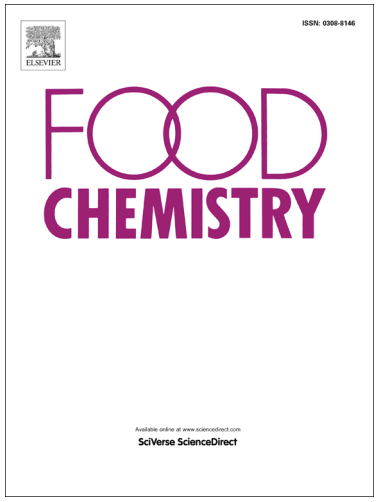

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PII: S0308-8146(18)32108-3
DOI: https://doi.org/10.1016/j.foodchem.2018.12.013
Reference: FOCH 23972

To appear in: *Food Chemistry*

Received Date: 10 September 2018
Revised Date: 13 November 2018
Accepted Date: 1 December 2018

Please cite this article as: Gao, W., Tian, J., Huang, K., Yang, Z., Xu, W., Luo, Y., Ultrafast, universal and visual screening of dual genetically modified elements based on dual super PCR and a lateral flow biosensor, *Food Chemistry* (2018), doi: https://doi.org/10.1016/j.foodchem.2018.12.013

Ultrafast, universal and visual screening of dual genetically modified elements based on dual super PCR and a lateral flow biosensor

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Running title: Ultrafast and visual screening of GMO elements based on DSPCR with LFB

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Number of tables: 0

No figures in color: 5

Abstract

In this study, a cascade screening system has been developed combining Dual Super Polymerase Chain Reaction (DSPCR) with the universal Lateral Flow Biosensor (LFB) for the ultrafast, universal and visual screening of dual GM elements, taking P-*35s* × T-*nos* for example. In the design of DSPCR for universal screening, gene-specific forward primers were labelled with biotin and gene-specific reverse primers were tagged with Cy5 and digoxin, respectively. In 2.5-min, DSPCR effectively amplified the dual target fragments through our prototype facility. Then, through specific antigen-antibody binding, a universal lateral flow biosensor exported visually dual-amplified results simultaneously without cross contamination. After optimization, the detection limit allowed 0.05% GM maize, corresponding to nine copies in maize. The entire detection process could be achieved in 10 min without any large-scale instrumentation. This method may be useful for the ultrafast, universal and visual screening of dual GM elements (P-*35s* × T-*nos*) in GM crop lines and is expected to be of great promise for rapid GMO screening and point-of-care tests.

Key words: Genetically Modified Organism (GMO), screening, Dual Super Polymerase Chain Reaction (DSPCR), Lateral Flow Biosensor (LFB)

Introduction

Genetically Modified Organisms (GMOs), mainly refer to plants, are those whose genetic material has been modified by the insertion of heterogenous genetic construct(s) (Griffiths, Partis, Croan, Wang, & Emslie, 2002; Persley, 1999). Recently, thresholds for labeling are incorporated for the reason of safety concerning GM crops in many countries. With the increasing worldwide commercialization, the success of the labeling schemes is dependent upon the efficiency with the results of GMOs detection(Holst-Jensen, Rønning, Løvseth, & Berdal, 2003). Moreover, GMOs have been booming recently. Until November in 2018, 498 GMOs events in 30 crops in 44 countries have been approved for cultivation, for instance, 229 events in Maize, 61 events in Cotton, 40 events in Soybean and 48 events in Potato, according to International Service for the Acquisition of Agri-biotech Applications (ISAAA). Indeed, the vast detective workload will come with constantly emerging GM events（http://www.isaaa.org/）. To minimize the analytical effort and to protect consumers' freedom of choice, screening methods should be paid more attention in preselection for further event-specific analysis. The application of screening analysis to be validated and standardized will not only contribute to unify detective performance for GM plants already in interlaboratory, but also be helpful to authorize upcoming commercial GM crops in the market (Waiblinger, Grohmann, Mankertz, Engelbert, & Pietsch, 2010).

In the screening of GMOs, PCR technology is the first choice of most analytical methods owing to the high sensitivity and specificity(Holst-Jensen, Rønning, Løvseth, & Berdal, 2003). Since most GM plants have been transformed containing Cauliflower Mosaic Virus (CaMV) *35s* promoter (P-*35s*) and the *Agrobacterium tumefaciens* nopaline synthase terminator (T-*nos*). Genetic elements, which were the P-*35s* and T-*Nos*, are widely used in PCR screening assays for GMOs(Holst-Jensen, Rønning, Løvseth, & Berdal, 2003). However, more than 2 h of thermal cycles in conventional PCR process and traditional

ethidium bromide-contained gel analysis after PCR are much more complicated, polluted, time-consuming and indistinguishable. Isothermal amplification reactions, such as loop-mediated isothermal amplification lamp (LAMP) and recombinase polymerase amplification (RPA), have been expected for GMOs screening analysis(Chen, Cheng, Xu, Huang, Luo, & Xu, 2016; Cheng, Shang, Xu, Zhang, Luo, Huang, et al., 2016). They only consume 30-60 min for amplification, yet have been blamed for systematic complexity, contaminative vulnerability and less specificity. Real-time PCR and chip-based detection methods, which are free of gel analysis, have difficulty in time- and cost-saving. Consequently, much more post-amplification efforts have to be made to satisfy the quick, sensitive need for GMOs screening analysis.

Encouragingly, recent studies showed that the reaction time of PCR could be shortened to several minutes by changing instrumental factor and biochemical factor. For the apparatus, researchers simplified the thermal device by two or three water baths(Farrar & Wittwer, 2014; Wong, Wong, Chan, Hsieh, & Wong, 2015). For the biochemistry, the speed was improved by increasing the concentration of primers and DNA polymerase(LN & CT, 2013; Montgomery & Wittwer, 2014). With the development of rapid PCR, relevant detective technology has achieved many progresses, such as the detection of pathogens(Pecchia & Da, 2018; Tian, Huang, Luo, Zhu, Xu, & Xu, 2018) and human gene(Farrar & Wittwer, 2014). However, the application of rapid PCR was hindered because of the difficult of visualization, complexity of gel analysis.

Accordingly, the Lateral Flow Biosensor (LFB) has been proposed to solve such problems above, due to the excellent traits of user-friendly, visual results interpretation, pollution-free, rapid and multiplex signal output in resource-limited setting(Ge, Asiri, Du, Wen, Wang, & Lin, 2014; Hu, Wang, Wang, Li, Pingguanmurphy, Lu, et al., 2014; Sajid, Kawde, & Daud, 2014; Z, Y, J, Z, JG, & Y, 2010). However,

inferior sensitivity of LFB based on gold-labelled antibody limited its further application (Hou, Hsiao, Lin, Yen, & Chang, 2012; Parolo, Escosura-Muñiz, & Merkoçi, 2013), thus much efforts toward nucleic acid amplification strategy (Chen, Cheng, Xu, Huang, Luo, & Xu, 2016; Cheng, et al., 2016), nucleic acid hybridization based capture strategy (Cheng, Xu, Luo, Zhu, Zhang, Huang, et al., 2017) and nanomaterial-based strategy(X. Wang, Choi, Cheng, Ko, Chen, & Choo, 2017; Y. Wang, Yan, Fu, Hu, Wang, Xu, et al., 2018) have been made for the detection of GMO(Cheng, Shang, Xu, Zhang, Luo, Huang, et al., 2017), pathogens(Y. Wang, Li, Wang, Zhang, Xu, & Ye, 2017; Y. Wang, et al., 2018; Zhan, Wang, Iradukunda, & Zhan, 2018),DNA(Sun, Guo, Li, Chen, Liu, Wong, et al., 2018) and RNA(Ying, Ju, Sun, Li, Chang, Song, et al., 2017).

Yet intricate reaction system, complicated operation and longer detective time still need to be addressed. Herein, an ultrafast, universal and visual screening method with the simpler system, easier manipulation for the determination of dual GM elements is planning to be established, by the integrated innovation combined the homemade Dual Super PCR within 2.5-min with the lateral flow biosensor within 5-min, for the discrimination of GMOs in a time-efficient way universally and visually.

Method and Material

Reagents and Apparatus

The GM plant events (59122 maize, GA21 maize, MON809 maize, 32138 maize, 3272 maize, CBH-351 maize, MON87411 maize, and 33121 maize) and non-GM plants (maize) were provided by Sigma-Aldrich Trading Co., Ltd. (Shanghai, China), the DuPont Company (Boston, MA, USA) and the Monsanto Company (St. Louis, MO, USA). Real samples of maize products (maize kernel, maize Oil, and maize Powder) were purchased from a local market in Beijing, China.

All synthetic DNA sequences in Table S1 were synthesized at KareBay Biochem (Ningbo, China). rTaq DNA polymerase, 10× PCR Buffer (20 mM Mg^{2+} Plus) and dNTP Mixture (2.5 mM each) were purchased from Takara (Beijing, China). Gold Chloride Trihydrate, Tri-Sodium Citrate Dihydrate, phosphate buffer saline (PBS, 0.01M), Bovine Serum Albumin (BSA), Tween 20 (polyoxyethylene-20-sorbitan monolaurate), the anti-Cy5, anti-digoxin, and anti-biotin were obtained from Sigma (St. Louis, MO). The plastic backing, conjugate pad, Nitrocellulose membranes, sample pad and absorption pad were acquired from Millipore (Billerica, MA). Ultrapure water was obtained from a Milli-Q water purifying system (resistivity ≥ 18.2 MΩ cm^{-1}, Milli-Q, Millipore). A portable test strip reader (DT2050), which connected to a laptop, was purchased from Shanghai Goldbio Tech. Co., Ltd. (Shanghai, China) to collect assay signals from LNF. Other chemicals were of analytical grade and used without further purification.

Fabrication of Dual Super PCR

A hot water bath (HH-1, Junsi) of 95 °C and a warm water bath of 58 °C were used to change the temperature of reaction containers. A stepper motor (42JSF630AS-1000, Just Motioin Control) powered by switching the power supply (S-100-24, Elecall) and controlled using an AC Servo Motor Driver (YZ-ACSD608, Moving) and the Labview Software (version 2014). We used a thermocouple (T-type, HXW) that was centered in a dedicated control tube to measure the temperature. Thermocouple amplification and linearization were transmitted using a temperature transmitter (SBWR-2260, K, Yuancheng) and performed using an Arduino UNO v1.0 chip. The analog signal was digitized using the Arduino UNO chip and processed with Arduino IDE (version 1.8.1). Sample containers for PCR were Light Cycler capillaries (20 μL, 04 929 292 001, Roche). Samples were spun down to the bottom of each tube by brief centrifugation and fixed on the 3d-printed plastic stent(Tian, Huang, Luo, Zhu, Xu, & Xu, 2018).

The 20-μL DSPCR reaction system contained 1×PCR Buffer, 250 μM of dNTP mixture, genome DNA extractions of Maize lines (different GM proportion), 1.5 U/μL rTaq DNA polymerase, and 2.0 μM of primers. The reaction system was prepared on ice, quickly transferred to the instrument, and immediately amplified without delay. DSPCR was triggered at 98 °C for 2 s and 37 °C for 3 s in one thermal cycle with the stepper motion controlled to obtain the 30 thermal cycles in 2.5 min. The amplification results were inspected using 2% ethidium bromide (EB)-stained agarose gel electrophoresis at 130 V for 25 min and were conducted on a Molecular Imager Gel Doc XR (Bio-Rad, Beijing, China).

Assemble of the universal lateral flow biosensor

Gold nanoparticles (AuNPs) were synthesized according to a method described previously(He, Zhang, Zhang, Baloda, Gurung, Xu, et al., 2011). The preparation of AuNPs-labelled antibody was conducted referring to the literature(Chen, Cheng, Xu, Huang, Luo, & Xu, 2016).

A universal lateral flow biosensor was made of a plastic backing, the conjugate pad, the NC membranes, the sample pad and the absorption pad. After spraying the AuNPs-labelled anti-biotin antibody and drying at room temperature, the conjugates pad was prepared. To reduce two kinds of DSPCR products-mixture in the same AuNPs, we applied the optimized concentration of antibody (1.0 mg/mL) in our previous multiplex and universal lateral flow biosensor(Chen, Cheng, Xu, Huang, Luo, & Xu, 2016). Therefore, the NC membrane was sprayed by the purified digoxin antibodies (1.0 mg mL^{-1}), anti-biotin antibodies (1.0 mg mL^{-1}) and Cy5 (1.0 mg mL^{-1}) within several micro-liter to form two test lines and one control line with the 5 mm interval, respectively, and then dried in the air at 37℃ for 12 h. Next, the universal lateral flow biosensor assembled with the plastic backing pasted by the membrane, covered with the absorption pad and the conjugated pad on each side, and was cut into 3mm wide test strip and stored 4 ℃ for use.

148

149 **Screening of dual GM elements with DSPCR-based LFB assay**

150 A 50 μL detecting buffer (4 × SSC, 2%BSA, 0.05 % Tween-20, pH 7.0) was mixed with 20 μL DSPCR
151 products. Followed that, a test strip was conducted and assayed according to the published
152 protocols(Chen, Cheng, Xu, Huang, Luo, & Xu, 2016). And the determination of GM copy numbers in
153 Maize lines is as follows:

154 GM copy number/μL= $(6.02\times10^{23})\times10^{-9}\times$ c (ng/μL)/ DNA length×660 × GM proportion

155 c: the concentration of Maize genome, 50 ng/μL

156 DNA length: 2300 M

157 **Result and Discussion**

158 **3.1 Design Principle**

159 **3.1.1 Dual Super PCR**

160 To realize while-you-wait dual screening of GMOs, the biosensor which combined amplification of Dual
161 Super PCR with versatile and visual results interpretation on a lateral flow biosensor was developed. As
162 it occurred to the author that the process of conventional PCR was restricted by instrumentation as well
163 as biochemistry. Conventional PCR is blamed by the time cost due to the temperature variation of
164 traditional thermocycler. To realize rapid thermal cycles, a versatile prototype was constructed of hot
165 water bath, warm water bath and a shaft module. 20-μL samples in thin glass capillary tubes flipped
166 between two water baths, proceeding two-step PCR process, which were composed of 80-90℃ in one-
167 fold times for template denaturation and 55-75 ℃ in two-fold times for primer annealing and polymerase
168 extension (Figure S1). With respect to biochemistry of conventional PCR, high concentrations of two
169 critical reactants, the DNA polymerase and the specific primers, have been reported to increase yield in

rapid-cycle(Higuchi, Fockler, Dollinger, & Watson, 1993; Wittwer, Fillmore, & Garling, 1990). To realize universality and to reduce cost, common recombinant Taq polymerase was selected. As shown in Figure 1A, two forward primers were labeled with biotin and two reverse primers were labeled with Cy5 and Digoxin, respectively. After fast thermal cycles of denaturation, annealing and extension, dual PCR amplified numerous biotin- and Cy5-labeled duplex DNAs and biotin- and digoxin-attached duplex DNAs. Therefore, Dual Super PCR assays are much faster compared with conventional PCR assays.

(Here Figure 1)

3.1.2 Universal visual lateral flow biosensor

The visual and universal results interpretation of the DSPCR products were performed on a LFB, and the principle is illustrated in Figure 1B. Anti-biotin-labeled AuNPs, the anti-biotin antibody, anti-Cy5 antibodies and anti-digoxin-antibodies were immobilized on the conjugate pad, control line (CL) and two test lines (TLs) of the LFN, respectively. When the LFN was dipped into the running buffer-DSPCR products-mixture, the solution migrated along the strip via capillary force, passed the conjugate, rehydrating the anti-biotin-AuNPs conjugates and generating plentiful antigen-antibody-AuNPs compounds (Cy5-DSPCR products-biotin-anti-biotin-AuNPs compounds and digoxin-DSPCR products -biotin-anti-biotin-AuNPs compounds). Then, the compounds were captured on the test line by the affinity interaction to form anti-Cy5-Cy5-DSPCR products-biotin-anti-biotin-AuNPs compounds and anti-digoxin- digoxin- DSPCR products -biotin-anti-biotin-AuNPs compounds. The AuNPs on the test line accumulated to form red band, prone to visual interpretation. Meanwhile, excess anti-biotin-AuNPs conjugates were intercept by the secondary antibody on the control line, forming another red band. In the absence of GM elements, there was no DSPCR products in the running solution. A red band only

192 appeared on the control line rather than on the test line due to the lack of captured anti-biotin-AuNPs
193 conjugates on the test lines.

194 **3.2 Characterization of DSPCR**

195 PCR detection is the mainstream technology for screening GMOs elements with relatively long reaction
196 process with 2-3 h. To achieve while-you-wait assays, a prototype device was put up to speed up PCR
197 process. As shown in Figure S2, the device was composed of two water baths. One is hot water bath at
198 95 ℃, the other is warm water bath at 58 ℃. In the reaction, PCR samples in capillaries constantly
199 reciprocated from hot water bath to warm water bath for to complete 30 cycles controlled by pre-designed
200 procedure. In the 2.5 min-reaction, a wire thermocouple in the control capillaries could record the
201 temperature in real time.

202 To meet the requirements of the fast PCR amplification, the concentrations of two critical factors, the
203 DNA polymerase and primer pairs, were increased to 60-fold (1.5 U/μL) and 5-fold (2 μM), respectively
204 (Table S2 & Table S3). Compared with traditional PCR reaction system with conventional thermocycler
205 (Table S4), fast PCR not only solely amplified expected 195-bp segment of *35s* gene in lane 9 and 151-
206 bp section of *Nos* gene in lane 11 with much brighter bands than corresponding amplification products
207 in lane 3 and lane 5 (conventional PCR), respectively, but also dually obtained amplified fragments of
208 *35s* and *Nos* gene in lane 12 simultaneously and successfully in Figure 2, Meanwhile, nonspecific
209 amplification of large fragment conducted by conventional PCR, nearly 1800 bp in lane 5 and nearly 700
210 bp in lane 6, were averted due to the restriction of polymerase extension in a flash. In addition, no bands
211 of primer dimers generated in the electrophoretogram. Therefore, dual fast amplification of two GM
212 elements has been perfectly prepared.

213 **(Here Figure 2)**

To acquire shorter reaction time and higher amplification efficiency, different DSPCR time was optimized. As shown in Figure 3, 30 thermo-cycles were completed in 10-min, 9-min, 7.5-min, 6-min, 5-min, 2.5-min and 1-min, respectively. Dual amplification products, 195-bp *35s* fragments and 151-bp *Nos* fragments, were obviously observed after10-min, 9-min, 7.5-min, 6-min, 5-min, 2.5-min and 1-min in lane 2, 3, 4, 5, 6 and 7. While DSPCR products were not generated after 1-min amplification. To achieve while-you-wait screening, 2.5-min was confirmed as optimal DSPCR reaction time.

(Here Figure 3)

3.3 Analytical Performance of DSPCR-LFB

3.3.1 Sensitivity

Under optimal experimental conditions, non-GM maize spiked with CBH-351 maize at different percentage (weight of GM / weight of non-GM, w/w) was assayed to investigate the detective sensitivity. As demonstrated in Figure 4 (Figure S3), the red color intensity of two test lines in photo images increased in response to increasing proportions of the standard GM maize samples. In terms of the lowest proportion with a visible test line, the limit of detection (LOD) was observed as 0.05%, corresponding to nine copies on the basis of genome size of maize. Therefore, the proposed screening method is fair sensitive to screen GM content up to the threshold for GM labeling of 0.9% in the European Union (European Commission Regulation, 2003), 3% in Korea (Ministry of Agriculture and Forestry of Korea, 2000) and 5% in Japan (Food and Marketing Bureau, Ministry of Agriculture, Forestry and Fisheries of Japan, 2000). Compared with other methods for GMO detection, our proposed method is competitive (Table S6).

(Here Figure 4)

3.3.2 Selectivity

To investigate the selectivity of the ultrafast, universal and visual biosensor, a series of GM maize including GA21 maize, 59122 maize, MON809 maize, 32138 maize, 3272 maize, CBH-351 maize, MON87411 maize, 33121 maize. Each with a concentration of 0.5%, were tested after DSPCR with LFB. As shown in Figure 5 (Figure S4), GA21 maize, 59122 maize and Bt176 maize showed one test lines and MON809 maize and CBH-351 maize showed two test lines, attributed to the specific amplification of DSPCR and the specific affinity of antigen-antibody interaction. So, it indicated the biosensor was of high selectivity.

(Here Figure 5)

3.3.3 Reproducibility

In addition to high sensitivity and selectivity, the proposed biosensor also exhibited excellent reproducibility. The reproducibility of the biosensor was evaluated by testing the amplification sample solutions in the presence of MON809 maize (GM proportion 0.5%) 6 times. As shown in Figure 6 (Figure S5), similar responses can be obtained each time. The corresponding RSD values of optical responses were 1.3%, indicating the excellent reproducibility of the proposed method.

(Here Figure 6)

3.3.4 Application

To further confirm the feasibility in practical samples, the ultrafast, universal and visual biosensor was

challenged to screen the elements containing *35s* and *Nos* in 8 samples, including 2 raw maize kernels, 4 highly processed foods and 2 artificially mixed samples with spiked Bt11 maize kernel. As shown in Table S7, the artificially mixed samples were dual-positive, neither raw maize kernel samples nor highly processed food samples containing GM elements, confirming the reliability of the proposed biosensor for screening GM elements.

Conclusion

In general, an ultrafast, universal and visual screening method has been developed, by which the dual GM elements (containing *35s* and *Nos*) can be firstly amplified via DSPCR and then visually interpreted by the universal lateral flow biosensor sensitively and selectively with a total time of 10 min. This proposed biosensor is able to allow nine copies (0.05%, w/w) in maize and is appropriate for dual screening of two GM elements in raw maize kernels and in highly processed food samples. In addition, the universal method can be extended to assay other dual targets with a little modification, showing great promise for while-you-wait detections for customs.

Acknowledgments

This work was supported by the National GMO Cultivation Major Project of New Varieties (Grant No. 2018ZX08012001).

Conflict of interest

The authors declared that they have no conflicts of interest to this work.

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Figure Caption

Figure 1. Principle of Dual Super PCR coupled with Lateral flow biosensor. (A)Schematic illustration of the formation of Cy5 and digoxin and biotin attached products based on DSPR. (B) Schematic illustration for the visual detection of DSPCR products on a LFB.

Figure 2. Comparison between single- and dual- conventional PCR and single- and dual- super PCR.

Figure 3. Amplification time Optimization of DSPCR. Lane 1: D2000 Marker. Lane 2: 10-min. Lane 3: 9-min. Lane 4: 7.5-min. Lane 5: 6-min. Lane 6: 5-min. Lane 7: 2.5-min. 8: 1-min.

Figure 4. Typical images of dual LFB in response to various proportion of CBH-351 maize. The proportion of targets: (A) 100%, (B) 50% (C) 5%, (D) 0.5%, (E) 0.05%, (F) 0%. Each test was performed in duplicates.

Figure 5. Typical images of dual LFB in response to a serious of GM maize (GM proportion 0.5%), including (A) 59122 maize, (B) GA21 maize, (C) MON809 maize, (D) 32138 maize, (E) 3272 maize, (F) CBH-351 maize, (G) MON87411 maize, (H) 33121 maize. Each test was performed in duplicates.

Figure 6. Typical images of the analytical reproducibility of dual LFB in response to MON809 maize (GM proportion 0.5%) 6 times. These tests occurred every other week.

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399 biosensor.

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413

414 **Highlights:**

415 1- An ultrafast, universal and visual screening method has been developed;

416 2- GM elements can be detected based on super PCR and lateral flow biosensor in 10-min;

417 3- Dual GM events can be amplified via Dual Super PCR in 2.5-min;

418 4- Visual results can be interpreted by the universal lateral flow biosensor.

419 5- The LOD of the biosensor achieves nine copies in maize.

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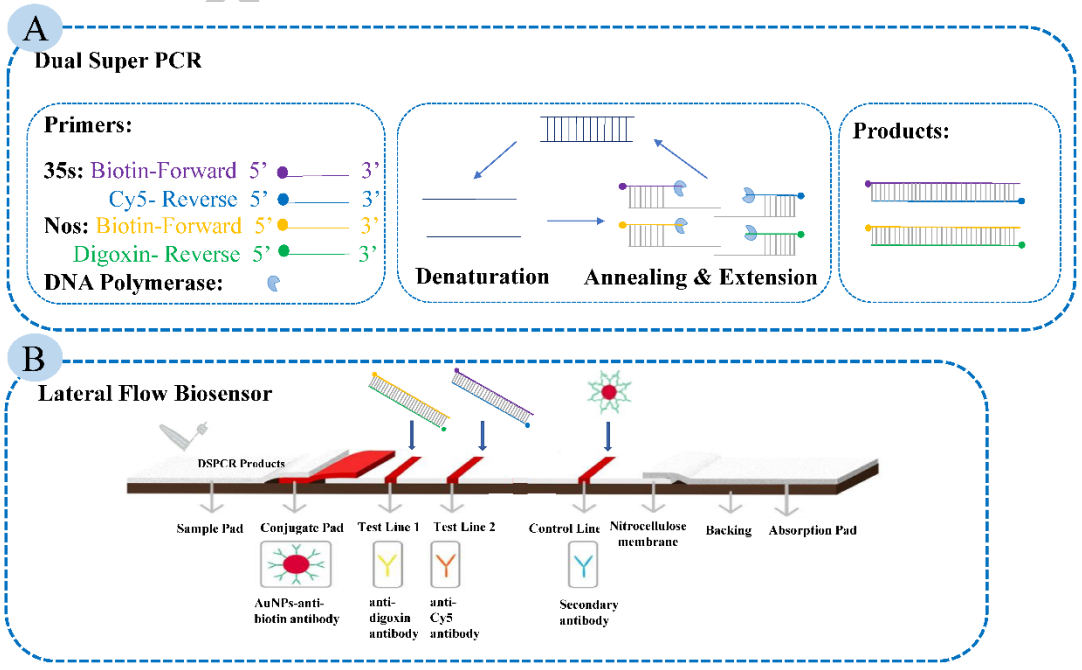

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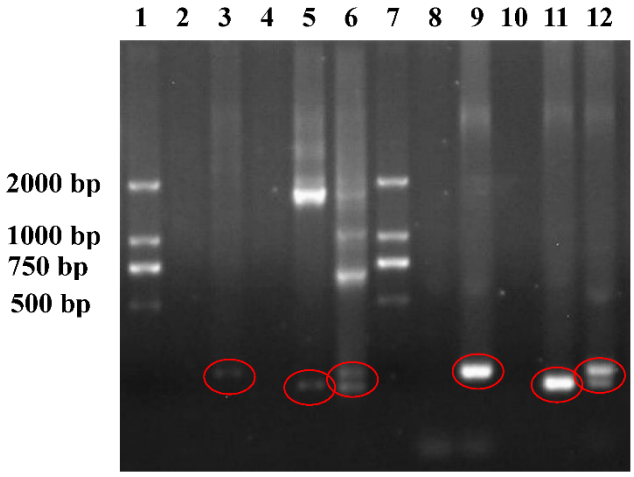
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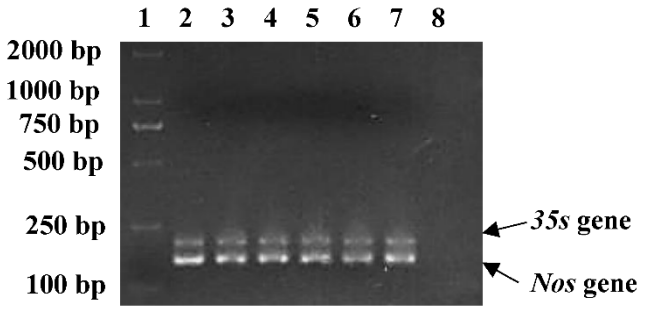
1000 bp
750 bp
500 bp

424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1000 bp
750 bp
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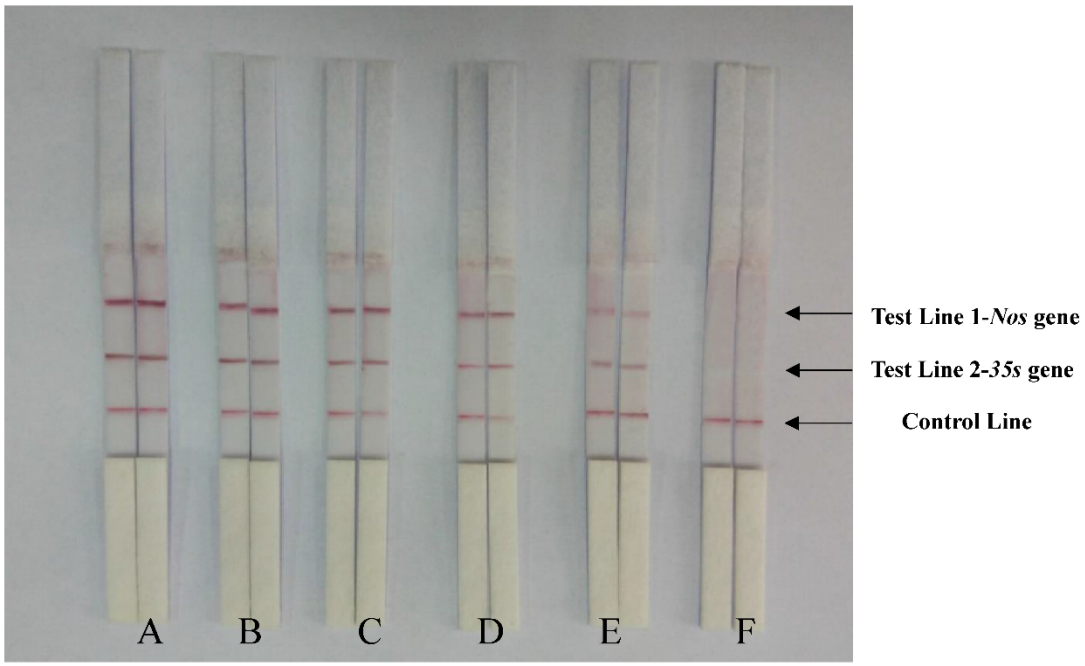
250 bp
100 bp
35s gene
Nos gene

425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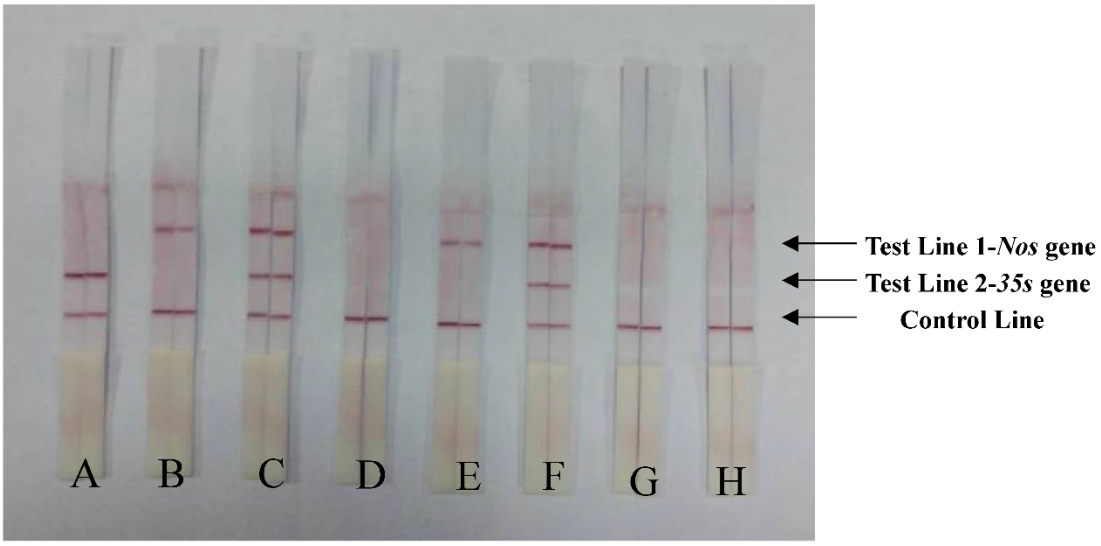
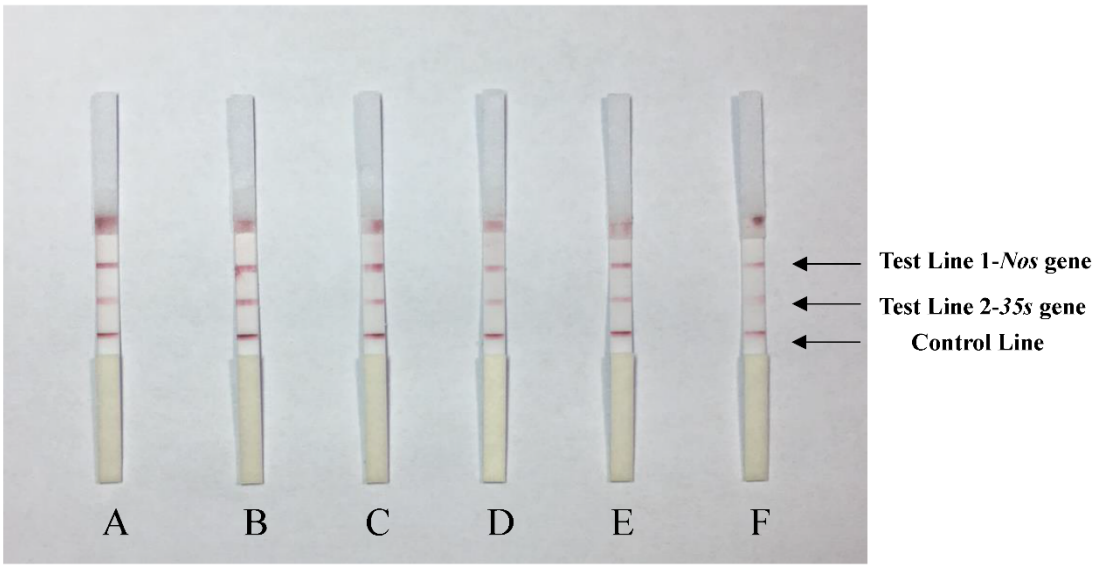
Test Line 2-35s gene
Control Line
A
B
C
D
E
F

426





Declaration of interests

☒ 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.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: