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Additional Information

Parallel solid-phase isothermal amplification and detection of multiple DNA targets in microliter-sized wells of a digital versatile disc

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ABSTRACT

An integrated method for the parallelized detection of multiple DNA target sequences is presented by using microstructures in a digital versatile disc (DVD). Samples and reagents were managed by using both the capillary and centrifugal forces induced by
15 disc rotation. Recombinase polymerase amplification (RPA), in a bridge solid phase format, took place in separate wells, which thereby modified their optical properties. Then the DVD drive reader recorded the modifications of the transmitted laser beam. The strategy allowed tens of genetic determinations to be made simultaneously within <2 h, with small sample volumes (3 µL), low manipulation and at low cost. The method
20 was applied to the high-throughput screening of relevant safety threats (allergens, GMOs and pathogenic bacteria) in food samples. Satisfactory results were obtained in terms of sensitivity (48.7 fg of DNA) and reproducibility (below 18%). This detection scheme warranted cost-effective multiplex amplification and detection, and was perceived as a viable tool in the massive screening of nucleic acid targets.

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Keywords: Multiplex PCR; DNA; Recombinase polymerase amplification (RPA);
30 Compact disc; High-throughput screening; Food safety

Running title: micro solid-RPA on DVD for high-throughput screening of DNA targets

Graphical Abstract text:

35 A low-cost, portable simple method is presented for the high-throughput screening of multiple DNA sequences. Parallelized isothermal amplification takes place in microwells integrated into a digital versatile disc. Optical detection is performed by a disc drive.

40

Multiplex PCR is one of the most widely used methods to detect DNA because of its capability to simultaneously amplify different targets in a single reaction mixture.[1] Many of the most well-established high-throughput methods are based on its combination with oligonucleotide microarrays.[2] However, due to the intrinsic interference and competition between primer pairs, targeted sequences are seldom amplified equally, and even result in the absence of some products in the final mixture. Thus multiple optimization rounds are required, which result in time-consuming and labor-intensive protocols.[3]

To overcome this limitation, several strategies, such as algorithms for primer design or the use of universal primers, have been proposed.[1] Better results have been achieved by physically isolating primer pairs, and the compartmentalization of amplification reactions is the most innovative approach.[1][4] In the literature, the generation of water-in-oil droplets, named digital-PCR,[5] or the fabrication of reactors[1][6][7], have been described to isolate reagents, and allow the simultaneous increase in the number of assays, which improves amplification efficiency and reduces reaction volumes. Nevertheless, these systems may present several drawbacks. First, the high temperature requirements of PCR may form gas bubbles, which leads to volume variations and amplification failures. Second, most systems employ fluorescent detection, or detection outside the device. This entails costly hardware equipment or labor-consuming procedures to collect amplified products before detection, and some present complex fabrication manifolds or equipment, such as external valves and pumps.[1][6][8]

Solid-phase amplification approaches have proven to be a promising alternative to avoid post-amplification treatments of PCR products. Here one primer or both is/are grafted onto a solid support, while other reaction components remain in the liquid phase. The enzymatic extension of primers directly produces a tethered amplification product onto the substrate surface. This format offers several advantages, such as spatially resolved products, high throughput, easy operation and specific detection.[9][10] The introduction of 3D chips has been seen to provide higher surface-density ratios, to increase primers immobilization density and to enhance target accessibility. However, imprecise heat distribution can lead to poorer amplification efficiency.[2] Examples of 3D systems include gel-based chips from polyacrylamide gel pads, conical dendrons, micropillars, single threads, bead microarrays or microreactors.[2][6][11]

Isothermal amplification methods, in which nucleic acids are synthesized at a constant temperature, can overcome the above-discussed thermal constraints. Among isothermal
75 reactions, recombinase polymerase amplification (RPA) has many interesting properties, such as short reaction times (20-60 min), robustness and low energy requirements (close to room temperature). Several research works have demonstrated that RPA is readily compatible with integrated systems, such as solid-phase amplification combined with silicon microring resonance,[12] lateral flow strips,[13]
80 electrochemical detection modes,[14] or microfluidic devices based on fluorescence detection.[15]

Recently, our research group developed two approaches that perform heminested solid-phase RPA (sp-RPA) in the 2D-chip format using compact disc technology as a support and detector. In the first study, spherical sessile droplets were dispensed onto the
85 surface of the disc to simultaneously detect three genes of *Salmonella* spp.[16] In the second approach, the single detection of genetically modified organisms (GMOs) was performed with a microfluidic system, fabricated in pressure-sensitive adhesive bound to a DVD.[17]

In this study, a support with 3D structures, composed of channels and wells embedded
90 in a standard DVD disc, is presented for parallel DNA detection. The device combines centrifugal microfluidics to automatically meter, split and dispense reagents with DVD drive detection. The main challenges involved were manufacturing these structures, maintaining optical disc performance, and carrying out a high-multiplexing DNA assay.

As proof-of-concept, five food safety threats were simultaneously detected. The selected
95 targets (peanut, GMOs and pathogen microorganisms) are intensively controlled along the food production chain, and according to the recommendations of the United Nations Food and Agriculture Organization and the regulations of many countries. Reviews on this subject have recently appeared. [18]-[20]

100

EXPERIMENTAL

Oligonucleotides and samples. The specific primers of the selected genes are shown in Table 1. All the primers were successfully checked for reliable inclusivity (specific target analyte detection) and exclusivity (lack of interference from non target analytes).

Salmonella Typhimurium group B (CECT 443) and *Campylobacter jejuni* (CCUG17696) were used as reference strains. Bacterial isolation and inoculation assays were prepared as described by Santiago-Felipe *et al.*[21] The certified reference materials (CRM), which contained 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 and food samples with the DNeasy Blood & Tissue Kit (Qiagen, Inc., CA, www.qiagen.com).

Fabrication of microstructured DVDs. The analytical platform was composed of a DVD disc with the microfluidic structures and a sealing layer. The channels and microliter-sized wells were fabricated on store-brought DVD-R discs. For this purpose, a computer numerical control drilling machine (Bungard CCD, Karo 5410, Germany, www.bungard.de) was used. The feed speed and rotational rate of the tungsten carbide drill were 2,000 $mm \cdot s^{-1}$ and 48,000 rpm, respectively. The precision of the fabrication process was ± 0.1 μm. Fluidic structures were holed radially (three radii) to enable 18 assays on a single disc (Figure 1). Each structure was combed-shaped to aliquot the reagents solutions to five microwells. The aliquoting structure was formed by an inlet chamber (1.5 mm in diameter and 0.4 mm in depth) and a primary channel (1 mm in width and 0.4 mm in height), connected to the wells by five other distribution channels, which acted as hydrophobic valves (5 mm long, 0.8 mm wide and 0.4 mm high). The primary channel lengths were 35, 45 and 50 mm, which gave volumes of 17, 21, and 23 μL, for the internal, middle and external radii, respectively. Microwells were drilled at 35 mm, 45 mm and 55 mm from the disc center, and their dimensions were 2 mm in diameter and 1.1 mm in depth.

The sealing layer was fabricated using pressure sensitive adhesive (PSA) (ARcare 90445, Adhesives Research, Ireland, www.adhesivesresearch.com) affixed to an acetate sheet, including access and emptying through-holes. The structures in PSA foil (0.08

mm depth) were made with a cutter-plotter (Graphtec, Japan-Graphtec CE-2000, www.graphteccorp.com). Square chambers (3.2 mm × 3.5 mm) were holed by
135 replicating the shapes made in the DVD disc.

The fabrication uniformity and quality of the microstructured disc were checked under a Dino-Lite Digital Microscope (BigC.com, California) at a resolution of 1.3 megapixel (1280x1024 pixels), and under a SMZ-1B Stereoscopic Microscope (Nikon Corporation, Japan, www.nikon.com) at a total magnification of 16-70x and a real field
140 of 13.1-3 mm. Surface contact angles were calculated by dispensing microdrops at room conditions in quintuplicate.

Before layer bounding, biotinylated primers were immobilized on the streptavidin-modified microwell surface. For this purpose, a mixture of streptavidin (20 $mg \cdot L^{-1}$) and the forward and reverse primers of a single targeted gene (200 nM) in 50 mM carbonate
145 buffer at pH 9.6, was dispensed in each microwell (2.5 µL) and incubated overnight at 4 ºC. Wells were washed with PBS-T (phosphate-buffered saline containing 0.05% (v/v) tween 20, pH 7.4), plus deionized water. Then the sealing layer was aligned to the coated DVD and affixed to enable a bubble-free flat disc. Assembled discs were stored at -20 ºC.

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Assay principle. Parallel isothermal amplification is based on an innovative assay, called bridge solid-phase RPA (bridge sp-RPA), and performed on a centrifugal microfluidic disc. The sample and other reaction components were dispensed thought the inlet hole. The disc was designed to enable to take simple steps at low spinning rates
155 (< 1,500 rpm), which are easily achieved by the stepper motor integrated into commercial DVD drives. As the disc spinned, each sample simultaneously split into five aliquots and was dispensed into microwells for parallel amplifications. Hence each specific reaction took place in a physically separated compartment.

In the solid-phase amplification format, a nucleic acid bridge was formed between two
160 extended strands.[25][26] To go about this, forward and reverse primers were 5'-end immobilized in microwells to allow the 3'-end free for DNA synthesis (Figure 2). The genomic DNA hybridized with the immobilized primers and the recombinase enzymes and single-stranded DNA binding proteins (gp32) facilitated the strand exchange of the non template strand and the primer. Then primers were elongated by *Bsu* DNA

polymerase I to form surface-bound amplified products. These surface-bound copies can also hybridize to other attached primers in the vicinity, which allows bridge-formation amplification. The generated products, labeled during amplification and developed by an immunoenzymatic reaction, were detected by a DVD drive.

According to specifications, a standard DVD disc is composed of two 0.6 mm-thick PC substrates, with a middle layer of highly reflective metallic material (thickness 1000-1500 Å). The bottom PC layer has an injection molded spiral microguide (0.74 mm track pitch) across the whole disc for surface scanning and data storage. Microwells are fabricated to cross the metallic layer from the top PC substrate and none of the other microfluidic structures go through it. The laser beam intensity emitted by the DVD pick-up is transmitted only through the transparent wells. Disc surface scanning is possible because the DVD drive contains an optical system with a laser (=650 nm) and a servo focus/tracking system to center and focus the beam on the spiral track. In addition, the DVD drive integrates an error correction system to avoid burst errors caused by dust, fingerprints or imperfections on disc surfaces like scratches. Thus the presence of microwells to perform biochemical reactions does not disturb disc scanning with a disc drive.

Assay protocol: amplification and development. Amplification mixtures, developing reagents and washing solutions were dispensed with a micropipette on primary channels through the inlet hole (17-23 µL). The stepper motor of the DVD drive rotated the disc, and controlled the spinning rate and the reagent flow to microwells.

Amplification enzymes, nucleotides and buffer (TwistDx, UK, www.twistdx.co.uk) were reconstituted, mixed with 10 µM digoxigenin-dUTPs and 6 ng of genomic DNA from the extracted sample to be loaded onto the disc. Outer holes were tape-sealed (Corning, USA) and spun slowly (500 rpm for 3 s) by the DVD drive to lead the solutions to the reaction wells. Next the disc stored in a water-saturated atmosphere container (standard DVD plastic box) was introduced into a laboratory oven (Memmert, model UF30) to carry out solid-phase amplification reactions at 37 ºC for 45 min. After removing the sealer tape, microwells were emptied by spinning at 1000 rpm for 5 s by the DVD drive. Wells were washed 3 times with 0.1× washing solution (SSC, 1× saline

sodium citrate: NaCl 150 mM, sodium citrate 15 mM, pH 7) and water by dispensation and emptying through centrifugation, as described above.

The enzymatic developing reaction was a mixture of an anti-digoxigenin antibody produced in sheep (1/4,000) and anti-sheep conjugated with horseradish peroxidase
200 (1/500) in PBS-T. After a washing step with PBS-T and deionized water, a mixture of 3,3',5,5'-tetramethylbenzidine (TMB) (0.25 $g \cdot L^{-1}$) and hydrogen peroxide (0.002 M) in citrate buffer at pH 5.5 was dispensed and incubated at room temperature for 10 min.

Assay protocol: DVD reading and data analysis. The equipment consisted in a
205 standard DVD drive (LG Electronics Inc., USA) provided with an upper planar photodiode (model SLCD-61N2, Silonex, USA) and a data acquisition board (DAQ, model USB-2527, Measurement Computing Corporation, USA). The planar photodiode (5.1 mm × 5.1 mm) had a spectral sensitivity of 0.55 $A \cdot W^{-1}$ at 940 nm, a spectral range between 400 and 1,100 nm, and an acceptance half-angle of 60°. The photonic system
210 detected the transmitted laser beam and converted it into an analog electrical signal. Then the DAQ digitized the analog signals from the detection areas and transferred them to the computer for processing.

The disc was scanned at a rotation speed of 4× (equivalent to 13.46 $m \cdot s^{-1}$) with an acquisition signal of 26 dB gain and a sample rate up to 1.7 Msps. Then microwell
215 images were created (tagged image file format, gray-scale with 16 bit-color depth, scale 0-3) and optical intensity signals were related with the amount of reaction product. When no reaction took place, the maximum intensity of the transmitted beam was collected by the photodiode (background signal). When the laser hit the colored product, the intensity of the laser beam that reached the photodiode diminished, which
220 corresponded to the microwell signal. Used discs were discarded following the same laboratory safety guidelines as the ELISA plates.

Optical disc drive control (centrifugation and detection) and image analyses were performed by custom software, written in Visual C++, and run on a laptop connected to it via a USB2.0 universal serial bus interface. Reading and image processing (feature
225 gridding, addressing, segmentation and quality assurance) were automatically done in 14 min by a disc. An assay was considered positive when the optical response was

higher than the cut-off value, calculated as 3 times the standard deviation of the negative control measurements.

Statistical package Statgraphics Centurion for Windows v. 16.1.15 was used for the data
230 analysis.

RESULTS AND DISCUSSION

Assay optimization. The first experiments focused on the set-up of the microfluidic disc. Repetitive comb structures embedded io the DVD disc were designed to hold
235 loading reagents, distribute the solution, and fill all the microliter-sized wells or reactors for the amplification reaction (Figure 1). According to a first prototype, the properties of microfluidic structures and rotation rates were optimized (see the Electronic Supporting Material). Under the selected design conditions, buffered solutions were loaded in the primary channel through the inlet, spontaneously moved forward along by capillary
240 action, and stopped at the junctions of the narrower distribution channels. At this point, the surface tension turned out to be a retarding force that stopped the fluid from moving forward by acting as a capillary valve. DVD spinning allowed proper aliquoting and samples were then completely transferred to wells to perform the reaction. The positions and dimensions of the micro-sized wells were selected to ensure appropriate tracking
245 during DVD reading and the greatest transmitted light intensity.

Regarding assay format, the following parameters were optimized: (a) choice of solid-phase approach; (b) immobilization mode of primers; (c) addition of reagents to prevent non specific adsorptions; (d) developing conditions. The respective data and figures are given in the Electronic Supporting Material. The following experimental conditions
250 were found to give the best results: (a) a bridge solid-phase approach where both primers are attached to the support; (b) indirect adsorption via streptavidin/biotin recognition; (c) no addition of blocking reagent; (d) immunoenzymatic reaction in a homogeneous format.

Lack of cross contamination among the microliter-sized wells was analyzed to ensure
255 success in multiplexing applications. To that end, primers were alternately coated in adjacent wells, and the sample was loaded and amplified to produce alternately positive and negative reactions. The results showed that all the negative reactions produced

signals below the limit of detection, which confirmed that no liquid transfer or diffusion between wells took place (t-test: p-value<0.01).

260

Analytical performance. The determination of the targeted genes (peanut, p35S, Bt-11, *Salmonella* spp. and *Campylobacter* spp.) was examined by a large-scale screening approach.

Assay sensitivity was determined by analyzing serially diluted genomic DNA (10-folds
265 dilution) in triplicate. A correlation between the concentration of the DNA template and the optical signal measured by the DVD detector was found for each analyte (Figure 4). Limits of detection (LODs) were calculated as the smallest amount of DNA able to produce a distinguishable signal from the cut-off value (optical density of 0.8). The smallest detected amounts of extracted genomic DNA ranged between 50 and 900 fg,
270 depending on the nature of the sample. These values corresponded to 335-810 $\mu g \cdot g^{-1}$ for allergen and GMOs, and to 92-176 $CFU \cdot mL^{-1}$ for pathogenic bacteria. These results comply with EU legal requirements for GMOs content (0.9 %), and are useful for allergen detection and pathogen screening. Assay reproducibility, expressed as the relative standard deviation (RSD), was determined from the optical density of the
275 microwells from the samples analyzed in triplicate. The intra-day RSD varied from 4.6 to 16.2%, and the inter-day RSD from 10.0 to 18.4%.

In summary, the attachment of both primers to the surface provided slightly worse analytical performances than those obtained by other solid-phase approaches.[16][17] These results were probably due to the lower amplification yield of the bridge
280 format.[10][11] However, the assay offered advantages, such as higher multiplexing capability, null cross contamination, non primer interferences and less handling since a single reaction mixture sufficed to analyze all the target genes.

Proof of concept. The method was applied to simultaneously screen common food
285 threats in representative samples to demonstrate its capability in a high-throughput DNA analysis. In a disc for 18 samples (90 parallel reactions), five genes were simultaneously amplified-detected with just one pipette shot per sample.

The samples that contained analytes at high concentrations were easily identified visually according to microwell color (Figure 5). A more accurate measurement was

290 taken by recording optical intensities by a DVD drive. The results were satisfactory compared to the declared composition of foods and those obtained by reference methods, as analyzed individually (Table 2). The targeted genes were detected in all cases, even at trace levels (12 cases), and negative results were obtained for all the samples with no genes (48 cases).

295 The selected application demonstrated that large-scale DNA screening was possible, this being a competitive solution, as shown in Table 3. First, most of the described approaches are based on PCR amplification, which required accurate temperature control. The use of isothermal RPA simplifies the procedure, cuts the analysis time, avoids bulky thermocyclers, and are suitable for *in situ* applications. Second,
300 fluorescence detection in current methods requires specific expensive equipment (fluorescence scanners, fluorimeters), which greatly limits their costs and portability. Hence the main advantage of our system stems from the detector since DVD drives are cheap, portable, sensitive, offer high-throughput and are robust devices manufactured according to high quality massive-production standards. As they are lightweight (<500
305 g) and small (a few centimeters), they can be implemented in different settings. Third, the current approach is a completely different concept to that involved in typical centrifugal microfluidic devices, [30] some of which also include sample extraction steps. Essentially, they use circular platforms, incorporate chambers, channels, etc., and common detectors adapted to the shape of plastic discs, which read color or
310 fluorescence signals. Thus analytical performance, such as size, portability and price, cannot be compared with our proposal. Fourth, this approach performed on 3-D structures improves other DVD-methods used for DNA detection. Although it is necessary to physically modify the disc, more sensitive developing reactions are compatible (homogeneous formats). Therefore, the combination of the selected DNA
315 methodology with powerful detection technology offers a powerful system with no loss of analytical performance (LODs, working range, etc.).

CONCLUSIONS

Screening analyses require tools where multiple samples and targets can be analyzed
320 with small sample volumes, in fast reaction times and with high-throughput detection. Several elements have been integrated to achieve this goal: bridge solid-phase RPA,

microfluidic structures embedded in the analytical platform (disc) and a detection approach using a DVD drive. This low-cost, fast, simple and parallel format improves the way to work with commercially affordable analytical systems, like those demanded by end users and the market. For instance, prospective costs (<3 € disc and <500 € reader) cheaper than the state of the art; i.e. qPCR plates and fluorescence-thermocyclers. Compared to similar technologies, some drawbacks are reported, such as not fully automated, dependence on an exclusive supplier of RPA reagents, or an improvable analytical platform fabrication process. Nevertheless, increased multiplexing and automation are possible, due to the scalability of DVD technology and associated methodologies. Likewise, the use of more accurate instruments, such as CO_2 laser cutters, can improve the fabrication process. Another bridgeable limitation is the heating system for out-of-lab applications. The integration of a heating device into the DVD reader should offer improved analytical performances, mainly reproducibility and portability.

The potential of a versatile and economical platform has been demonstrated by its excellent analytical performance achieved for studied targets, and the approach should also be suitable for many health, forensic, or environmental applications.

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Figure 1. (a) Picture of the microfluidic DVD platform for 18 samples (5 genes) (b) Picture of three samples loaded into the primary channel through the inlet hole. (c) Picture of three samples after spinning the DVD at 500 rpm, which opens the capillary valve and samples are aliquoted into parallel microwells, where solid-phase amplification and detection occur. Note: the buffer was colored with blue ink to achieve better quality photographs.

Figure 2. Scheme of the amplification assay (bridge sp-RPA). (a) Initial microwells embedded in the DVD disc that contained tethered primers for five targeted genes. Insert shows the reaction mixture that contained the sample with different DNA molecules (targets or not) and RPA reagents. (b) Final microwells with immobilized products (bridge structures). Dig-dUTPs: digoxigenin-labeled deoxynucleotide, FP: forward primer, RP: reverse primer.

Figure 3. Calibration curve of peanut DNA (n=3). Inserts: Microwell images of the developed products that correspond to the indicated DNA concentration.

Figure 4. Images from the analysis of six food samples: (a) Muesli cookies; (b) Chocolate wafer; (c) MON 810 maize; (d) Bt-11 maize; (e) Powdered infant formula; (f) Chicken carcass. Microwells that contained the primers for the specific detection of (from left to right): peanut, p35S, Bt-11, *Salmonella* spp. and *Campylobater* spp.

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Table 1. Primer sequences used for the amplification procedures.

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Table 2. Results of the analysis of the different food samples by DVD technology (n=3).

Table 3. Advantages and limitations of the methods reported for the determination of multiple targets

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