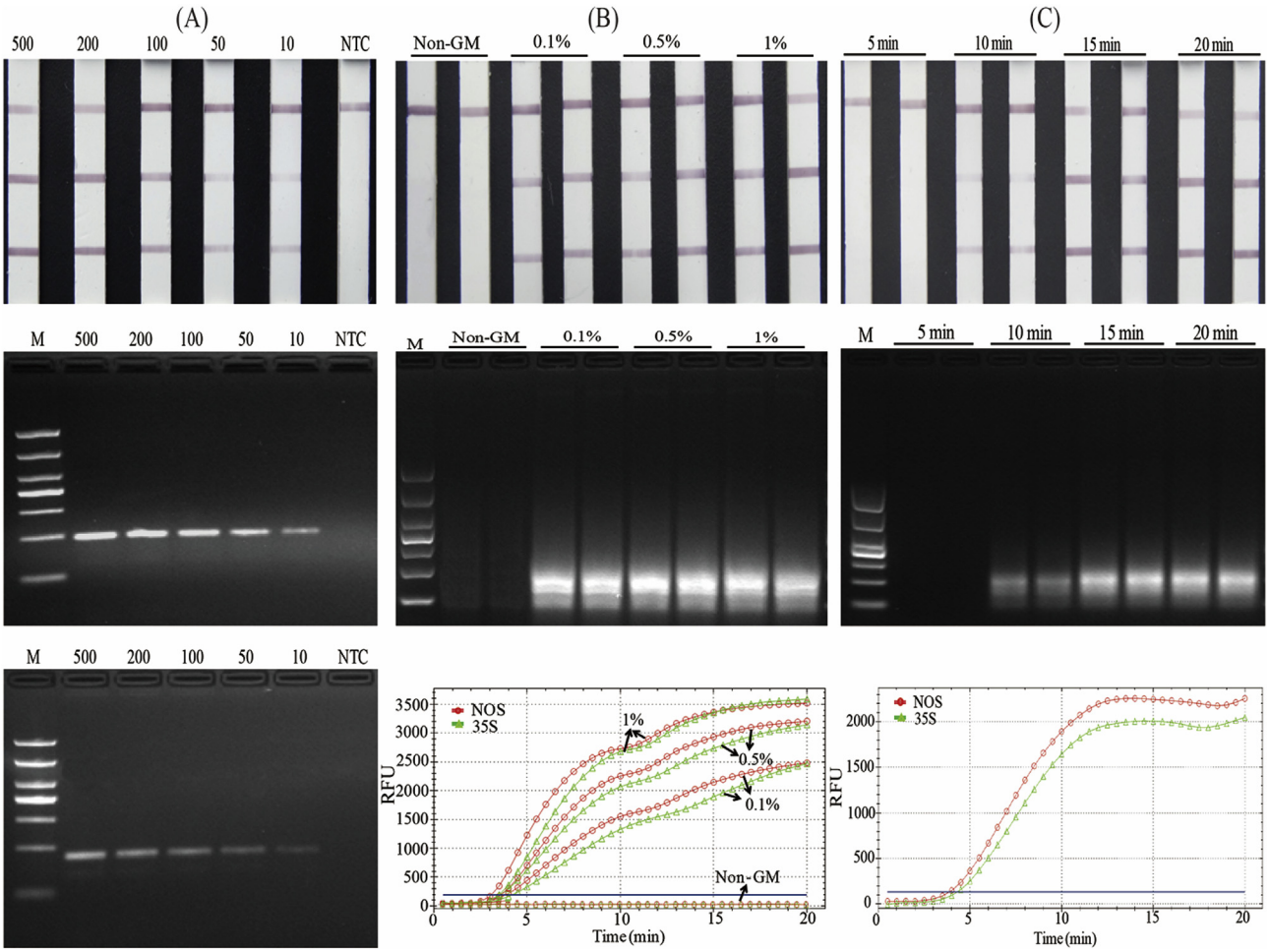


Fig. 3. Sensitivity of the 35S and NOS assays. (A) Sensitivity performance of DRPA-LFB and conventional PCR with different amounts of genomic DNA. Top is DRPA-LFB results. Middle panel shows the sensitivity of 35S using conventional PCR. Bottom panel shows the sensitivity of NOS using conventional PCR. (B) Sensitivity performance of DRPA-LFB with 100 ng genomic DNA containing 1%, 0.5%, or 0.1% GST 40-3-2 as the template. Top panel shows the DRPA-LFB results. Middle panel shows DRPA amplification products on an agarose gel. Bottom panel shows the fluorescence signal of the DRPA products. (C) DRPA results using 100 ng genomic DNA of 1% GST 40-3-2 as the template amplified for the indicated periods of time. Top panel shows the DRPA-LFB results. Middle panel shows DRPA amplification products on agarose gel. Bottom panel shows the fluorescence signal of the DRPA products.

and the control line indicate the tested sample contained only 35S, or two red lines appearing in the T1 line and the control line indicate the tested sample contained only NOS. Only one red line appearing in the control line indicates that the tested sample did not contain 35S or NOS. In all test results, for the test to be valid, the control line must appear.

3.3. Optimization of the RPA-LFB method

The concentrations of the primers and probes play a critical role in the efficiency of the RPA reaction, especially in multiplexed RPA [33,34]. A total of six combinations (I-VI) of various final concentrations of primer/probe for 35S and NOS were tested (Fig. 2A). The LFB results indicated that both 35S and NOS were amplified in all six combinations, although the intensity of the red color in the three detection lines differed with each combination (Fig. 2B). The best LFB result was obtained from combination IV, which exhibited equal and high signal intensity for the T1 and T2 lines. The products of the six combinations were also analyzed by agarose gel electrophoresis (Fig. 2C). The results indicated that DNA was amplified in all six combinations. Theoretically, there should be four amplified bands in the DRPA (191 bp, 169 bp, 141 bp, and 92 bp), but it was difficult to resolve the amplification products with such a small difference in length. To further confirm the optimal concentrations of primers and probes, a fluorescent probe having the same structure as the DRPA-LFB probe was used, except that a fluorophore and a quencher were inserted on each side of the THF. The THF residue was cleaved by endonuclease *nfo*, thus separating the fluorophore and quencher and generating a fluorescent signal (Fig. S2C). The probes for 35S and NOS were labeled with different fluorophores so the amplification of the two targets in the DRPA could be monitored simultaneously. As the concentrations of the primers and probes increased (combinations I to IV), the fluorescence intensity of 35S and NOS increased (Fig. 2D). At higher concentrations (V and VI), the fluorescence intensity of 35S and NOS decreased. According to these results, the highest amplification fluorescence of 35S and NOS was obtained with combination IV, and thus 500 nM primers and 120 nM probes of 35S and NOS were used for the subsequent DRPA-LFB experiments.

To determine the optimal temperature, we performed the DRPA reactions at different temperatures with the optimal concentrations of primers and probes. In the LFB, reaction temperatures ranging from 30 to 44 °C produced all three detection lines, with 36–40 °C producing the greatest intensity of red in the T1 and T2

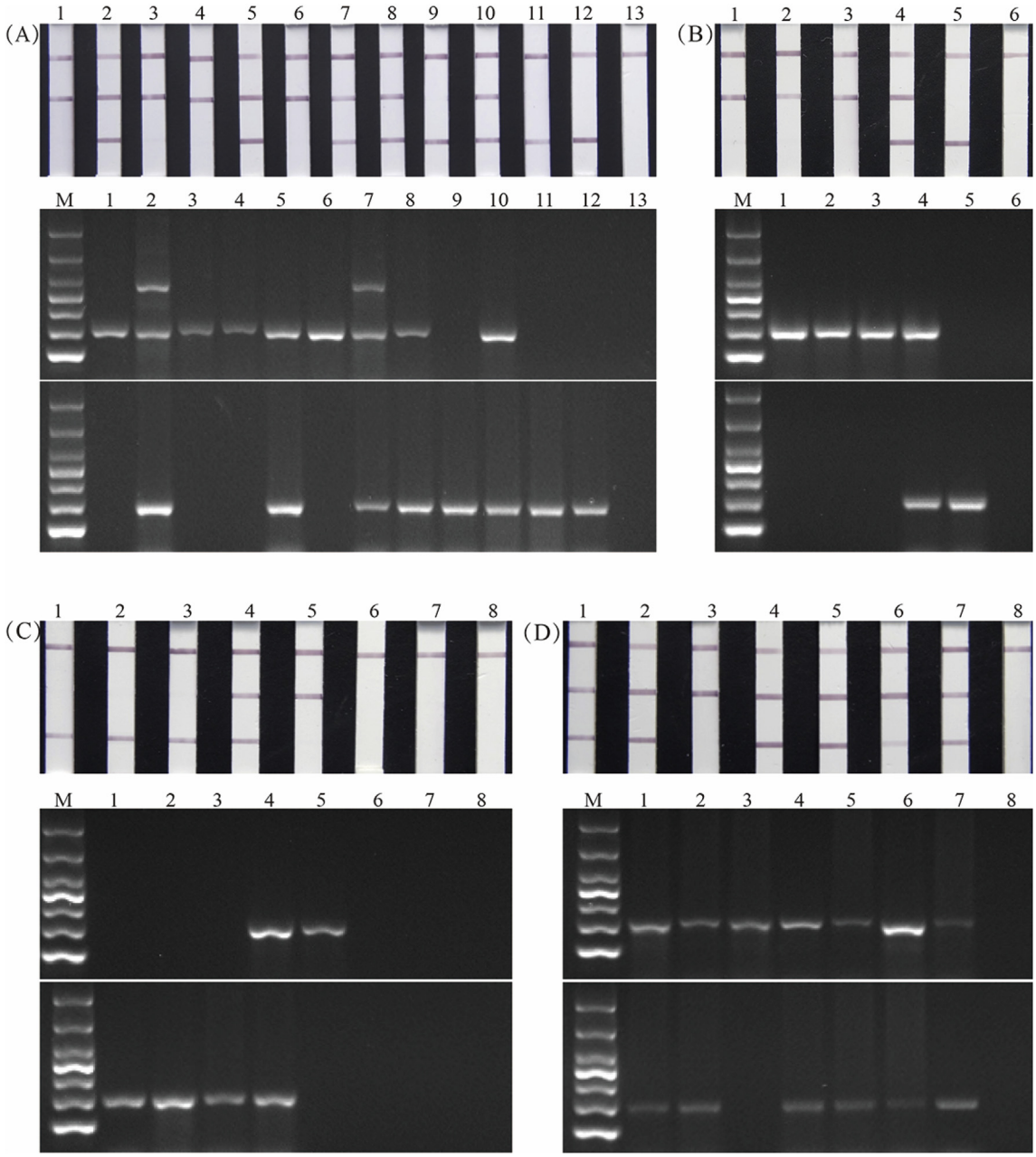


Fig. 4. Selectivity of the DRPA-LFB system. (A) The GM maize results. Lanes 1–13: 59122, NK603, MON810, BT176, MON89034, T25, MON88017, MON863, MIR162, BT11, GA21, 3272, and non-GMO-maize. (B) The GM soybean results. Lanes 1–6: A2704-12, A5547-127, 356043, GTS40-3-2, FG72, and non-GMO-soybean. (C) The GM canola results. Lanes 1–8: MS1 × RF1, MS1 × RF2, MS8 × RF3, OXY235, T45, Topas19/2, MON88302, and non-GMO-canola. (D) The GM cotton results. Lanes 1–8: MON1445, LLcotton25, MON88913, MON15985, GHB119, T304-40, MON88701, and GHB614. Lane M: DL1000 DNA marker. Top panel shows the detection results of DRPA-LFB. Middle and bottom panels show the results of conventional PCR for 35S and NOS, respectively.

lines (Fig. 2E). At higher temperatures (48–50 °C), only the control line was observed. These results were consistent with the results of the agarose gel electrophoresis. The amplification bands became sharper as the temperature was increased from 30 to 40 °C. When the temperature was 44 °C, the amplification band intensity decreased; furthermore, when the reaction temperature was 48 or 50 °C, there were no amplification bands detected (Fig. 2F). These results indicated that the RPA can be used in a wide range of reaction temperatures, consistent with findings from previous reports [35,36]. In this study, 39 °C was selected as the optimal temperature for the DRPA reaction.

3.4. Sensitivity and selectivity of the RPA-LFB method

The LOD of the DRPA-LFB method was assessed by testing several dilutions of GTS 40-3-2 genomic DNA, at concentrations of 10, 50, 100, and 200, and 500 copies per reaction. The results showed that the lowest detection level was 10 copies/reaction for both 35S and NOS, and a similar LOD for 35S and NOS was obtained with the conventional PCR (Fig. 3A). According to previous reports [4,5], the LOD of a conventional PCR and a 7-plex digital droplet PCR (ddPCR) assay for detection of GTS 40-3-2 was 8 copies and 10 copies, respectively. The LOD of DRPA-LFB was similar to the LODs of conventional PCR and ddPCR. In addition, the sensitivity of

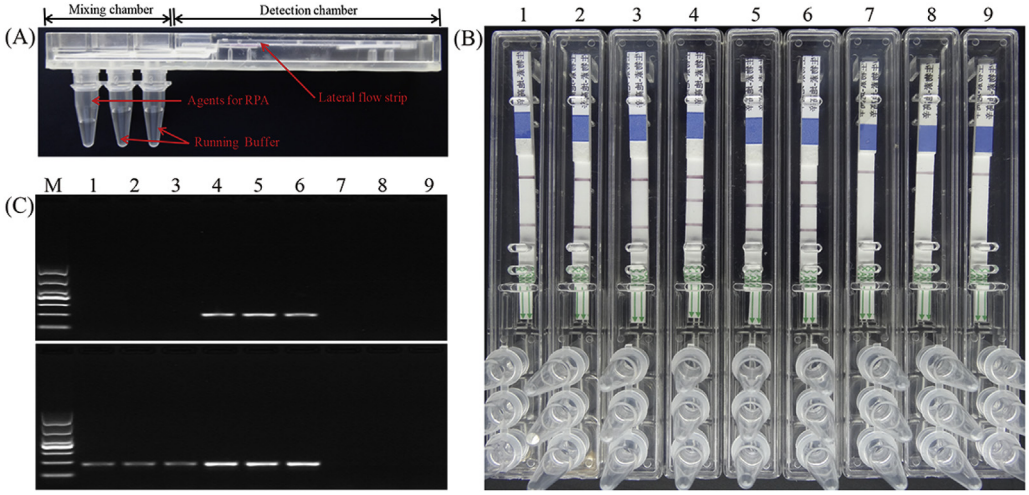


Fig. 5. Results of on-site testing of GM rice. (A) Enclosed disposable cassette integrated with RPA amplification and LFB visual detection. (B) Detection results using our system. (C) Detection results using conventional PCR. Top and bottom show the results of conventional PCR for 35S and NOS, respectively. Lane M: DL1000 DNA marker. Lane 1–3: TT51-1, Lane 4–6: KMD1, Lane 7–9: Non-GMO-Rice.

Table 1
Comparison of different methods for detection of GM crops-the case of GST40-3-2.

Analytical method	target	[a] Instrument	[b] Time for detection	Sensitivity	Suitability for on-site testing	Ref.
Protein strip test	Protein	No need	3–5 min	0.1%	Suitable	[8]
ELISA	Protein	Need	>5 h	0.25%	Unsuitable	[7]
Western blotting	Protein	Need	>5 h	0.25%	Unsuitable	[7]
Conventional PCR	DNA	Need	>3 h	8 copies	Unsuitable	[4]
Real-time PCR	DNA	Need	>2.5 h	1 copy	Unsuitable	[6]
ddPCR (7-plex)	DNA	Need	>3 h	10 copies	Unsuitable	[5]
LAMP	DNA	No need	About 2 h	4 copies	Unsuitable	[24]
LAMP-LFD	DNA	No need	About 2 h	2.4 copies (plasmid)	Unsuitable	[25]
DRPA-LFB	DNA	No need	About 30 min	10 copies	Suitable	This study

[a] It refers to professional or large instruments.
[b] It refers to entire detection time from extraction of protein or DNA to the final determination of the target.

DRPA-LFB was evaluated using samples containing 1%, 0.5%, and 0.1% GST 40-3-2 soybean and non-GM soybean. DNA (100 ng) from these samples was used as the template in the DRPA. After amplifying for 20 min, the 35S and NOS targets were all observed by LFB and agarose gel electrophoresis. The fluorescence dynamics of the DRPA are also presented in Fig. 3B. These results illustrated that the DRPA-LFB system could detect GM constituents in amounts as low as 0.1%. Furthermore, to evaluate the sensitivity of the DRPA-LFB within a shorter amplification time, 100 ng DNA from 0.1% GST 40-3-2 was used as the template in the DRPA. The DRPA amplification product was detected in the LFB at 5 min, 10 min, 15 min, and 20 min (Fig. 3C). The control line of the LFB for all assays was observed, and the T1 and T2 lines were present on all LFBs except for the DRPA amplified for 5 min. The red intensity of T1 and T2 line for the DRPA amplified for 10 min was slightly weaker than those from the DRPA amplified 15 and 20 min, but this difference did not affect the final visual judgment. No distinct differences were observed between the results of LFB for DRPA amplified 15 min and 20 min. These results were consistent with the results of the agarose gel electrophoresis (Fig. 3C). At 5 min, no amplification products were observed. When the amplification time was extended to 10 min and 20 min, the typical DRPA amplification product bands appeared and the intensity of the bands increased. The fluorescence dynamics of 35S and NOS in the assay are shown in Fig. 3C. From 0 to 5 min, i.e., the preparation phase for initiating RPA, only a small amount of fluorescence signal was observed. From 5 to 10 min, RPA obtained exponential amplification of the products and the fluorescence intensity increased as the reaction time increased. From 10 to 20 min, the RPA reached a plateau phase, and the fluorescence intensity stabilized. The result was consistent with the detection results of LFB and agarose gel electrophoresis. The results demonstrated that the DRPA system exhibited extremely high amplification efficiency and 10 min amplification was sufficient to detect 0.1% GST 40-3-2.

The selectivity of the DRPA-LFB assay was verified using 32 samples of GM crops and 3 samples of non-GM crops (Table S4). As expected, the detection results of the RPA-LFB were consistent with those obtained using conventional PCR (Fig. 4, Table S4). In the result of conventional PCR for 35S, the tested samples of Lanes 2 and 7 were NK603 and Mon88017, respectively. The two GM events were employed an enhanced 35S, which was formed by two 35S in series. So the conventional PCR results of the two GM events have two PCR products (Fig. 4A). These results indicated that the duplex DRPA-LFB assay has high selectivity and is suitable for screening GM crops.

3.5. On-site detection of GM rice using this system

The system we designed has rapid DNA extraction, rapid DNA amplification, and visual inspection. The applicability of this system was tested for on-site detection of GM rice TT51-1 and KMD1. Furthermore, to eliminate the risk of carryover contamination, a disposable, convenient, and enclosed cassette was employed as reported in our previous work [20,37]. The cassette comprise a mix

chamber and detection chamber, mixing chamber for mixing reagents, and a lateral flow strip pre-inset in the detection chamber (Fig. 5A). Three common PCR tubes are connected with the mixing chamber. In this study, the DRPA reaction was placed in one of the PCR tubes, 100 μL of running buffer was added into another two PCR tubes, respectively, and after amplification with a metal heater, the mixture of DRPA reaction and running buffer was directly poured into the mixing chamber. Next, the end of detection result is displayed on the lateral flow strip. For on-site tests, the two GM rice events were successfully detected using the developed system. 35S was detected in the KMD1 field sample and not detected in the TT51-1 field sample, NOS was detected in both of the field samples of KMD1 and TT51-1 (Fig. 5B). The test results were consistent with the detection results using conventional PCR (Fig. 5C). These results indicated that the novel DNA extraction method and DRPA-LFB system were suitable for on-site screening of GM crops.

We compared previously reported methods for detecting GM crops (Table 1). Unlike the PCR method, the DRPA-LFB system does not require an expensive thermal cycler. The rapid and convenient process of the DRPA-LFB system is comparable to that of protein test strips used for GM crop testing, but our system has a wider range of detection targets than are currently available for protein test strips, whose targeted proteins are limited. The DRPA-LFB system is faster than the other INAA methods, such as LAMP methods, due to their lack of a rapid DNA extraction step.

4. Conclusions

In summary, we developed a highly integrated system consisting of a rapid DNA extraction method and DRPA-LFB assay for rapid DNA amplification and visualization of the results to meet the requirements of on-site detection of GM crops. Using the novel rapid DNA extraction method, DNA extraction from the seeds and leaves of various crops required only about 5 min. The established DRPA-LFB assay required 10–20 min for RPA amplification and 5 min for LFB visualization, with the entire detection process accomplished in approximately 20–30 min. The developed system exhibited high selectivity and sensitivity (LOD 10 copies/sample). The simple detection system combining a rapid DNA extraction method with a DRPA-LFB assay for rapid DNA amplification and visualization will meet the demand for rapid on-site testing of GM crops.

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.

CRediT authorship contribution statement

Xiaofu Wang: Writing - original draft. **Yu Chen:** Validation. **Xiaoyun Chen:** Validation. **Cheng Peng:** Validation. **Liu Wang:** Methodology. **Xiaoli Xu:** Visualization. **Jian Wu:** Methodology. **Wei Wei:** Visualization. **Junfeng Xu:** Writing - original draft.

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Appendix A. Supplementary data

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