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RECOMBINASE POLYMERASE AND ENZYME-LINKED IMMUNOSORBENT ASSAY AS A DNA AMPLIFICATION-DETECTION STRATEGY FOR FOOD ANALYSIS

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ABSTRACT

Polymerase chain reaction in conjunction with enzyme-linked immunosorbent assay (PCR-ELISA) is a well-established technique that provides a suitable rapid, sensitive, and selective method for a broad range of applications. However, the need for precise rapid temperature cycling of PCR is an important drawback that can be overcome by employing isothermal amplification reactions such as recombinase polymerase amplification (RPA). The RPA–ELISA combination is proposed for amplification at a low, constant temperature (40 ºC) in a short time (40 min), for the hybridisation of labelled products to specific 5'-biotinylated probes/streptavidin in coated microtiter plates at room temperature, and for detection by colorimetric immunoassay. RPA-ELISA was applied to screen common safety threats in foodstuffs, such as allergens (hazelnut, peanut, soybean, tomato, and maize), genetically modified organisms (P35S and TNOS), pathogenic bacteria (*Salmonella* spp. and *Cronobacter* spp.), and fungi (*Fusarium* spp.). Satisfactory sensitivity and reproducibility results were achieved for all the targets. The RPA-ELISA technique does away with thermocycling and provides a suitable sensitive, specific, and cost-effective method for routine applications, and proves particularly useful for resource-limited settings.

Keywords: *isothermal amplification; ELISA; allergen; GMO; pathogen; food safety*

1. Introduction

Analytical methods for fast, reliable, sensitive and cost-effective detection are highly demanded in many areas, including food safety. Methods based on the detection of nucleic acids offer interesting benefits because DNA molecules show constant concentrations, stability, and better extraction yields (even from processed and heat-treated samples) than proteins [1-2]. Furthermore, the amplification by polymerase chain reaction (PCR) ensures the required sensitivity levels. Consequently in recent years, the PCR-based methods, e.g. RT-PCR, digital PCR or microarray, are the gold standard for the analysis of nucleic acids [2-4].

The demand for sequence-specific approaches that do not require laborious or expensive detection technologies has led to the hyphenation of PCR with enzyme-linked immunosorbent assay (PCR-ELISA) [5-7]. This combines the high selectivity of DNA-based methods with ELISA sensitivity. This integration involves the hybridisation of labelled amplification products to specific captured probes in each microtiter well, as well as their immunodetection. Although sensitivity can be improved using fluorometric or chemiluminiscent substrates, PCR-ELISA methods normally employ colorimetric detection because better reproducibility, cost-by-assay, and stability are achieved [6-8]. Therefore, colorimetric PCR-ELISA is a method that is capable of processing up to 96 or 384 assays simultaneously, it is potentially automatable, and only requires the basic instruments present in any diagnostic laboratory.

The thermal PCR technique has its limitations, such as requiring precise temperature control and rapid thermocycling steps among the temperatures of dissociation (95 ºC), annealing (55-65 ºC) and elongation (70 ºC) [6]. Yet the use of other enzymes (or a combination of enzymes) to mimic DNA replication *in vivo* has emerged as a solution to conventional PCR polymerases [9-10]. At the moment, two isothermal amplification reactions in combination with ELISA have been described: loop-mediated isothermal amplification (LAMP-ELISA) [11] and helicase-dependent amplification (HDA-ELISA) [12]. They do not require expensive amplification equipment and have been seen to be very flexible and capable of simultaneously processing up to several hundreds of samples in a few hours. However, these reactions are performed far from room temperature (60 – 65 ºC), require an initial denaturation step at 95 ºC (HDA) or need complex primers design (LAMP).

An innovative isothermal amplification called recombinase polymerase amplification (RPA) offers interesting advantages [13-14]. This technique facilitates the

binding of oligonucleotide primers to template DNA. Primers are elongated by a strand-displacing DNA polymerase, called *Bsu* polymerase, while single-stranded DNA-binding proteins stabilise amplification reaction intermediates. Compared to other amplification enzymes, *Bsu* polymerase maintains similar activity in inhibiting environments, requires a shorter incubation time, operates at lower temperatures, is easy to use and the amplified products do not need post-amplification treatment. The integration of DNA amplification and the detection step entails adjusting several critical variables. For instance, RPA buffer contains Carbowax 20M, a high-molecular-weight polyethylene glycol, as a crowding agent to influence the recombinase kinetics. However, it is known that these molecules may affect the solubility, melting temperature or viscosity of the reaction mix, and hence the amplified product [15].

In this study, the hyphenation of the RPA and ELISA methodologies (RPA-ELISA) is developed for food safety applications. The technical implementation of integrated screening methods in food industry can vastly help to simplify the process and to reduce costs, thus making analytical procedures friendlier [1]. Here the simultaneous detection of different common food threats is proposed. Hazelnut, peanut and soybean have been selected as representative examples of the allergens included in priority lists [16]. Tomato and maize have also been included because there is growing concern about their allergenicity associated with their current widespread use [17-18]. Promoter 35S and terminator NOS are widely used for screening genetically modified organisms (GMOs) since seventy-two percent of GMOs contains at least one of these sequences [19-20]. *Salmonella* spp., *Cronobacter* spp. are frequently detected as being responsible for food contamination. According to the Food and Agriculture Organization of the United Nations and the World Health Organization, they are considered to be included among the most relevant pathogens and their absence is required in food safety analysis [21-22]. Finally, *Fusarium spp.* is one of most frequent fungi found in food and feed derivates, and it produces mycotoxins that cause serious health problems in both humans and livestock [23-24].

2. Experimental

2.1 Target genes

The selected genes for hazelnut, peanut, soybean, tomato, maize, GMO promoter-P35S, GMO terminator-TNOS, *Salmonella spp*., *Cronobacter spp.*, and *Fusarium spp.* are shown in Table 1. All the primers and probes were successfully checked for relevant homologies by a BLASTNr search (http://blast.ncbi.nlm.nih.gov/).

2.2 Bacterial and fungal strains, foodstuff and DNA extraction

Salmonella typhimurium group B (CECT 443) and *Cronobacter sakazakii* (ATCC BBA-894) were used as reference strains. They were isolated and provided by the GAIKER Technology Centre (Bizkaia, Spain). Viable samples were obtained by overnight culture on nutrient agar plates (0.5% Peptone, 0.3% beef extract, 1.5% agar, 0.5% NaCl, pH 7) at 37 ºC. Bacterial inoculation assays were prepared by adding 10-fold serial dilutions of an 18-hour culture of each pathogen in sterile saline solution (0.8% NaCl), covering a range from 0 to $4 \cdot 10^4$ CFU mL^{-1}. *Fusarium moniliforme* (CECT 2982) was used as the reference fungal strain. It was isolated and provided by the Instituto Agroforestal Mediterráneo (IAM), Universitat Politècnica de València (UPV). Viable samples were obtained by culturing 4 days on nutrient agar plates at 25ºC. Fungi inoculation assays were prepared by adding fungal mycelium (10^3-10^5 µg of mycelium per g of food) from a 4-day culture. The certified reference materials (CRM) containing 0.05% of transgenic Bt11 maize (ERM-BF412f) and 0.01% of transgenic RRS soybean (ERM-BF410gk) were purchased from the Institute for Reference Material and Measurements (Geel, Belgium). Food products were bought in local stores. Genomic DNA was extracted from bacterial cultures, fungal mycelium, and food samples using the DNeasy Blood & Tissue Kit (Qiagen, Inc., CA).

Inoculation assays were assessed after taking into account their common concentration in contaminated foods (e.g., *Salmonella* spp. $>10^2$ CFU/mL) [28].

2.3 RPA-ELISA

RPA assays were carried out in a total volume of 25 µL using the TwistAmp Basic kit (TwistDX, Cambridge, UK). Reactions contained 480 nM of each 5'-digoxigenin labelled primer (Table 1), 15 ng of genomic DNA, 14 mM of Mg acetate, and 1× rehydration buffer. Firstly, all the reagents except for the DNA template and Mg

acetate were prepared in a master mix, which was distributed into each 0.2 mL reaction tube containing the enzyme and the nucleotides in a dried pellet. Then, DNA was added into the tubes, and Mg acetate was dispensed lastly. Since the RPA reaction starts as soon as magnesium is added, the tubes were immediately placed into a heating oven (Memmert, model UF30, Germany) at 40 ºC for 40 min.

Amplification products were analysed in 96-well microtiter ELISA plates (Corning, USA). For this purpose, 100 µL of streptavidin (0.2 mg L^{-1}) and biotinylated probes (20 nM), diluted in coating buffer (50 mM carbonate buffer, pH 9.6) and were incubated overnight at 4 ºC. Double labelled oligonucleotide (5'-biotin and 3'-digoxigenin) was used as the positive control and non-target biotinylated oligonucleotide was the negative control (not complementary to any target). Microtiter plates were washed 3 times with PBS-T (phosphate-buffered saline containing 0.05% (v/v) tween 20, pH 7.4) plus deionised water, and were stored at 4 ºC to become stable at less 1 month. Amplified products (1 µL) were mixed with 99 µL of 5× hybridisation buffer (SSC, 1× saline sodium citrate: NaCl 150 mM, sodium citrate 15 mM, pH 7) and heated at 95 ºC for 5 min to denature into single strains. Then denatured products (100 µL) were dispensed into each well and incubated at 37 ºC for 45 min. After washing the plate 3 times with PBS-T and deionised water, 100 µL per well of anti-digoxigenin antibody labelled with horseradish peroxidase (anti-Dig-HRP) solution in PBS-T (1:2000) were dispensed and incubated at room temperature for 25 min. After a washing step with PBS-T and deionised water, 100 µL of TMB solution (0.25 g L^{-1} of 3, 3', 5, 5'-tetramethylbenzidine and 0.002 M of hydrogen peroxide in citrate buffer, pH 5.5) were dispensed and incubated at room temperature for 10 min. Finally, the reaction was stopped with 50 µL of 2.5 M sulphuric acid and absorbance was measured at 450 nm (reference wavelength: 650 nm) with a microtiter plate reader (Wallac, model Victor 1420 multilabel counter, Finland). A sample was considered positive when the optical response was higher than the cut-off value.

2.4 PCR-ELISA

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 $MgCl_2$, 200 µM dNTPs, 1.25 units of Taq DNA polymerase (Roche, Germany) and 400 nM of each 5'-digoxigenin labelled primer (Table 1). Reactions were carried out in a TC-400 thermocycler (Bibby Scientific, Staffordshire, UK) and applying the thermal programme: denaturation (95

ºC, 5 min) followed by 40 cycles of denaturation (95 ºC, 30 s), annealing (62 ºC, 30 s) and elongation (72 ºC, 30 s), with a final elongation step (72 ºC, 5 min). The immunoassay was performed as is described above.

2.5 Comparison with other detection techniques

Amplification products were checked by electrophoresis on a 3% (w/v) agarose gel at 110 V and room temperature. Gels were stained for 30 min with 0.5× TBE buffer (Tris/Borate/EDTA) containing fluorophore Real-Safe (Real Laboratories, Spain) at 0.01% (v/v), and bands were visualised with a UV transilluminator. Product size was determined by comparison with a 50 bp ladder (Fermentas, Lithuania). Amplification yields were calculated from the fluorescence measurements with SYBR-Safe at 0.01% (v/v) in a microtiter plate reader.

2.6 Comparison of developing strategies

Other developing immunoassay steps were also tested independently. o-Phenylenediamine dihydrochloride (OPD) was used as an alternative colorimetric substrate to TMB. The addition of digoxigenin-dUTPs (35 µM) in the RPA mixtures was an alternative to obtain labelled amplified products. Three antigen-antibody recognition assays were also compared. The first approach was based on digoxigenin-labelled primers, an anti-digoxigenin antibody produced in sheep (anti-Dig) as a primary antibody at the 1/20,000 dilution, and anti-sheep conjugated with horseradish peroxidase (anti-sheep-HRP) as a secondary antibody at the 1/4,000 dilution. The second approach used Cy5-labelled primers, an anti-Cy5 antibody produced in mouse (anti-Cy5) at the 1/2,000 dilution, and an anti-mouse conjugated with horseradish peroxidase (anti-mouse-HRP) at the 1/500 dilution. The third approach was based on Dig-labelled primers, sheep anti-Dig antibody as a primary antibody at the 1/5,000 dilution, and anti-sheep conjugated with alkaline phosphatase (anti-sheep-AP) as a secondary antibody diluted at 1/250, using the nitro-blue tetrazolium/5-bromo-4-chloro-3'-indolyphosphate solution (BCIP/NBT) as the colorimetric substrate. Data analysis was performed with the statistical package SPSS for Windows, v. 16.0

3. Results and discussion

3.1 Adaptation of the RPA protocol

The reaction conditions (primer concentrations, temperature and time) were studied to achieve the same amplification conditions for ten analytes: hazelnut, peanut, soybean, and maize seeds, tomato fruit, P35S and TNOS from the CRM, and pure cultures of *Salmonella* spp., *Cronobacter* spp., and *Fusarium* spp. The optimal RPA conditions were 480 nM for the forward and reverse primers, and incubation at 40 ºC for 40 min. A single protocol was achieved for the parallel amplification of all tested analytes, which considerably cut the total analysis time. Amplified products were characterised by agarose gel electrophoresis for product size determination. RPA reactions generated the predicted product length, according to the proposed primers, these being: 109 bp for hazelnut, 82 bp for peanut, 81 bp for soybean, 92 bp for tomato, 136 bp for maize, 123 bp for P35S, 118 for TNOS, 152 bp for *Salmonella* spp., 190 bp for *Cronobacter* spp., and 180 bp for *Fusarium* spp.

3.2 Hybridisation assays. Integration of RPA and ELISA

Several factors influence hybridisation efficiency to the specific probe immobilised on the microplate, and later the detection response. For this purpose, multi-variable experimental designs were made to optimise the main parameters during the assays.

The hybridisation process on solid supports, including polystyrene used in microplate wells, depends on probe coverage density. An indirect immobilisation reaction (streptavidin/biotin-labelled probe) was chosen. To that end, coating conditions were optimised by varying the streptavidin concentration from 0.002 to 2 mg L^{-1}, and the probe concentration from 0.2 to 200 nM, and 0.2 mg L^{-1} of streptavidin and the 20 nM probe were selected (Figure 1A). A streptavidin concentration above 0.2 mg L^{-1} increased the signal, but drastically reduced assay reproducibility. Probe coverage was calculated using double labelled oligonucleotides (5'-biotin and 3'-Cy5) and a homemade surface fluorescence reader. Probe density was 0.08 fmol mm^{-2}. Additionally, the hybridisation yield changed according to temperature. Hybridisation time was tested every 10 min for 1 h, and the temperature range was 25-50 ºC. The maximum signal was achieved after 45 min at 37 ºC (Figure 1B and 1C).

The effect of the reaction volume (12.5-200 µL/well) and product dilution (1/50 to 1/5,000) was also evaluated (Figure 1D). The best results were obtained at the 1/100 dilution of amplification product and using 100 µL as the reaction volume. Higher concentrations of amplification product reduced the signal. This effect can be explained by the presence of Carbowax 20M (5%) in the RPA buffer. No other effects of amplification reagents or the washing protocol were observed, so it was unnecessary to perform further treatments with the RPA products in the proposed method. After testing some solutions (water, SSC, PBS-T, and PBS) and cycles (1-5), three cycles with PBS-T and one cycle with deionised water were chosen as the appropriate washing protocol.

3.3 Optimisation of the detection step

The ELISA assays based on the recognition digoxigenin-labelled RPA products by an anti-Dig-HRP antibody were optimised by analysing the DNA extracted from hazelnut, soybean seeds and transgenic maize. The highest signal was achieved at the 1/2,000 dilution, and no significant signal improvement was accomplished at higher concentrations (Figure 1E). The antibody incubation time was optimised, with the best results obtained at 25 min (Figure 1F). Three PBS-T washing steps and further deionised water rising were sufficed to eliminate any excess reagents.

For the immunoenzymatic detection of the hybridisation complex, a colorimetric reaction (λ = 450 nm, $\lambda_{background}$ = 650 nm) was employed by comparing two common HRP substrates, 3,3',5,5'-tetramethylbenzidine (TMB) and o-phenylenediamine dihydrochloride (OPD), at different concentrations up to 2 g L^{-1}. As no remarkable differences were observed during the achieved analytical performances between both substrates, the use of TMB at 0.25 g L^{-1} was selected because it offers advantages, such as low cost, easy manipulation, greater stability and lower toxicity [8].

Alternative strategies to the digoxigenin-labelled primers/anti-Dig-HRP/TMB system were adopted and compared to conventional methods: electrophoresis and in-well-fluorescence by the SYBR-Safe DNA stain (Figure 2). Firstly, digoxigenin-dUTPs in the PCR mixtures were tested as an example of nucleotide labelling. This option, either combined or not with digoxigenin-labelled primers, increases assay sensitivity, but at a slightly higher cost-by-reaction [8]. Secondly, the immunoassay can be performed using different combinations of primary and secondary antibodies. Two approaches were tested: digoxigenin-labelled primers/sheep anti-Dig/anti-sheep-HRP and Cy5-labelled primers/mouse anti-Cy5/anti-mouse-HRP. By keeping digoxigenin as

the label group, the use of anti-Dig-HRP or anti-Dig/anti-sheep-HRP reagents yielded similar results. Yet the incorporation of Cy5 as the labelling group provided significantly poorer responses, probably due to the less effective antigen recognition of the anti-Cy5 antibody. Thirdly, the enzyme conjugated to the antibody is another open field that extends the number of available enzymatic substrates, such as antibodies conjugated with alkaline phosphatase. To that end, the digoxigenin-labelled primers/sheep anti-Dig/anti-sheep-AP/BCIP/NBT approach was tested. The conjugation of antibodies to AP gave worse than HRP conjugation results. The statistics study of the results by an ANOVA test revealed that there were significant differences among the detection formats. All the RPA-ELISA approaches provided better sensitivity than the electrophoresis and fluorescence methods. The three systems based on digoxigenin-labelling and HRP/TMB detection gave better results than those obtained with the Cy5-labelled primers or with AP/BCIP/NBT detection ($F=39.49>F_{6, 14}=2.85$, p-value < 0.05).

3.4 Analytical performances

The analytical performances of RPA-ELISA (selectivity, limit of detection and reproducibility) were established and compared to those of PCR-ELISA. Firstly, assay selectivity was excellent, showing no cross-reactivity in any case, which reinforces its use for screening purposes.

Assay sensitivity was determined by analysing serially diluted DNA extracts. Genomic DNA, extracted from each ingredient (hazelnut, peanut, soybean and maize seeds, fruit tomato and both CRMs) or pure culture (*Salmonella* spp., *Cronobacter* spp., and *Fusarium* spp.), was 10-fold diluted with free-analyte extracts (wheat flour), and the total DNA concentration remained constant (30 ng μL^{-1}). Mixtures were amplified using both methods and were detected by ELISA. The limits of detection for each analyte were 1.3 - 5.3 $\mu g\ g^{-1}$ for ingredients and 6 - 13 CFU mL^{-1} for pathogen cultures without an enrichment step (Table 2). The results were similar, or even better, than others obtained by the techniques available only in full-equipped facilities, such as real-time PCR or DNA microarrays for foods and for microorganisms [4,8,29,30].

Assay reproducibility was also determined from the optical density of replicates. The intra-day and inter-day RSDs for RPA-ELISA were lower than 6.6% and 12.0%, respectively. The results were similar to PCR-ELISA, demonstrating that the isothermal approach is a powerful alternative that does not compromise analysis quality. Therefore,

292 the analytical performance of the RPA-ELISA method mean that it is suitable for
293 routine DNA-based analyses in a broad range of applications.
294
295 *3.5 Analysis of food samples*
296 Twelve commercial foodstuffs were studied in order to evaluate the reliability of
297 the method for its application in large-scale screening. The evaluation set was selected
298 by including several categories and a large variety of food-processing methodologies
299 (e.g., raw, baked, etc.) (Table 3).
300 All the samples were negative for pathogenic bacteria (*Salmonella* spp. and
301 *Cronobacter* spp.) and fungi (*Fusarium* spp.), but the samples from the inoculation
302 assays were positive. Although colorimetric responses increased with analyte
303 concentrations, it was not possible to obtain an exact quantification of the samples,
304 rather only an approximate result was obtained. This was probably due to the variability
305 associated with the DNA extraction process or with the end-point amplification
306 technique. Therefore as previously described, cut-off values were established from the
307 negative control readings [6,12]. The absorbance values higher than or equal to 0.10
308 were considered positive for RPA-ELISA. The occurrence of the different analytes was
309 simultaneously determined in a single plate by common recognition and developing
310 reagents (Table 3). Nevertheless, the naked-eye detection of food threatens is also
311 possible, as shown in Figure 3 (an example of the microplate image). The yellow colour
312 appeared for positive samples, whereas negative samples showed a non-colour.
313 Positive results were observed in all cases for the analytes declared, even at trace
314 levels, or in spiked samples (40/40). Thus, traces of hazelnut in chocolate wafer, soya in
315 chocolate wafer and soup, and maize in cookies gave a signal with low absorbance
316 values (<0.2). Low absorbance signals corresponded to minor ingredients, such as
317 peanut in chocolate wafer or soya in muesli cookies. Greater absorbance signals (>0.5)
318 were recorded for major ingredients such as tomato or maize in ketchup. In the
319 inoculated samples, low absorbance signals were related to concentrations of up to
320 $4 \cdot 10^1$ CFU mL^{-1} in bacteria and of up to 10^3 µg g^{-1} (0.1%) in fungus. This was observed
321 for *Salmonella* spp. in chocolate wafer and for *Fusarium* spp. in ketchup. The higher
322 absorbance values corresponded to concentrations above $4 \cdot 10^3$ CFU mL^{-1} in bacteria,
323 which occurs for *Salmonella* spp. in tomato or feed samples, and for *Cronobacter* spp.
324 in skimmed powdered milk or powdered infant formula. In fungal inoculations, higher

absorbance values corresponded to concentrations of up to 10^5 μg g^{-1} (10%); for example, in muesli cookies or baby food.

Negative results were found in most of the samples declared to be analyte-free (78/80). The one exception was muesli cookies in which, despite not having declared any GMO, positive results were obtained for both the P35S and TNOS analyses. Their detection can be explained because it is not required to declare GMO ingredients for European food labelling unless they are above 0.9% [31].

The reliable and sensitive results achieved indicate that the proposed RPA-ELISA method is useful for the detection of the most important food threats in a broad set of samples.

4. Conclusions

RPA, as an isothermal amplification method, offers numerous advantages. This reaction does away with the need for thermocycling and allows the use of simple technology such as heaters or ovens, is inexpensive and allows minimal maintenance control. Specifically, RPA has proven to have interesting properties, such as tolerance to temperature fluctuations, working near room temperature, cost-effectiveness, short amplification time, reliability and simplicity. Besides, its combination with ELISA for the detection of nucleic acid amplified products offers other advantages, such as sensitivity enhancement. Two other approaches that combine isothermal DNA amplification with ELISA detection have been described. However, RPA has demonstrated to provide equal or better analytical performance with greater simplicity of operation (one single and a lower temperature, and easier primer design).

The present method has demonstrated its usefulness in the food safety area as a screening assay capable of detecting target genes of potential food threats, such as allergens, GMOs, pathogens, or undeclared food intolerance ingredients. This fast, low-cost technology for semi-quantitative analyses has shown excellent analytical performances (selectivity, sensitivity, reproducibility, and high throughput). After the DNA extraction step, the assay can be performed in 2 hours and all the samples can be processed simultaneously with only one amplification condition and the same detection technique. It is worth mentioning that our approach also proves flexible to help significantly increase the number of analyzed samples and/or replicates, or to simultaneously detect agents of a different nature. The results are also obtained by naked-eye examination in some applications. Therefore, the proposed method is

especially suitable for screening applications in point-of-control facilities and does not compromise analytical performance.

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FIGURE CAPTIONS

Figure 1. RPA-ELISA optimization: effect of different experimental variables on optical intensity (A) Coating conditions (streptavidin and probe concentrations); (B) Hybridisation time; (C) Hybridisation temperature; (D) Hybridisation solution (dilution RPA solution and total volume); (E) Developing agent dilution (dilution of anti-Dig-HRP antibody); (F) Developing time (antibody incubation). Signal corresponds to 1.5 ng of target genomic DNA.

Figure 2. Comparison of detection strategies for RPA products. Bt-probe: biotinilated probe; anti-Dig: anti-digoxigenin antibody produced in sheep; anti-sheep: anti-sheep antibody; anti-Cy5: anti-Cy5 antibody produced in mouse; anti-mouse: anti-mouse antibody; HRP horseradish peroxidase; AP: alkaline phosphatase. Signal corresponds to the sensitivity of each format (calculated in CFU/mL), determined by analysing serially diluted DNA extracts from *Salmonella* spp.

Figure 3. Naked-eye results of commercial food samples in microplates. Each sample (row) is tested for each analyte (columns): (1) hazelnut; (2) peanut; (3) soybean; (4) tomato; (5) maize; (6) P35S; (7) TNOS; (8) *Samonella spp.*; (9) *Cronobacter spp.*; (10) *Fusarium spp.* Highlighted rectangles indicate positive samples (absorbance > cut-off value).

TABLE CAPTIONS

Table 1. The primers, probes, and control sequences used for amplification procedures

Table 2. Comparison of limits of detection and reproducibility obtained by RPA-ELISA and PCR-ELISA

Table 3. Screening results of the analytes in commercial food samples analysed by RPA-ELISA

461 **GRAPHICAL ABSTRACT**

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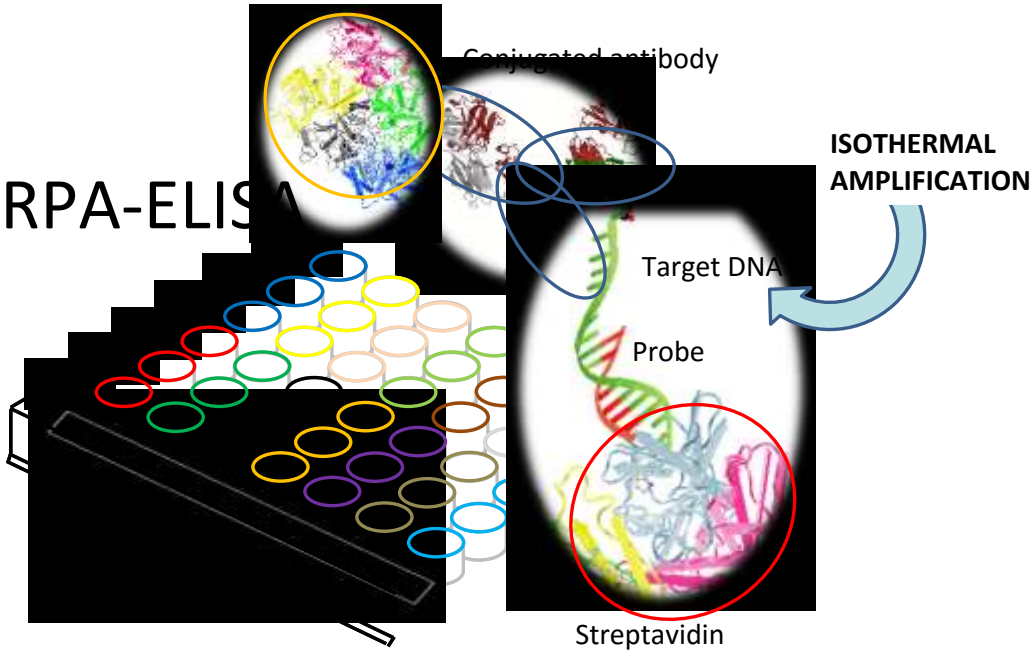

463 Figure 1.

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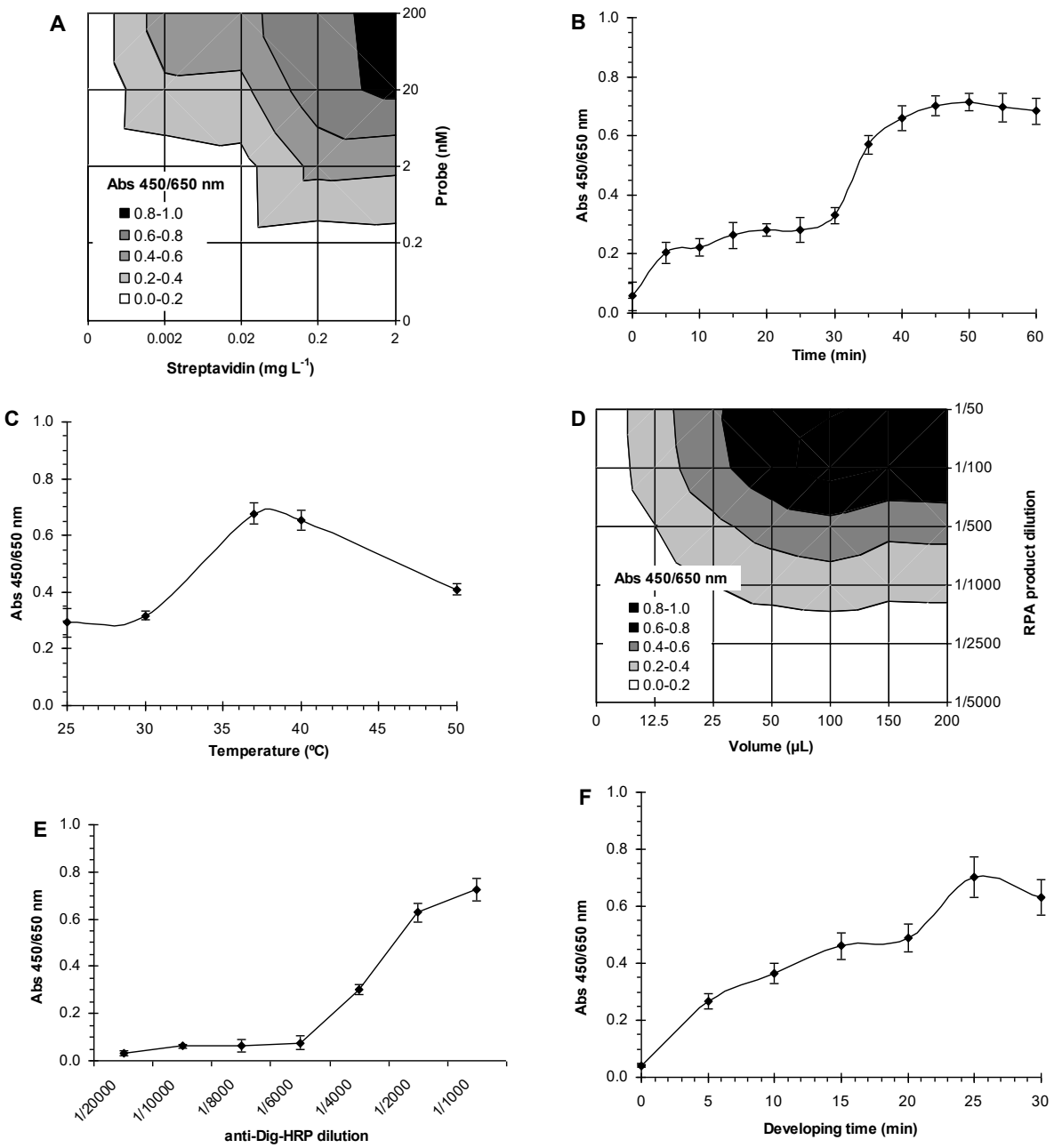

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467 Figure 2

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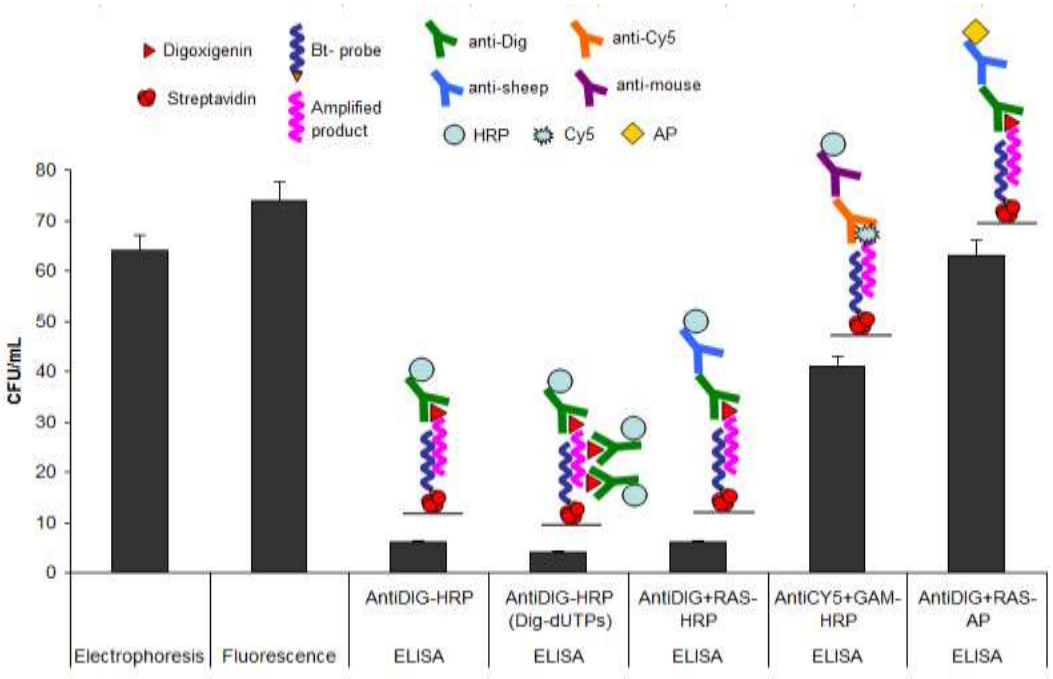
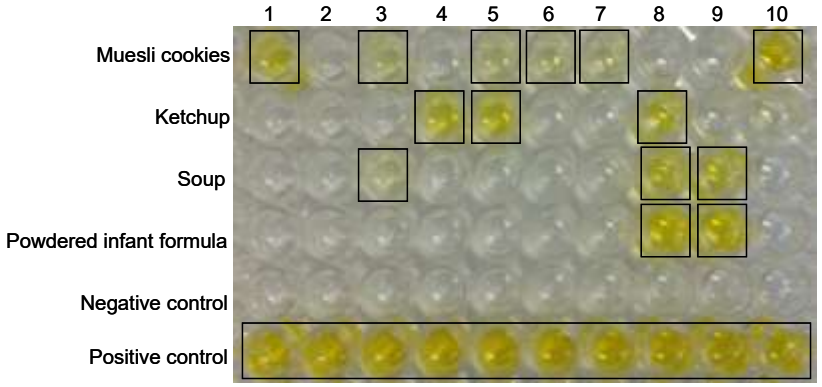


469 Figure 3



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472 **Table 1.**

Target		Sequence 5'-3'	Tm (ºC)	Amplicon size (bp)	Reference
Hazelnut	FP	Dig-ACTACATAAAGCAAAAGGTTGAAG	53.5	109	[4]
Cor a1 gene	RP	TCGTAATTGATTTTCTCCAGTTTG	55.2		
	Probe	BtnTg-TTTTTCGGACAAAGCATCGCCTTCAATCA	67.1		
Peanut	FP	Dig-CTAGTAGCCCTCGCCCTTTT	59.9	82	[4]
Ara h2 gene	RP	GGCATCTTCTGTCTCCTTGG	59.8		
	Probe	BtnTg-TTTTTAGTTCCCACTGCTGCCTC	62.6		
Soybean	FP	Dig-TCCACCCCCATCCACATTT	59.2	81	[4]
Le gene	RP	GGCATAGAAGGTGAAGTTGAAGGA	58.8		
	Probe	BtnTg-TTTTTTTTTTCGAAGCTGGCAACGCTACCGGTT	74.1		
Tomato	FP	Dig-AGACCACGAGAACGATATTTGC	66.8	92	[25]
Lat 52 gene	RP	TTCTTGCCTTTTCATATCCAGACA	58.4		
	Probe	BtnTg-TTTTTACTCTCTTTGCAGTCCTCCCTTGGG	57.6		
Maize	FP	Dig-CGTCGTTTCCCATCTCTTCCTCC	64.2	136	[26]
adh 1 gene	RP	CCACTCCGAGACCCTCAGTC	63.5		
	Probe	BtnTg-TTTTTCCTCACCAGTTACGAAACCAATCGATCCAA	67.1		
GMO promoter	FP	Dig-CCACGTCTTCAAAGCAAGTGG	59.8	132	[27]
35S gene	RP	TCCTCTCCAAATGAAATGAACTTCC	59.7		
	Probe	BtnTg-TTTTTTTATATAGAGGAAGGGTCTTGCGAAGGATA	64.8		
GMO terminator	FP	Dig-GCATGACGTTATTTATGAGATGGG	59.3	118	[27]
NOS gene	RP	GACACCGCGCGCGATAATTTATCC	64.4		
	Probe	BtnTg-TTTTTTTTGCGCGCTATATTTTGTTTTCTATCGCG	64.8		
Salmonella spp.	FP	Dig-TACCAAAGCTAAACGCGCAGCT	62.1	152	[8]
hns gene	RP	TGATCAGGAAATCTTCCAGTTGC	61.1		
	Probe	BtnTg-TTTTTTTTTTTTTGATTACAGCCGGTGTACGACCCT	75.9		
Cronobacter spp.	FP	Dig-GTTGGATCACCTCCTTACCTGC	64.2	190	[8]
16S-23S rDNA gene	RP	AGTTAAACCTCTTCAACTCCTG	58.4		
	Probe	TGTGAGCACGCGAGGTTGTATCTTGCA-TTTTTTTTTT-BtnTg	64.0		
Fusarium spp.	FP	Dig-CCGAGTTTACAACTCCCAAA	62.7	180	[5]
ITS 1 gene	RP	ACAGAGTTTAGGGGTCCTCT	58.4		
	Probe	BtnTg-TTTTTTTTTTTTACCGGGAGCGGGCTGAT	67.4		
Positive control	Probe	Dig-TTTTTTTTTTTTTTTGTCATGGGCCTCGTGTCGGAAAACC-BtnTg	81.0		
Negative control	Probe	BtnTg-ACCGTCGCGCACTATCTGATTTCAAA	73.3		

473 FP: forward primer, RP: reverse primer, Dig: digoxigenin-labelled, Btn-Tg: biotin labelled

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475 **Table 2.**

		RPA-ELISA	PCR-ELISA
Limit of detection	Hazelnut (µg g^{-1})*	1.29	5.80
	Peanut (µg g^{-1})	11.21	13.27
	Soybean (µg g^{-1})	2.01	1.47
	Tomato (µg g^{-1})	9.45	6.63
	Maize (µg g^{-1})	14.36	2.00
	P35S (µg g^{-1})	8.36	1.24
	TNOS (µg g^{-1})	19.74	6.69
	Salmonella spp. (CFU mL^{-1})	6.00	5.00
	Cronobacter spp. (CFU mL^{-1})	13.00	12.00
	Fusarium spp. (µg g^{-1})	5.93	31.96
Mean Reproducibility ** **(%)**	Intra-day	1.4 - 6.6	3.4 - 7.2
	Inter-day	7.9 - 11.3	8.5 - 14.5

476 * µg g^{-1} refers to µg of analyte per g of food
477 ** Reproducibility was calculated from the samples containing 0.1 % of analyte (n=3)
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Table 3.

	Declared analyte[a] / Detected analyte[c]							Spiked analyte[b] / Detected analyte[c]		
Food	Hazelnut	Peanut	Soybean	Tomato	Maize	P35S	TNOS	*S. spp*	*C. spp*	*F. spp*
Muesli cookies	+ / ++	- / nd	+ / +	- / nd	± / +	<0.9% / +	<0.9% / +	- / nd	- / nd	10% / +++
Chocolate wafer	± / +	+ / +	± / +	- / nd	- / nd	<0.9% / nd	<0.9% / nd	$4 \cdot 10^1$ / +	- / nd	- / nd
Ketchup	- / nd	- / nd	- / nd	+ / ++	+ / ++	<0.9% / nd	<0.9% / nd	$4 \cdot 10^3$ / ++	- / nd	0.1% / +
Feed	- / nd	- / nd	- / nd	- / nd	+ / +	+ / ++	+ / +	$4 \cdot 10^4$ / +++	- / nd	1% / ++
Tomato	- / nd	- / nd	- / nd	+ / +++	- / nd	+ / ++	+ / ++	$4 \cdot 10^3$ / ++	$4 \cdot 10^2$ / ++	- / nd
Baby food	- / nd	- / nd	- / nd	- / nd	+ / +++	<0.9% / nd	<0.9% / nd	$4 \cdot 10^4$ / +++	- / nd	10% / +++
Soup	- / nd	- / nd	± / +	- / nd	- / nd	<0.9% / nd	<0.9% / nd	$4 \cdot 10^2$ / ++	$4 \cdot 10^2$ / ++	- / nd
Skimmed powdered milk	- / nd	- / nd	- / nd	- / nd	- / nd	<0.9% / nd	<0.9% / nd	$4 \cdot 10^2$ / ++	$4 \cdot 10^4$ / +++	- / nd
Powdered infant formula	- / nd	- / nd	- / nd	- / nd	- / nd	<0.9% / nd	<0.9% / nd	$4 \cdot 10^4$ / +++	$4 \cdot 10^3$ / +++	- / nd
CRM (RRS 5%)	- / nd	- / nd	+ / +++	- / nd	- / nd	+ / ++	+ / +	- / nd	- / nd	- / nd
CRM (Bt11 Maize 5%)	- / nd	- / nd	- / nd	- / nd	+ / +++	+ / ++	+ / +	- / nd	- / nd	- / nd
Sweet corn	- / nd	- / nd	- / nd	- / nd	+ / +++	<0.9% / nd	<0.9% / nd	- / nd	- / nd	1% / ++

[a] Declared: + analyte listed; - analyte not listed; ± may contain trace levels; <0.9% labelling not required (GMO-EU regulation).
[b] Spiked analyte correspond to *Salmonella spp. (S. spp), Cronobacter spp. (C. spp),* and *Fusarium spp. (F. spp).*
[c] Used code: +, detected at low level; ++, detected at medium level; +++, detected at high level; nd, non detected.