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Original Research Article

Multiple recombinase polymerase amplification and low-cost array technology for the screening of genetically modified organisms

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ABSTRACT

The identification of different transgenic products that are potentially present in foods is a priority given their impact on environmental safeness and health care. In this context, reliable, fast and inexpensive detection methods are demanded to screen the compliance of product labelling and traceability regulations. We herein developed a method that combines recombinase polymerase amplification (RPA) in a multiple format and a hybridisation assay. This system was optimised for the simultaneous amplification/detection of the 35S promoter, the NOS terminator and taxa, soya (*Glycine max*), corn (*Zea mays*) and potato (*Solanum tuberosum*), to denote the transgenic ingredients present in samples and to help to identify their source. As proof-of-concept, compact disc technology automated the optical sensing of RPA products. Discs worked as an analytical platform in the microarray format, and the reader/recorder as a detector. The analysis of the food mixtures containing genetically modified organisms up to 0.2 % showed excellent selectivity (no false-positives), reproducibility (relative error <20 %) and sensitivity (0.04 ng). The isothermal method was validated using certified reference materials and successfully compared to PCR-ELISA. The results of food products also confirmed it as an effective high-throughput tool for supporting simple, low-cost food safety controls, which makes it ideal for laboratories with limited resources.

1. Introduction

Traceability regulations require genetically modified organisms (GMOs) to be tracked throughout the production chain, from seed production and crop harvest to food products on markets. Therefore, developing reliable, rapid analytical methods for the detection and quantification of GMOs is most important (Qian et al., 2018). The introduction of real-time PCR or digital PCR technologies has significantly improved the detection limits compared to detection based on agarose gel electrophoresis or by capillary electrophoresis (Marmiroli et al., 2008). Despite its high potential in GMO testing, the use of both methods has its limitations (Querci et al., 2010). The most important drawbacks are associated with high-technology costs and the risk of false-negative results, especially if an intercalating fluorescent molecule is used as the reporter. In this context, microarray technology offers substantial advantages, such as automation of analytical processes and higher multiplexing capability, which allow the simultaneous detection of many sequences to detect GMOs (Von Götz, 2010; Brod et al., 2014).

The need for simplicity and speed in GMO screening has also stimulated the development of several methods based on low-cost portable technologies (e.g. biosensors) (Huang et al., 2015; Zhang et al., 2020). However, the polymerase-based amplification reaction imposes constraints that technical requirements must meet, including precise temperature control and rapid thermocycling. In fact, temperature fluctuations during reactions strongly influence amplification yields and assay specificity.

In the last decade, the use of other enzymes, or the combination of enzymes, to mimic DNA replication *in vivo* has progressed and become a real alternative (Qian et al., 2018; Singh et al., 2019; Liu et al., 2020a,b). Most studies that have successfully applied for GMO screening are based on loop-mediated isothermal amplification (LAMP) combined with an electrochemical sensor (Ahmed et al., 2009) or naked-eye assays (Shao et al., 2017; Kaygusuz et al., 2019). However, LAMP operates far from room temperature, the design of primers is complex, and multiplexing

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capability is limited. Isothermal recombinase polymerase amplification (RPA) is an interesting approach because this reaction provides excellent yields when operating at 37 °C (Lobato and O'Sullivan, 2018). Furthermore, exponential amplification can be accomplished in a short time (40−60 min) and is more tolerant to some inhibitors. Several biomolecular techniques have enabled the identification of RPA products from GMO targets, such as fluorescent labelling (Wang et al., 2017), real time-RPA (Wang et al., 2020a), ELISA-RPA microplates (Santiago-Felipe et al., 2014) or lateral flow strip (Liu et al., 2020a,b; Wang et al., 2020b). These highly integrated approaches are adequate for the on-site detection of genetically modified products, but their screening capability is limited to a single target per assay.

A few methods have been proposed for RPA-based assays to overcome the drawbacks of isothermal methods to amplify several targets in a single reaction. The variant called solid-phase RPA separates different reactions on a solid support in a microarray format (Tortajada-Genaro et al., 2015a,b). One primer of each GMO target is attached on a solid support, while the other remains in the liquid phase, and then enzymatic extension directly produces a tethered amplification product. However, solid-phase RPA generally requires elucidating the optimal surface chemistry for the efficient immobilisation of selective primers (Del Río et al., 2017). A parallelised lateral flow RPA assay has been reported to detect three genetically modified corn events simultaneously (Li et al., 2020). Another interesting approach for the multiplex detection of RPA products is their specific recognition by probes anchored to planar chips (solid-phase hybridisation). This principle has been successfully demonstrated with products from both allele-specific RPA (Yamanaka et al., 2017) and multiplex ligation/universal RPA (Lázaro et al., 2019).

The multiple RPA-based biosensing technology is herein addressed for the simultaneous sensitive screening of five regions. They are the two commonest transgenic genes, 35S-promoter from cauliflower mosaic virus (P-35S) and nopaline synthase terminator (T-nos), to allow the detection of most authorised or unauthorised lines. Plant-specific elements detect certain host genome regions, such as lectin (*lec*) for *Glycine max* (soya bean), alcohol dehydrogenase 1 (*adh1*) for *Zea mays* (corn) and UDP-glucose pyrophosphorylase (*ugp*) for *Solanum tuberosum* (potato). The novelty lies in the precise amplification reaction control and a later hybridisation on a chip for optical detection purposes. As proof-of-concept, the assay was performed by compact disc technology. The methodology involves similar steps to those based on glass slide microarrays, but discs act as polymeric supports for carrying out assays and the disc drives is the imaging system (Hwu and Boisen, 2018). The potential advantages arise from a sophisticated precise optical-sensing detector, but a very simple operation (end-user friendly) that is mass-produced (consumer electronic device), inexpensive (costs less than €350) and portable (2.7 kg). Therefore, the resulting image pattern can be related to a precise genetic profile of the studied food.

2. Experimental

2.1. Reagents and materials

The specific primers for RPA and specific probes for DVD-hybridisation are listed in Table SI.1. All the oligonucleotides used in this study were purchased from Eurofins (Luxembourg). Printing solution contained 1 % glycerol (v/v)10 mg/L streptavidin, and 50 mM carbonate buffer at pH 9.6. Phosphate buffered saline with Tween (PBS-T) was prepared at 10 mM sodium phosphate, 0.15 M NaCl, 0.05 % Tween 20, pH 7.4. The hybridization buffer was saline sodium citrate (NaCl 150 mM, sodium citrate 15 mM, pH 7, 15 % formamide), containing 10 nM of positive hybridization control. The staining reagent was anti-digoxigenin antibody conjugated to horseradish peroxidase (Abcam) at 1:500 in PBS-T. The washing buffers were diluted hybridization buffer (1:10) and PBS-T. The developing reagent was 3,3′,5,5′-tetramethylbenzidine (ep(HS)TMB-mA, SDT). Standard digital versatile disc (DVD) were used (CD Rohling-up GmbH).

For hybridization assay, functionalized chips were prepared from bulk standard DVDs. Biotinylated probes (50 nM) prepared in printing solution were transferred to the disc (50 nL) with a non-contact arrayer (AD 1500 BioDot, Inc., CA). The working temperature and relative humidity were controlled at 25 °C and 90 %, respectively. The incubation time was 9 h. The layout was 9 × 9 spots per array and 12 arrays per DVD, being the distances between flanking spots of 1.5 mm. Hence, each sequence has nine replicate spots corresponding to each screening element (P-35S and T-nos), plant-specific target (*lec*, *adh1* and *ugp* genes), two positive control (hybridisation and staining), and two negative controls (immobilisation and hybridisation). The spot diameter was 500 ± 10 μm (Fig. 1).

2.2. Foodstuff

Certificated reference materials (CRM), plant materials and trial food samples were used. CRMs were purchased from the Institute for Reference Material and Measurements (Geel, Belgium), and included four transgenic corn (Bt11, Bt-176, MON810, GA-21) and one soya bean (GTS40-3-2). The seeds of the non-GM foods (corn, soya bean, tomato, rice), GM corn (MON-810) and GM tomato (NahG) were kindly supplied by the COMAV research Institute (UPV, Spain). Food products, bought in local food stores, were checked to confirm that GM events were lacking by a single PCR method. The flours containing defined percentages of GMO material were prepared from the GMO and non-GMO seeds, ranging from 0 % to 100 % (weight percentage). Genomic DNA was extracted from samples with the GMO extraction kit (Applied Biosystems). Genomic DNA quality was confirmed by spectrophotometry and gel electrophoresis (Fig. SI.1).

2.3. RPA amplification

A duplex primer mixture for screening elements (mixture A) was prepared, with 4 μM for P-35S and 5 μM for T-nos. A triplex primer mixture for the plant-specific amplification (mixture B) was prepared, with 3 μM for *lec*, 4 μM for *adh1* and 3 μM for the *ugp* genes. Two multiplex reactions (reaction A and B) were carried out in a total volume of 10 μL using the kit TwistAmp Basic (TwistDx, UK). In each polypropylene phial, the genomic DNA from the extracted sample (10 ng) and the corresponding primer solution (1 μL of mixture A or B) were added to the reconstituted solution of the amplification enzymes, nucleotides and buffer. After the addition of magnesium acetate (280 mM), reactions were carried at 40 °C for 50 min in an oven or heater block. The control samples were included in addition to the tested samples to each assay: water control, positive sample (known GMO seed) and negative sample (non-GMO seed).

2.4. Hybridisation-detection

Both RPA products (1 μL) were mixed with 50 μL of hybridisation buffer per triplicate. Subsequently, the solution was denatured by heating at 95 °C for 5 min and transferred to the DVD surface. After incubation for 60 min at 37 °C, the disk was washed with diluted hybridisation buffer (0.1×) and then rinsed with deionised water. Next 1 mL of staining reagent was dispensed for 30 min, followed by rinsing with washing buffer, dispensing 1 mL of developing reagent for 8 min, and then rising with water. The results were directly read by the DVD drive and surface scanning occurred at 4× speed, with a gain of 18 dB. The image processing diameter to calculate the optical density of each microarray dot was 250 μm (460 pixels per spot).

3. Reference methods

3.1. Analysis of amplification products

Amplification yields were calculated from the fluorescence

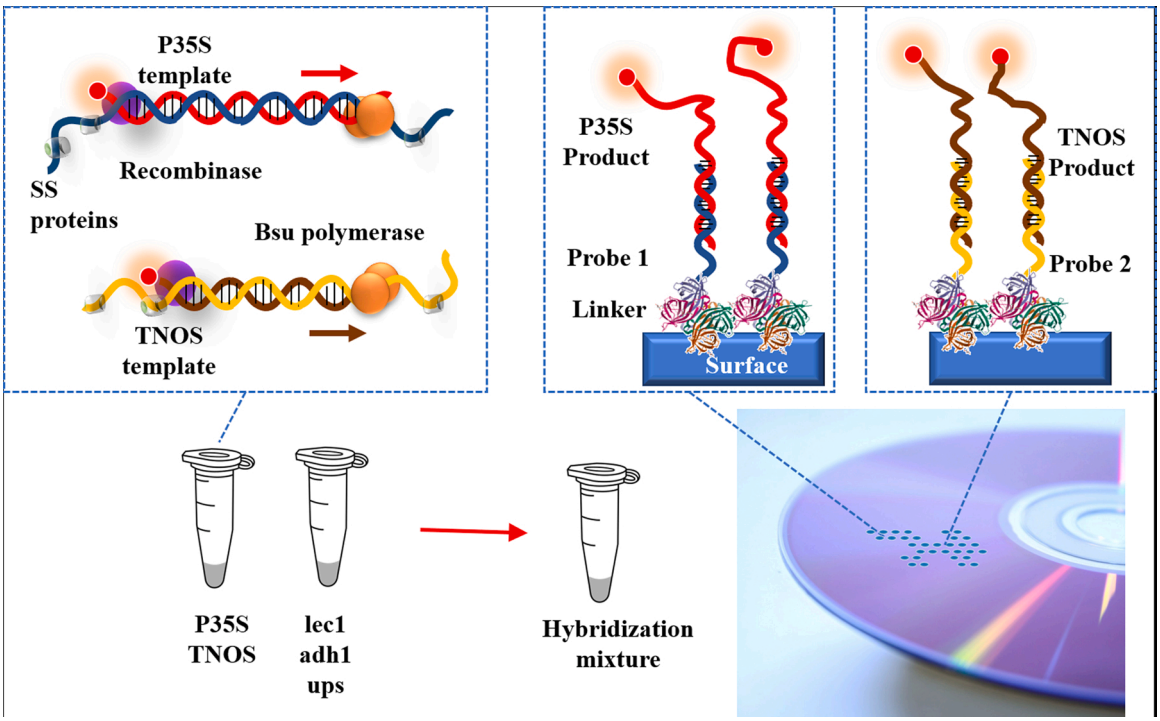

Fig. 1. Scheme of the assay for GMOs detection based on multiplex RPA amplification (left) and hybridisation assay in the array format (right).

measurements in a microtitre plate reader (Wallac, model Victor 1420 multilabel counter, Finland). The RPA products were purified by silica-gel membrane adsorption (PCR purification kit, Jena Bioscience, Germany). An aliquot was mixed with loading buffer and the solution was loaded on 3 % (w/v) agarose gel. The size of amplicons was determined by comparing to a 50 pb ladder, and electrophoresis was carried out with 1 × TBE buffer at 120 V at room temperature. Gels were stained for 30 min with 1 × TBE containing SYBR-Safe at 0.01 % (v/v), and bands were visualised on a UV transilluminator.

3.2. PCR-ELISA

The single-PCR mixtures (25 μL) contained 15 ng of extracted genomic DNA, 1× Tris–KCl buffer (100 mM Tris–HCl, 500 mM KCl, pH 8.3), 2 mM of $MgCl_2$, 300 μM of dNTPs, 1.25 units of Taq DNA polymerase (Roche, Germany), 300 nM of the 5′-digoxigenin forward primer and 300 nM of the reverse primer. Reactions took place in a Bibby Scientific (Staffordshire, UK) TC-400 thermal cycler programmed for an initial denaturation step of 5 min at 95 °C, and 40 cycles consisting of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C and a final extension of 5 min at 72 °C.

The biotinylated probes (20 nM) were immobilised on 96-well microtitre ELISA plates (Corning, USA) as described in Santiago-Felipe et al., 2014. PCR products (2 μL) were mixed with 99 μL of hybridisation buffer and heated at 95 °C for 5 min. Then the denatured products (100 μL) were dispensed into each well and incubated at 37 °C for 45 min. Immunostaining was achieved by employing an antidigoxigenin antibody labelled with horseradish peroxidase solution (1:2000) at room temperature for 25 min. The colorimetric substrate was tetramethylbenzidine and hydrogen peroxide (0.002 M) in citrate buffer (pH 5.5). After incubating for 10 min, the reaction was stopped with 50 mL of 2.5 M sulphuric acid. Absorbance was measured at 450 nm (reference wavelength: 650 nm) with a microtiter plate reader. A sample was considered positive when the optical response was higher than the cut-off value.

4. Results

4.1. Optimisation of multiplex amplification

The simultaneous amplification of several targets was studied by considering the critical variables. For the oligonucleotide design, primers were chosen from reference methods for their contrasted selectivity (Table SI.1). Non-specific cross-hybridisation was evaluated (Table SI.2). The structural stability of primer-primer duplexes was low in all the possible combinations ($\Delta G > -9$ kcal/mol, $Tm < 42$ °C), which confirmed their compatibility in an integrated reaction.

Compared to multiplex PCR, the RPA reaction mechanism is more restrictive as regards the set of target amplicons to be co-amplified and primer concentrations (Lobato and O'Sullivan, 2018). As DNA target copies differ in food samples, genes were grouped according to their relative abundance by dividing screening elements (duplex: P-35S and T-nos) and plant-specific regions (triplex: *lec*, *adh1* and *ugp* genes). Primer concentrations were studied within the 150–600 nM range. By changing the ratios of the amount of primers used in the reaction also prevented the rapid amplification of one target suppressing the detection of the rest. Therefore, all the targets reached detectable levels before reagents were depleted. The results indicated a ratio of 4:5 for the screening elements and one of 3:4:3 for the plant-specific reaction. Under the selected conditions (Table SI.3), comparable amplification rates were achieved for all the targets. Therefore, the developed method discriminated many different targets without a single target influencing the detection of one of the others, with acceptable sensitivity.

The amplification time was especially examined because of the length of the selected primers. Although they provided excellent reaction yields for the single PCR amplifications, short primers affect the RPA mechanism (Santiago-Felipe et al., 2014; Lobato and O'Sullivan, 2018). Oligonucleotides shorter than 30 nucleotides can still function as hybridisation primers even though their recombinase-mediated strand-invasion activity is slight. The experiments showed that saturation was reached after 50 min of heating reaction phials at 40 °C (Fig. 2a). Hence the recorded amplification kinetics were slower (a 10-minute delay) compared to the single RPA assays or longer primers (>30 nucleotides)

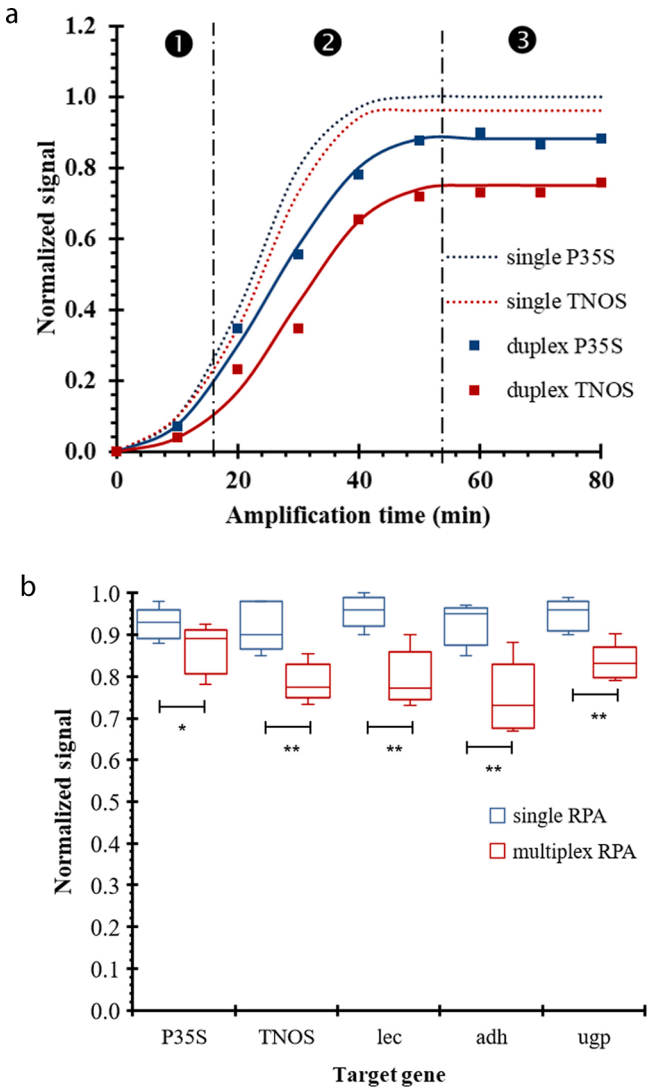

Fig. 2. Single RPA *versus* multiplex RPA (a) Recorded signal of the screening elements according to amplification time: ❶ linear, ❷ exponential, ❸ saturation. Values represented as the mean ± standard deviation (3 replicates). (b) Amount of formed product obtained from fluorescence quantification. * p-value <0.1; ** p-value<0.01.

(Wang et al., 2020a,b; Liu et al., 2020a,b).

The amplified samples were subjected to agarose gel electrophoresis to determine product size (Fig. SI.2). All the reactions generated products of expected lengths according to the proposed primers, with 81, 136, and 88 bp for genes *lec*, *adh1*, and *ugp*, and 123 and 118 bp for P-35S and T-nos, both respectively. Therefore, the experiments demonstrated the feasibility of isothermal amplification in the multiple format for avoiding heterogenous approaches or pre-amplification strategies (Tortajada-Genaro et al., 2015a,b; Li et al., 2020).

4.2. Optimisation of the hybridisation-detection assay

The detection principle of DVD-based biosensing (disc + drive) is described in the Supplementary Information (Fig. SI.3). Briefly, the reading was based on the variation of the disc surface's reflection properties, due to the presence of a solid deposit generated during the biorecognition process. In the presence of the product, the laser beam intensity changes at λ =650 nm in relation to the background signal (polycarbonate layer) recorded by the optoelectronic sensor. After appropriate treatment, the result is an image of the DVD surface with different spot intensities depending on the GMO amplification product concentration.

The proposed DNA assay was applied to detect RPA products following different hybridisation-detection strategies (Fig. 3). The first option (strategy I) consisted in labelling the target sequence during DNA amplification using the forward primer with a reporter (digoxigenin at 5′-end) to incorporate the sequence during replication, and later the simple hybridisation. The second option (strategy II) was based on a competitive hybridisation format between the unlabelled amplification product and an oligonucleotide complementary to the probe called a competitor. The third option (strategy III) was based on a competitive hybridisation format between the unlabelled amplification product and a nanoparticle with an oligonucleotide complementary to the probe. In this case, the specific nanogold oligo-functionalised particles were synthesised (Fig. SI.4) for direct silver enhancement to lead to a detectable solid product. Three approaches generated a resistant deposit to washing that remained stable over time without having to use fixing solutions and to, consequently produce a microarray image. The best responses were obtained with labelling during the amplification and simple hybridisation (strategy I) when considering both sensitivity and reproducibility.

Following the selected biorecognition strategy, the rest of the assay variables were revised by considering the thermodynamic properties of GMO sequences (Table SI.4). For this purpose, the immobilisation of probes, the hybridisation with the labelled target amplified products and chip staining were studied. The resulting array fulfilled the optical sensing requirement for spot features and signal-to-noise ratios (Tortajada-Genaro et al., 2015a,b; Yamanaka et al., 2017; Hwu and Boisen, 2018).

The products obtained from the multiple RPA reactions were detected by the developed methodology by identifying taxa and screening elements from pure flours or non-GMO materials. The amplified copies of the triplex and duplex RPA assays were hybridised on DVD surfaces in two arrays. Selective recognition was achieved by observing a high-intensity spot for the probes associated with the correspondent probe (Fig. SI.5 and SI.6). Both assays were integrated into a single array (9 × 9 pattern) as described in Fig. 1. According to the *t*-test (p-value < 0.05), the 5-plex hybridisation assay provided comparable spot intensities to previous approaches (3-plex and 2-plex). This method brought about a marked reduction in reagents, fast reaction times and high-throughput detection.

4.3. Analytical performances

The multiplex RPA/DVD-based assay was evaluated for the analysis of food mixtures. As non-specific amplification and cross-hybridisation would greatly impair the reliability of the detection results in the multiple assay, specificity was firstly examined. No unbiased amplification was detected, not even for the mixtures in which a GMO was present in a small amount and other GMO were in excess. Assay reproducibility, expressed as relative standard deviation, was determined from the optical density of the spots from samples (0.5 % GMO content) after each one was analysed in triplicate in three DVDs. Intraday deviation varied from 7% to 16 % and interday deviation from 8% to 20 %, which was lower than the 30 % acceptance criterion.

Regarding high-throughput capability, the number of samples that can be simultaneously determined in a single run. For array-based assay, the number of samples depends on the chip dimensions and the spot density. The sensing region of a standard DVD goes from a radius of 2 cm to one of 12 cm, with a usable surface of about 100 cm^2. A single disc can have approximately 6300 assays when considering 500 μm-diameter spots. As proof-of-concept, the disc was segmented for the simultaneous analysis of eight samples (or replicates) containing 9 × 9 arrays (646 spots), which is 6.75-fold higher than the assays performed in 96-plates. Due to the low required volume for spotting (50 nL per spot), the amount of reagent per assay is 10-fold lower, being the required time

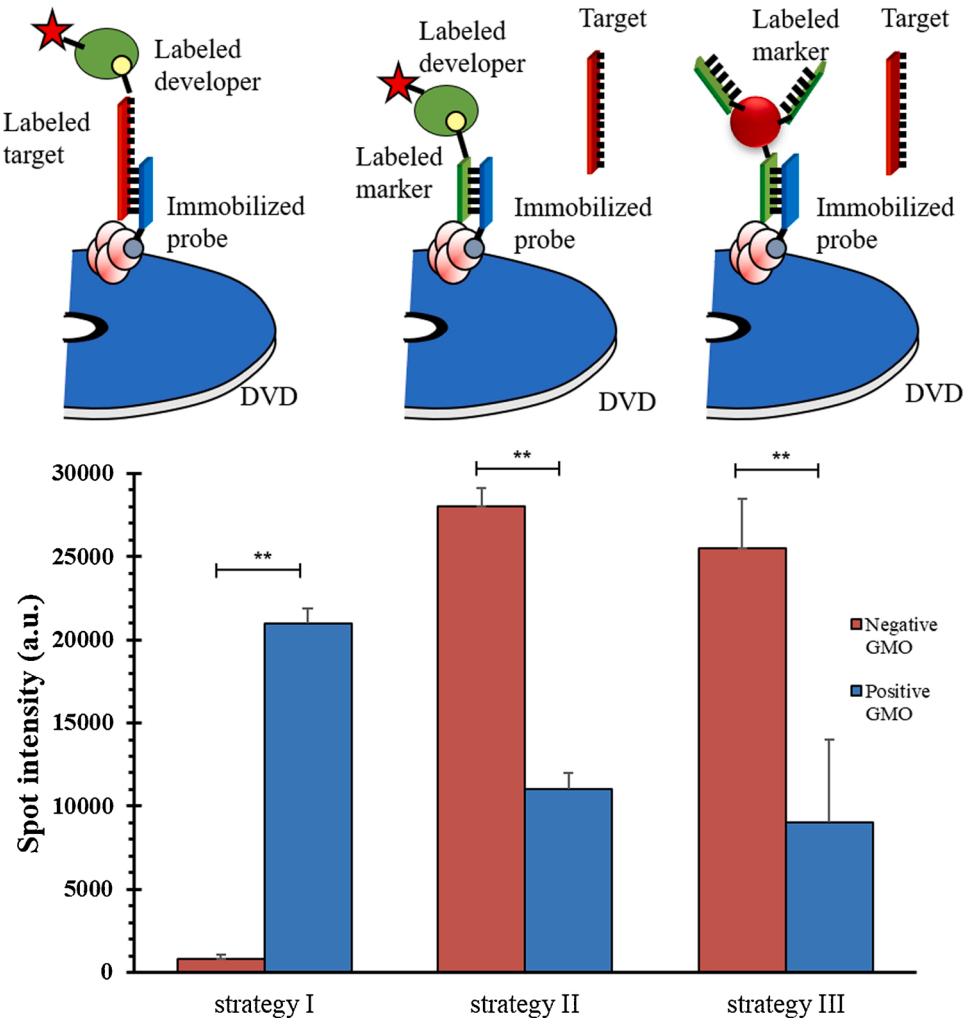

Fig. 3. Comparison of labelling strategies for DVD biosensing. I: simple hybridisation of the labelled amplification products (digoxigenin); II: indirect competitive hybridisation of the amplification products and labelled marker (digoxigenin); III: direct competitive hybridisation of the amplification products and nanogold-labelled marker. **: p-value < 0.01.

similar (Santiago-Felipe et al., 2014).

Multiplex assay sensitivity was determined in two ways: one by analysing the serially diluted DNA extracted from transgenic food; another by analysing the DNA extracted from a food sample by adding certain amounts of transgenic ingredient. In both cases, the free non-transgenic food samples were selected for obtaining blank responses. For the first approach, the amount of DNA extracted from the tested GMOs varied from 0 ng to 50 ng, and a total template of 50 ng was left per reaction. The signal-to-noise ratio calculated from the background signal was obtained, and positive responses were reported for a larger amount than 0.04 ng, which is equivalent to 17 copies (mean corn genome 2198 Mb). This sensitivity is consistent with previous studies of event-specific PCR assays, which report a detection limit of 10–20 initial template copies per reaction (Marmiroli et al., 2008; Peng et al., 2016).

For the second approach, multiplex method sensitivity was assessed by simultaneously determining samples with lowering concentrations of transgenic foods in relation to non-GMO foods from 0 % (w/w) to 20 % (w/w). The same mixtures were analysed by the PCR-ELISA method (Fig. 4). Adequate dynamic ranges were obtained for both approaches. Our experimental data indicated than multiplex-RPA was slightly less reproducible than single approach based on PCR-ELISA. The relative standard deviation was 16 % and 12 %, respectively. The detection limits were the smallest amount of transgenic ingredient capable of producing a signal that could be distinguished from the non-GMO food (signal-to-noise ratio >3). For the screening elements (P-35S and T-nos), the values were 0.2 % and 0.08 % for the RPA/DVD assay and PCR-ELISA, respectively. Thus, the system can reliably detect the relative GMO content to monitor, e.g., the European 0.9 % labelling system, which is a very restrictive regulation. The proposed technology has the potential to offer important contributions via the parallel detection of multiple GMO-related sequences per sample.

By way of conclusion, analytical performances were better than or comparable to the multiple PCR and isothermal amplification techniques (Park et al., 2015; Santiago-Felipe et al., 2014; Tortajada-Genaro et al., 2015a,b; Kaygusuz et al., 2019; Zhang et al., 2020). The main advantage was the multiplexing capability in relation to previously published studies on RPA (Wang et al., 2017, 2020a,b; Liu et al., 2020a, b).

4.4. Analysis of food samples

The proposed system's performance was validated by analysing certified reference materials. The screening elements and host genome were detected according to the recorded pattern (Fig. 5). The transgenic material of soya bean (Round ReadyTM Soya, ERM-410dk) provided a signal for both the promotor and terminator elements and the soya bean-specific gene (*lec*). The transgenic materials of corn were corn Bt176 (ERMBF411), corn Bt11 (ERM-BF412b and ERM-BF412f), corn GA21

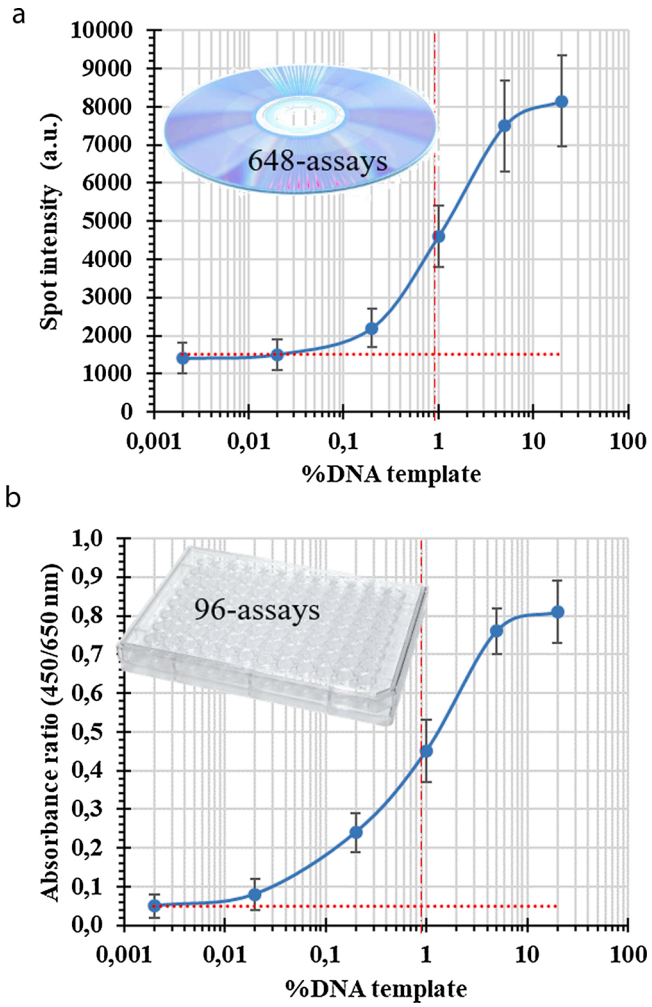

Fig. 4. Comparison of GMO detection capability based on multiplex-RPA/DVD biosensing (a) and the single PCR-ELISA method (b). Dotted lines indicate the corresponding detection limit and the labelling limit as regulated in the EU (0.9 % of GMO).

(ERM-BF414b) and corn MON810 (ERM-BF413ck). In all the assays, the corn-specific gene (*adh1*) was the only one detected among the plant-specific genes. As expected, the promotor was identified in three variants (Bt176, Bt11, MON810) and the terminator in two variants (Bt11 and GA21). These results agreed with the single PCR-based method (Table SI.5).

The method was applied for simultaneously screening in commercial food samples and samples with fortified transgenic ingredients to demonstrate its capability as a safety control tool. The developed technology was fast enough (< 3 h) to be run in routine screening. The output was eight images per disc (9 replicated spots), including five targets and internal quality controls (Fig. SI.7). Regarding taxa, the negative results were obtained for all the samples with no target ingredients (32 cases) (Table 1). The targeted genes were detected in all cases, even at trace levels (10 cases). In the food samples containing two transgenic ingredients or more, positive responses were detected on the expected probe (promoter, terminator, or both). These experiments confirmed the absence of matrix effects, exclusivity against GMO materials and the capability to work with short DNA fragments in highly processed food.

Demanded GMO detection specification in a routine diagnostic laboratory is excellent coverage against the growing number of variants. These studies have demonstrated that the developed method can be used as a screening tool to identify the presence of the most frequent promotor and terminator. Thus, it is quite likely that unknown GMOs will be detected (Qian et al., 2018; Shang et al., 2020). Additionally, the presence of specific genes related to abundant genetically modified plants supports the source identification (Querci et al., 2010). This multiplexing approach can narrow down the group of possible events being present by guiding additional testing based on reference methods (Huang et al., 2015; Wang et al., 2020b). The proposed methodology is flexible enough to improve the number of GMOs that can be detected

Table 1
Analysis of the commercialised food products and food mixtures.

	GMO content	P-35S	T-nos	*lec*	*adh1*	*ugp*
Wheat pasta	Not declared	–	–	–	–	–
Cereal bar	Not declared	–	–	–	–	–
Ketchup	Not declared	–	–	–	+	–
Vegetable soup	Not declared	–	–	+	–	–
Feed	Declared	+	–	–	+	–
Snack	Not declared	–	–	–	–	–
Snack + Bt11	5%	+	+	–	+	+
Biscuits	Not declared	–	–	–	–	–
Biscuits	5%	+	+	–	+	–
Biscuits	2%	+	+	–	+	–
Biscuits	0.5 %	+	–	–	+	–
Tomato	Declared	+	+	–	–	–
Soya sauce	Not declared	+	–	+	–	–
Soya sauce + RRS	2%	+	+	+	–	–

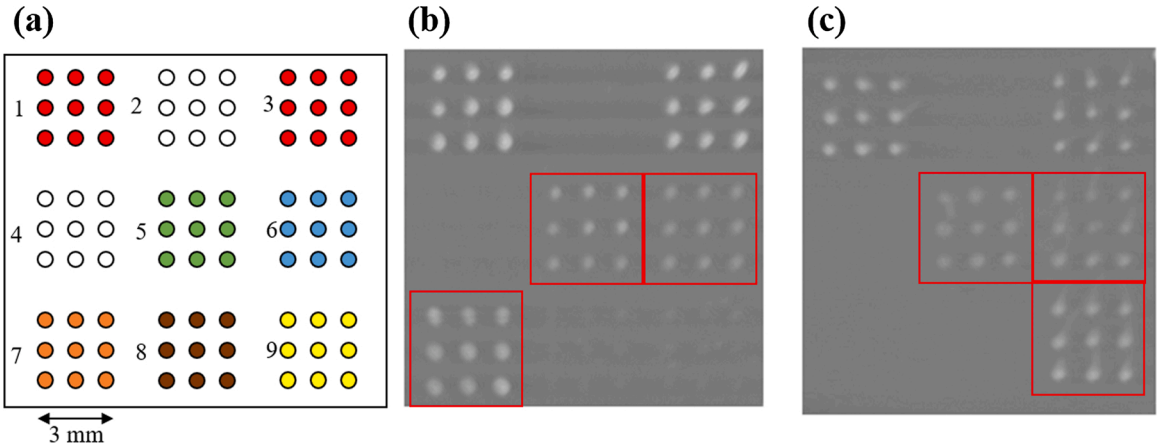

Fig. 5. Application to the analysis of certified reference materials. (a) Array layout. 1: positive control (hybridisation), 2: negative control (hybridisation), 3: positive control (staining), 4: negative control (staining), 5: terminator T-nos, 6: promotor P-35S, 7: soya bean, 8: potato, 9: corn. (b) DVD image for soya bean RRS 5%. (c) DVD image for corn Bt-11 5%.

and identified in a single technology. The following research step involves incorporating more sequences, including construct or event specific targets.

5. Conclusions

Reliable analytical methods are required to comply with regulations, and also for routine analyses throughout the food chain. Isothermal amplification methods provide interesting solutions for the fast screening of GMOs close to points-of-need. However, most described approaches provide information about a single target element per assay, which limits their coverage against authorised and non-authorised GMOs. Furthermore, despite the advantages of sensitive detection techniques, especially optical ones, they generally require expensive equipment, which is difficult to miniaturise, and are neither portable nor user-friendly. Thus, innovative solutions are still required for massive food safety control.

To address these issues, multiple RPA was explored and optimised for the reliable detection of the main GMO screening elements. This gave a method that simplifies the assay as reactions are shorter (1 h) and at a low constant temperature (37 °C), which minimises the instrumental complications generally associated with PCR or other isothermal reactions, including previous RPA-based methods. In this way, expensive thermal cyclers can be avoided and replaced with more widely used equipment, such as heaters, ovens, etc. Moreover, DVD-biosensing offers the advantages of consumer electronics (low-cost, simple-to-use, precise focusing, high-fidelity) for the selective recognition of RPA products. Consequently, the achieved detection limits are better than naked-eye approaches or other detection technologies, such as lateral flow strips, and are cheaper than fluorescence scanners.

In short, the complete system, including amplification reagents and instrument-software for readouts, can be considered a cost-efficient screening assay, e.g., a complementary tool to reference methods in the farm-to-fork strategy.

Author contributions section

L.A. Tortajada-Genaro: Conceptualization; Investigation; Data curation; Writing. **A. Maquieira**: Supervision.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jfca.2021.104083.

References

Ahmed, M.U., Saito, M., Hossain, M.M., Rao, S.R., Furui, S., Hino, A., et al., 2009. Electrochemical genosensor for the rapid detection of GMO using loop-mediated isothermal amplification. Analyst 134 (5), 966–972.

Brod, F.C.A., van Dijk, J.P., Voorhuijzen, M.M., Dinon, A.Z., Guimarães, L.H.S., Scholtens, I.M., et al., 2014. A high-throughput method for GMO multi-detection using a microfluidic dynamic array. Anal. Bioanal. Chem. 406 (5), 1397–1410.

Del Río, J.S., Lobato, I.M., Mayboroda, O., Katakis, I., O'Sullivan, C.K., 2017. Enhanced solid-phase recombinase polymerase amplification and electrochemical detection. Anal. Bioanal. Chem. 409 (12), 3261–3269.

Huang, L., Zheng, L., Chen, Y., Xue, F., Cheng, L., Adeloju, S.B., Chen, W., 2015. A novel GMO biosensor for rapid ultrasensitive and simultaneous detection of multiple DNA components in GMO products. Biosens. Bioelectron. 66, 431–437.

Hwu, E.E.T., Boisen, A., 2018. Hacking CD/DVD/Blu-ray for biosensing. ACS Sens. 3 (7), 1222–1232.

Kaygusuz, D., Vural, S., Aytekin, A.Ö., Lucas, S.J., Elitas, M., 2019. DaimonDNA: a portable, low-cost loop-mediated isothermal amplification platform for naked-eye detection of genetically modified organisms in resource-limited settings. Biosens. Bioelectron. 141, 111409.

Lázaro, A., Yamanaka, E.S., Maquieira, A., Tortajada-Genaro, L.A., 2019. Allele-specific ligation and recombinase polymerase amplification for the detection of single nucleotide polymorphisms. Sens. Actuators B Chem. 298, 126877.

Li, K., Luo, Y., Huang, K., Yang, Z., Wan, Y., Xu, W., 2020. Single universal primer recombinase polymerase amplification-based lateral flow biosensor (SUP-RPA-LFB) for multiplex detection of genetically modified corn. Anal. Chim. Acta 1127, 217–224.

Liu, H., Wang, J., Li, P., Bai, L., Jia, J., Pan, A., et al., 2020a. Rapid detection of P–35S and T-nos in genetically modified organisms by recombinase polymerase amplification combined with a lateral flow strip. Food Control 107, 106775.

Liu, H., Wang, J., Zeng, H., Liu, X., Jiang, W., Wang, Y., et al., 2020b. RPA-Cas12a-FS: a frontline nucleic acid rapid detection system for food safety based on CRISPR-Cas12a combined with recombinase polymerase amplification. Food Chem. 334, 127608.

Lobato, I.M., O'Sullivan, C.K., 2018. Recombinase polymerase amplification: basics, applications and recent advances. Trac Trends Anal. Chem. 98, 19–35.

Marmiroli, N., Maestri, E., Gulli, M., Malcevschi, A., Peano, C., Bordoni, R., De Bellis, G., 2008. Methods for detection of GMOs in food and feed. Anal. Bioanal. Chem. 392 (3), 369–384.

Park, S.B., Kim, H.Y., Kim, J.H., 2015. Multiplex PCR system to track authorized and unauthorized genetically modified soybean events in food and feed. Food Control 54, 47–52.

Peng, C., Wang, P., Xu, X., Wang, X., Wei, W., Chen, X., Xu, J., 2016. Development of a qualitative real-time PCR method to detect 19 targets for identification of genetically modified organisms. Springerplus 5 (1), 1–6.

Qian, C., Wang, R., Wu, H., Ping, J., Wu, J., 2018. Recent advances in emerging DNA-based methods for genetically modified organisms (GMOs) rapid detection. Trac Trends Anal. Chem. 109, 19–31.

Querci, M., Van den Bulcke, M., Žel, J., Van den Eede, G., Broll, H., 2010. New approaches in GMO detection. Anal. Bioanal. Chem. 396 (6), 1991–2002.

Santiago-Felipe, S., Tortajada-Genaro, L.A., Puchades, R., Maquieira, A., 2014. Recombinase polymerase and enzyme-linked immunosorbent assay as a DNA amplification-detection strategy for food analysis. Anal. Chim. Acta 811, 81–87.

Shang, Y., Zhang, B., Zhu, L., Huang, K., Xu, W., 2020. A novel quantitative technique in detecting stacked genetically modified plants by fluorescent-immunohistochemistry. J. Food Compos. Anal. 88, 103452.

Shao, N., Chen, J., Hu, J., Li, R., Zhang, D., Guo, S., et al., 2017. Visual detection of multiple genetically modified organisms in a capillary array. Lab Chip 17 (3), 521–529.

Singh, M., Pal, D., Sood, P., Randhawa, G., 2019. Loop-mediated isothermal amplification assays: rapid and efficient diagnostics for genetically modified crops. Food Control 106, 106759.

Tortajada-Genaro, L.A., Rodrigo, A., Hevia, E., Mena, S., Niñoles, R., Maquieira, Á., 2015a. Microarray on digital versatile disc for identification and genotyping of Salmonella and Campylobacter in meat products. Anal. Bioanal. Chem. 407 (24), 7285–7294.

Tortajada-Genaro, L.A., Santiago-Felipe, S., Amasia, M., Russom, A., Maquieira, Á., 2015b. Isothermal solid-phase recombinase polymerase amplification on microfluidic digital versatile discs (DVDs). RSC Adv. 5 (38), 29987–29995.

Von Götz, F., 2010. See what you eat—broad GMO screening with microarrays. Anal. Bioanal. Chem. 396 (6), 1961–1967.

Wang, R., Zhang, F., Wang, L., Qian, W., Qian, C., Wu, J., Ying, Y., 2017. Instant, visual, and instrument-free method for on-site screening of GTS 40-3-2 soybean based on body-heat triggered recombinase polymerase amplification. Anal. Chem. 89 (8), 4413–4418.

Wang, X., Chen, Y., Chen, X., Peng, C., Wang, L., Xu, X., et al., 2020a. A highly integrated system with rapid DNA extraction, recombinase polymerase amplification, and lateral flow biosensor for on-site detection of genetically modified crops. Anal. Chim. Acta 1109, 158–168.

Wang, X., Xie, S., Chen, X., Peng, C., Xu, X., Wei, W., et al., 2020b. A rapid and convenient method for on-site detection of MON863 corn through real-time fluorescence recombinase polymerase amplification. Food Chem. 324, 126821.

Yamanaka, E.S., Tortajada-Genaro, L.A., Maquieira, Á., 2017. Low-cost genotyping method based on allele-specific recombinase polymerase amplification and colorimetric microarray detection. Microchim. Ichnoanal. Acta 184 (5), 1453–1462.

Zhang, M., Ye, J., He, J.S., Zhang, F., Ping, J., Qian, C., Wu, J., 2020. Visual detection for nucleic acid-based techniques as potential on-site detection methods. A review. Anal. Chim. Acta 1099, 1–15.