Low-cost genotyping method based on allele-specific recombinase polymerase amplification and colorimetric microarray detection

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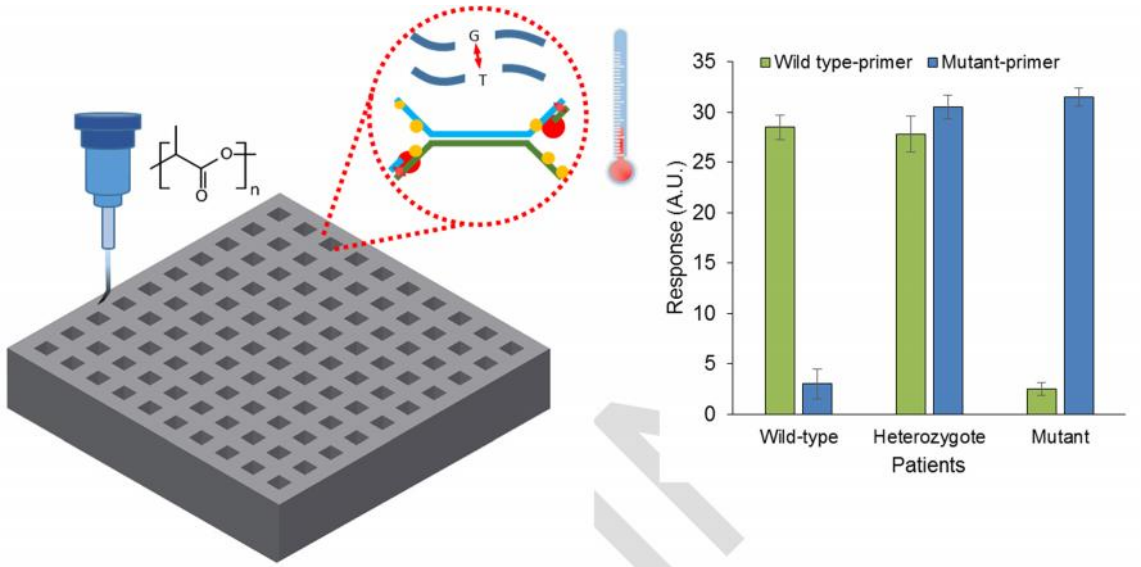
ABSTRACT

The costs of current genotyping methods limit their application to personalized therapy. The authors describe an alternative approach for the detection of single-point-polymorphisms (SNPs) using recombinase polymerase amplification (RPA) as an allele-specific technique. The use of short and chemically modified primers, locked nucleic acids (LNA), allowed the selective isothermal amplification of wild-type or mutant variants at 37 °C in 40 min. An amplification chip containing 100 wells was manufactured with a 3D printer and using thermoplastic polylactic acid (PLA). The platform reduces costs, reagent consumption, sample volume and allows assay parallelization compared to other amplification formats. As proof of concept, the method was applied to the genotyping of four SNPs that are related to the treatment of tobacco addiction. The target polymorphisms were rs4680 (*COMT* gene), rs1799971 (*OPRM1* gene), rs1800497 (*ANKK1* gene), and rs16969968 (*CHRNA5* gene). The capabilities of the method are demonstrated detecting the reaction products using a colorimetric technique in microarray format. The genotype populations can be well discriminated.

Keywords: pharmacogenomics; SNP genotyping; isothermal amplification; micro-well plate; microchip; tobacco addiction; 3D-printer; *COMT* gene; *OPRM1* gene; ANKK1 gene; *CHRNA5* gene.

Graphical abstract

A method based in the allele-specific recombinase polymerase amplification was developed for the genotyping of polymorphisms. The isothermal discrimination reaction was produced in a multi-well amplification chip manufactured with a 3D printer and using thermoplastic polylactic acid.



INTRODUCTION

Pharmacogenomics is currently considered one of the most active areas of the personalized medicine paradigm. However, numerous barriers have been encountered to launch DNA variation analyses, such as single-nucleotide polymorphisms (SNPs), in health systems [1]. Among other factors, available platforms have a marked technological limitation. Most genomic findings have been obtained from high-throughput technologies, such as Illumina and Affimetrix platforms. However, the incorporation of these methods into primary care centers is limited, and cost is the main drawback [2-4]. Dramatically cutting the turnaround times of these platforms is an important goal for implementing SNP testing into clinical scenarios. Therefore, the novel generation of simple diagnostic tools is absolutely necessary for the real adoption of personalized medicine [5,6].

A large family of high-potential methods to be developed in simple systems is that based on allele-specific (AS) amplification [7]. Polymerase chain reaction (PCR) uses primer pairs, deliberately designed at SNP sites. Primers have a single-base variation at the 3' end (allele-specific primers), so extension and amplification reactions take place with only perfectly-matched sequences of target regions. This approach has been successfully used as a pharmacogenomic tool combined with several detection systems [8-10]. Nevertheless, these techniques require particular thermal cycling, consequently there are several limitations for their future integration as point-of-care devices. PCR demands an accurate temperature control system to quickly heat/cool reaction solutions. The high temperature reached (up to 95 °C) leads to variations in the volume reaction due to water evaporation and gas bubble formation, which renders accurate process control necessary.

Several new technologies have emerged to improve DNA-based analyses [11]. Many efforts have been made that focus on developing low-cost systems to be used for point-of-care applications or in small laboratories located at the physician's office or in primary health centers.

A revolution in the development of new methods is currently being witnessed, and is associated with the application of isothermal solutions for microanalyses

[12]. These approaches are based on using proteins that separate DNA strands instead of thermal approaches so that target nucleic acids are synthesized at constant temperature. Nevertheless, the application of isothermal amplification for SNP genotyping is still minimum [13,14]. Among isothermal reactions, recombinase polymerase amplification (RPA) has many interesting properties; e.g., short reaction times (20-60 min), robustness and low energy requirements (close to room temperature) [15]. Recently, our research group has demonstrated that polycarbonate-based substrates can be used to support RPA assays in chip [16], micro-reactors [17], or dynamic formats [18]. A well array chip has been described to process miniaturized RPA assays, applied for pathogen detection in under 30 min [19]. The chip was manufactured from a silicon substrate by a complex fabrication technique, including photolithography and chemical treatment, and is only available in specialized laboratories.

The capability of RPA technology for SNP genotyping remains an unsolved issue. A recent study evaluated the influence of sequence mismatches on the amplification specificity of closely-related pathogens [20]. A proof of concept assay describes the discrimination of a single-point mutation of the HRAS gene [21]. To this end, DNA extracted from cell lines was selectively amplified and genetic variants were distinguished by measuring the wavelength shift on silicon micro-ring resonators. However, this technology is far from being adopted generally in health centers.

In the present study, the capability of allele-specific RPA (AS-RPA) is evaluated and a low-cost method is developed for the SNP genotyping of clinically relevant polymorphisms. In a first approach, amplification is performed in conventional polypropylene vials in a single format (detection of a single polymorphism). The advantages of this disposable format are its low cost and compatibility with a huge number of laboratory equipment. However, lab-on-a-chip or μ-total analysis systems offer important advantages for diagnostic devices, such as high-throughput and miniaturization, among others [22].

There are many ways to microfabricate plastic-based materials; e.g. laminate, embossing or injection molding. Additive manufacturing is being examined given its growing interest in the microfluidics field [23]. It is capable of producing

customized structures that range from a few microns to several centimeters in a single step. The main limitations of 3D printers are related to spatial resolution, dimensional fidelity, surface quality, biocompatibility, optical transparency, among others [24]. Advantages include low infrastructure costs and easy manufacturing compared to photolithography or soft lithography approaches. In order to evaluate the potential of this technology, a well array chip for performing AS-RPA was designed and developed with a commercial 3D printer. The objective was to demonstrate RPA's capability as a genotyping method and to compare its performance in an advanced platform compared to the standard format (vials).

As proof of concept, the solution was applied as a pharmacogenomics tool to treat smoking cessation and the highly addictive properties of nicotine [25,26]. In such diseases, the benefits of personalized medicine based on genotype populations has been demonstrated. However, the high cost of current genotyping technologies, compared to the cost of ineffective or erroneous treatment, compromises the application of the test. Therefore, the approach was designed by following the analytical quality and health system sustainability goals.

MATERIAL AND METHODS

Target genes

The target polymorphisms for the tobacco use disorder were rs4680 (*COMT* gene), rs1799971 (*OPRM1* gene), rs1800497 (*ANKK1* gene) and rs16969968 (*CHRNA5* gene). The wild-type variants are G, A, G, and G, and the mutant variants are A, G, A, and A, respectively. The pharmacogenomic information about these variants is included as Supplementary Material (Tables SI.1, SI.2 and SI.3). The human beta actin (*ACTB*) gene was selected as an endogenous control. The list of oligonucleotides for the genotyping of each SNP is found in Table SI.4.

Patient samples and reference discrimination method

Subjects (n=17) were recruited for the present study according to ethics and with informed consents. Buccal smear samples were collected using sterile swabs. They were submitted to digestion and purification steps with a PureLink Genomic DNA Mini Kit (Invitrogen, Thermo Fisher Scientific, USA, www.thermofisher.com). The isolated DNA extracts were eluted from the spin-columns of the kit with Tris-HCl buffer (10 mMTris, pH 8.6) and stored at -20 °C until analyzed.

Allele-specific PCR in a single format was applied as the reference genotyping method. Briefly, the extracted genomic DNA (4 ng) was amplified using two PCR master mixes (Biotools, Spain, www.biotools.eu) and employing 300 nM of each variant primer pair (reverse and allele-specific forward). To confirm amplification, products were diluted in 0.5x SyBR Safe (Invitrogen, Thermo Fisher Scientific, USA) and submitted to fluorescence measurements in a plate reader (Victor 3TM V1420, PerkinElmer, Finland, www.perkinelmer.com). Genotypes were also verified by 3% agarose gel electrophoresis, followed by dying with an intercalating agent (Real Safe, Durviz, Spain, www.durviz.com) and visualization under UV light.

Fabrication of structured platforms

The 100-well array chip was modeled with the Autodesk Inventor Professional 2015 software (Autodesk, USA, www.autodesk.com) and fabricated with a 3D printer (Ultimaker 2 Extended, UltimakerB.V., the Netherlands, www.ultimaker.com). Polylactic acid (PLA) filament (RS Pro, Spain; 2.85 mm diameter, www.rs-online.com) was employed as the printing material. Fabrication was carried out using a 0.4 mm diameter nozzle at 210 °C and a bed operation temperature of 60 °C. In order to evaluate the best printing conditions, prototypes were fabricated with different layer thicknesses (up to 0.2 mm) and at various printing speeds (up to 300 $mm \cdot s^{-1}$). Subsequently, printed

structures were cleaned with a 30-minute ultrasonic bath and dried with compressed air.

The fabrication quality of the PLA-chips was monitored by optical microscopy imaging. Surface pictures were captured (1.2x magnification) by an Olympus SZ61 stereo microscope (Olympus Co., Japan, www.olympus.com). Images were analyzed with the Image J software to provide an estimated roughness for each sample. Surface hydrophobicity was estimated from the contact angle data. Measures of the deionized water droplets (4 μL) were taken using a Dino-Lite Digital Microscope (AnMo Electronics Co., Taiwan, www.dino-lite.com) at the 1.3 megapixel resolution.

A mass loss study was also done to evaluate possible sample evaporation on the platform. The chip was loaded with 10 RPA samples (4 μL each) and placed in an oven at 37 °C. Mass measurements of the set were periodically taken during 4 h and compared with those of an unloaded reference chip.

Assay protocol: amplification

The amplification step was performed using a TwistAmp Basic RPA kit (TwistDx, UK, www.twistdx.co.uk). Eight allele-specific mixes (2 per SNP) were prepared with rehydration buffer, 14 mM of magnesium acetate, 480 nM of allele-specific forward primer and reverse digoxigenin-labeled primer, and the enzyme pellet. Mineral oil (8%) was also added to minimize sample evaporation. Solutions were loaded onto the 100-well array chip and the DNA template (2.56 ng) was added to allow the simultaneous amplification of eight different allelic variants for 10 patient samples and controls (human *ACTB* gene). The chip was then covered with a polyester plate sealer (Corning, USA, www.corning.com) and gently vortexed to mix reagents and samples. Amplification was carried out in a heating oven (Memmert UF30, Germany, www.memmert.com) at 37 °C for 40 min.

The AS-RPA reactions were also performed in 0.2 mL-polypropylene vials (Labbox, Spain, www.labbox.com) and polycarbonate home-made array chips. These chips were fabricated using a computer numerical control drilling

machine (Bungard CCD, Karo 5410, Germany, www.bungard.de). The feed speed and rotational rate of the drill were respectively 2 000 mm·s^{-1} and 48 000 rpm. The diameter of each well was 5 mm and their depth was 1.1 mm. The composition of the RPA mixtures was the same as that previously described, but volumes were 25 µL and 4 µL per reaction for vials and chips, respectively. The heating system used was a thermocycler (TC-4000, Techne, UK, www.techne.com) and an oven, respectively.

Assay protocol: detection and data analysis

The AS-RPA products were detected by a hybridization assay on polycarbonate chips, adapted from reference [10]. Briefly, the mixtures of the wild-type or mutant products for all four SNPs were prepared from the respective single RPA solutions. For this purpose, 2 µL of each amplification product were diluted in 16 µL of hybridization buffer composed of NaCl 225 mM, sodium citrate 22.5 mM, 10% formamide and 2.5x Denhardt's solution, pH 7. Subsequently, mixtures were heated at 95 °C for 10 min for denaturation and transferred to the chips with the immobilized probes in the microarray format. After 60 min of incubation at 37 °C, chips were washed with diluted hybridization buffer. The immunoreaction protocol with enzymatic labelling was followed to develop the duplex of the probe-RPA product, as described in reference [10]. The oxidized form of 3,3',5,5'-tetramethylbenzidine (substrate of horse-radish-peroxidase) produced a blue precipitate over the positive or control spots. Chips were then read with a desktop scanner (Epson Perfection 1640SU Office, Epson, Japan, www.epson.com).

Discrimination index

The resulting gray-scale images (Tagged Image File Format, color depth 16 bit) were processed by an in-house software for the microarray analysis. The optical intensity signals of each spot and local background were quantified by generating a data matrix of the signal-to-noise ratios. The genotype determination rule was constructed according to the replicated responses of the

specific probes for each polymorphism. A discrimination index was calculated from the signal of the wild-type (WT) and mutant (MUT) variants according to this equation: (WT − MUT)/(WT + MUT). The Statgraphics Centurion statistical package for Windows v.16 was used for the data analysis.

RESULTS

RPA capability as a genotyping tool

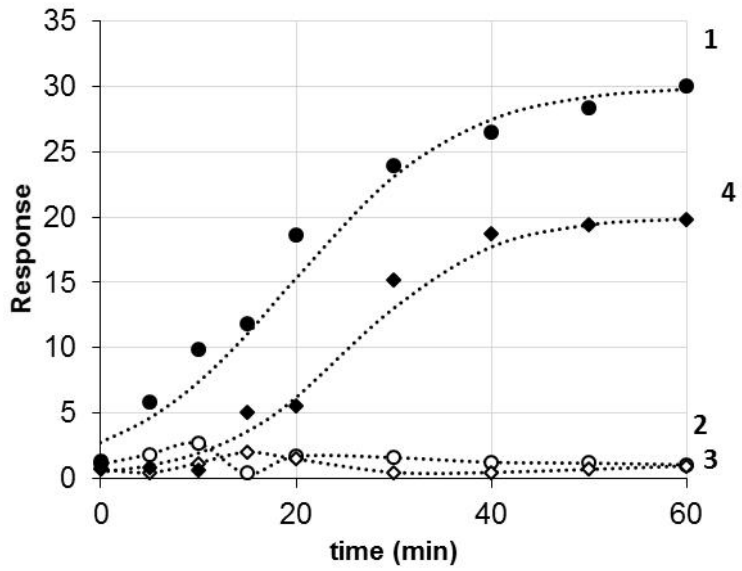
The use of RPA as an allele-specific amplification technique was analyzed by considering the role of each element in the process. A recombinase (T4 uvsX) recognizes targeted DNA templates and specific primers at a high affinity and catalyzes subsequent homologous pairing and strand exchange [20]. Polymerase produces the correct elongation of the perfect-annealed primer/template, and is the key reaction in the DNA duplication process [27]. Furthermore, the Pol I large fragment (Bsu polymerase) lacks exonuclease activity (3'→5') that may modify the target nucleotide. Therefore, we expected the presence of mismatches on their 3'-extreme to hamper the nonspecific reaction due to the combined action of two enzymes, even at a low working temperature.

Complementarily, oligonucleotide sets were carefully selected to satisfactorily amplify/detect the given template region. The *in silico* design restrictions were primer length, absence of secondary structures, and primer/template duplex stability. Both these last parameters were estimated from the thermodynamic models available for DNA duplexes [28]. Although the recommended length for RPA primers should be 30-35 bases long according to the manufacturer's instructions, shorter primers (19–21 mer) were chosen to improve selectivity. The free energy (ΔG) values for the self-annealing and hairpin structures were restricted to 1.0 $Kcal \cdot mol^{-1}$ (the equivalent to melting temperatures < 50 °C). The selected oligonucleotides produced primer/template duplexes with changes in free energy (ΔG) of -25.6±0.2 $Kcal \cdot mol^{-1}$ (the equivalent to a melting temperature of 75.4±0.1 °C) for totally complementary primers. The duplexes

between the template and mismatched primers were less stable (-24.0±0.6 Kcal·mol^{-1}, 72.7±0.6 °C).

The experiments focused on evaluating discrimination capability using the designed primers that differentiated at their 3'-endnucleotide. Figure 1a shows the kinetic curve to perform amplification in a homogeneous format (reaction volume of 25 μL). The expected positive signals were observed after 10-20 min following typical logistic regression (maximum response after 60 min). Under the selected conditions, a different behavior was observed depending on the added primer. Extension by polymerase was efficient when the 3' terminal base of a primer matched its target, whereas extension was inefficient or nonexistent when the terminal base was mismatched. These effects agree with the previously reported results about the reduction or inhibition of the RPA reaction due to the presence of a mismatch in the primer/template duplex [20].

(a)



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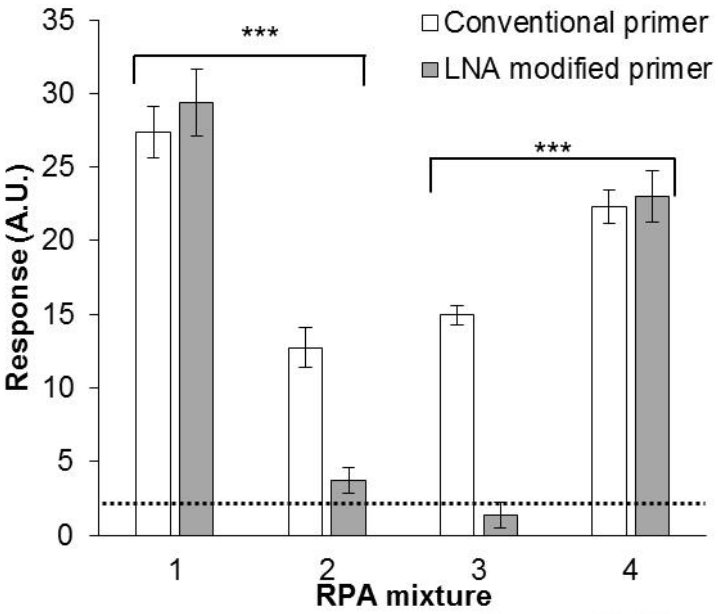

Figure 1. (a) Amplification kinetic curves of rs1799971 (*OPRM1* gene) depending on the RPA mixture: logistic regressions y = 30 / (1+exp(-2.28 – 0.12 t), R= 0.977 for the wild-type and y = 20 / (1+exp(-3.64 – 0.15 t)), R= 0.977 for the mutant variant. (b) Response depending on the primer nature and RPA mixture: statistical comparison compared to the perfect-match duplex (*** p<0.001). Mixture 1: wild-type template DNA and wild-type FP). Mixture 2: wild-type template DNA and mutant FP. Mixture 3: mutant template DNA and wild-type FP. Mixture 4: mutant template DNA and mutant FP. Four replicates

Conventional and chemical-modified primers, locked nucleic acids (LNA), were compared for AS-RPA. Figure 1b shows that nonspecific amplification took place for the mismatched duplexes between the primer and templates. Nevertheless, the amplification yield was significantly higher for the totally complementary duplexes (ANOVA, p-value<0.001). With the LNA primers, differences were more marked, and even nonspecific amplification was similar to the negative controls. These experiments demonstrated that the presence of this nucleoside at the 3' terminal base improved allelic discrimination.

A multiplex reaction was studied for the simultaneous amplification of more than one target in a single reaction. However, reaction yields were not satisfactory and there were sensitivity losses. One system displayed dominating and/or

inhibiting activity over other primers and amplicons, and even genotyping capability was lost. These results agreed with conventional RPA behavior and can be associated with their high sensitivity to the total primer concentration [16].

Design and fabrication of well array chip

The amplification assays, described in the previous section, were performed in 0.2-mL polypropylene vials. The next step was to reduce the reaction volume by performing the assay in a well array chip. This kind of platforms improves amplification capabilities, particularly high-throughput [29]. Fused filament fabrication was chosen as the additive manufacturing technology, with a biocompatible polymer, e.g., PLA, as the thermoplastic material. This technique was selected because it produces innovative bioanalytical platforms that can be customer-designed and fast prototyped by a 3D printer.

The first experiments focused on designing an array chip with 100 wells. The well dimensions (2.5 mm × 2.5 mm × 4 mm) were chosen to perform RPA in a reduced volume (<5μL). Edge-to-edge spacing (distance between wells) was 1 cm for RPA-mixture dispensation by a multi-channel micropipette. The deposition of filament layers, one on top of the other, built up the bottom and the walls of the chip. This additive technique produced grooved structured surfaces on chip walls to study the effect of the 3D printing parameters on chip quality (Supplementary Material/Figure SI.1). By increasing printing layer separation, groove thickness changed from 71±2 μm (0.06 mm) to 238±5 μm (0.2 mm). With a 0.02-mm layer height, the surface became irregular and did not produce visible grooves. Surface roughness, expressed in Rq, varied from 53.8 μm (0.02 mm) to 76.6 μm (0.2 mm). The effect of printing speed and working temperatures during the deposition process were negligible. The selected values were a layer height of 0.1 mm and a print speed of 50 $mm \cdot s^{-1}$, which resulted in a fabrication time of 480 $min \cdot unit^{-1}$. Figures 2a and 2b show the optical microscope images of the PLA-chips produced under the selected conditions. Sealing, performed with a polyester adhesive film and by adding

mineral oil, was effective for the tested range (up to 37 ºC and 4 h), with null leaking and evaporation (p-value< 0.001).

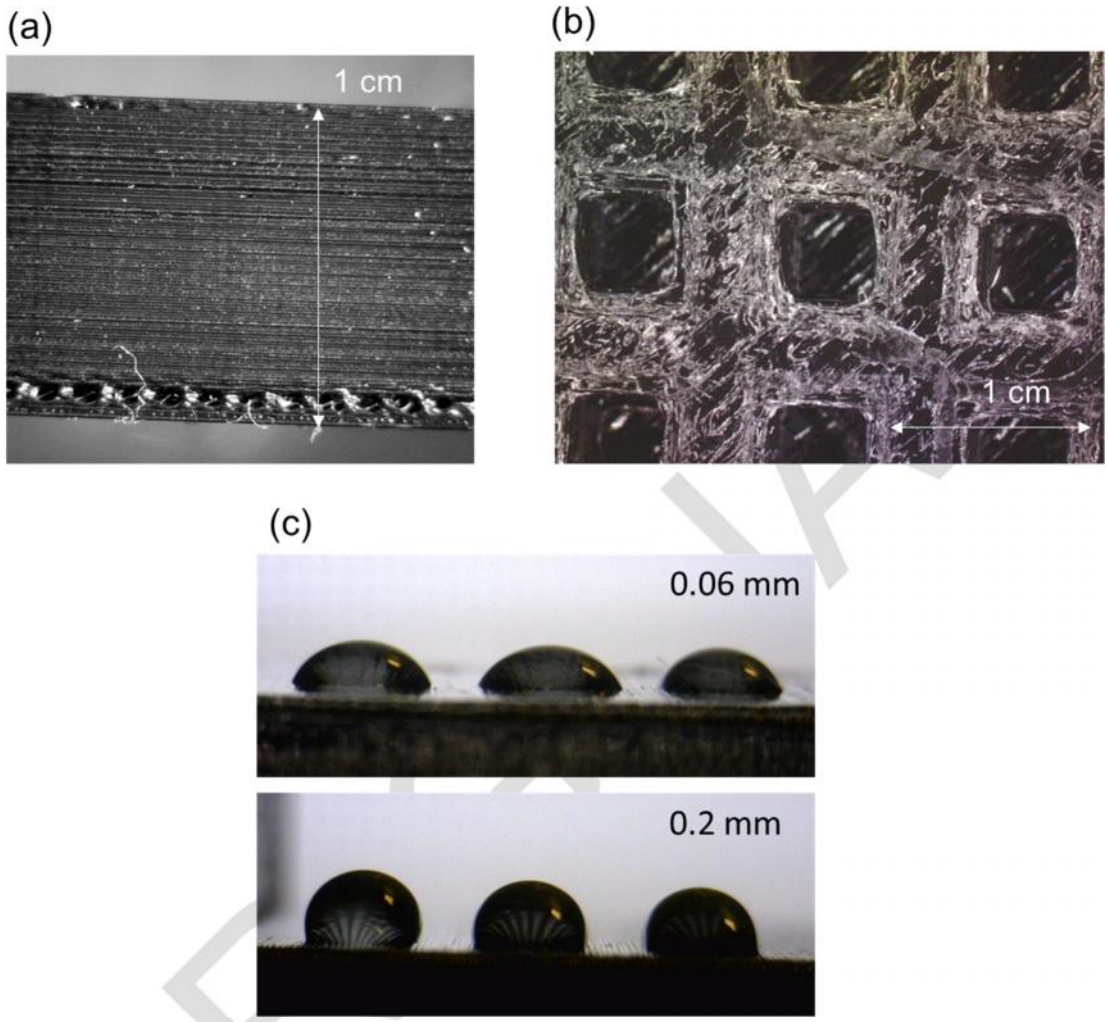

Figure 2. (a) Optical microscope image of the chip wall. (b) Optical microscope image of chip wells (top view). (c) Effect of printing layer height on the PLA contact angle

The hydrophilic/hydrophobic nature of the reaction vessel can affect the easy-filling of wells. Hence the wettability of the raw PLA-chips was estimated and the contact angle of well surfaces was measured. The results were 97±4 ° and 77± 2 ° for the wall and the bottom surface, respectively. These values indicated how the patterned topography modified the interfacial tension between the liquid and thermoplastic compared to the base material (PLA, contact angle of about 80 °). Two chemically modified chips (UV/ozone irradiation and PEG

passivation) were tested. The surface topographies, estimated by microscope image analyses, were comparable to those from the raw chip (p-value > 0.05). After applying soft UV/ozone irradiation, the contact angle was 71±2 °. This lower value indicated that some photo-oxidized polar groups on the surface were formed, consequently fiber adhesion increased (higher surface energy). Addition of PEG also produced a coating over all the active surfaces, and hydrophilicity increased (62±3 °). Regardless of the surface treatment, reagent solutions were easily loaded in wells.

The effect of the unpolished surfaces and chemical treatment on the amplification yield was studied. Replicate RPA reactions were performed using genomic DNA for native/mutant patients (order of magnitude: 10^3 pg of gDNA). Effective amplification was achieved in all the wells of the raw and chemically modified PLA-chips. Nevertheless, the PEG coating was chosen because this treatment can help block chip surfaces, and prevent nonspecific signals and sample losses through protein and amplification product adsorption [30].

Comparison of amplification platforms

The RPA performances for the reactions run in the PLA-3D printed chip were compared with two previously reported platforms (Table 1). The first reference platform was polypropylene vials (0.2 mL) (individual or tube strips), which are widely used for DNA amplification in conventional thermal cyclers. The second was micro-reactors fabricated in polycarbonate (PC) substrate by drilling because they are a low-cost alternative for reduced volume amplifications performed in ovens or other cheap thermal systems [17]. The analysis of variance (ANOVA) indicated that the responses for three platforms were comparable, with p-values of 0.63 for the negative controls, 0.27 for the reference gene (*ACTB* gene) and 0.23 for the target genes. The platform cost for 100-plex reactions using the current 3D-printed chips was the equivalent to those of the polypropylene vials. Nevertheless, the main advantages of this approach stemmed from volume reduction and the cost of the reagent; reagent consumption (and the amount of DNA) decreased by about 6-fold. Other advantages were reduced size, which was compatible with portable heating

systems (i.e. miniaturized Peltier-based devices), and facilitated their adaptation for field or doctor office applications [11,12]. These performances confirmed PLA-additive manufacturing to be a strategy for the rapid versatile low-cost prototyping of bioanalytical devices. The assay costs of each platform were estimated, considering their material, equipment and processing expenses (3D printing or CNC milling), as well as their number of parallel assays. The estimated platform cost per assay for the 3D printed PLA chip was similar to polystyrene vials, while 4-times lower than for the polycarbonate milled chips.

Our approach based on reaction vessels was compared with microfluidic chips in virtue of their high applicability as point-of-care systems [31]. The microfluidic platforms, generally based on poly(methyl-methacrylate) (PMMA) or similar polymers, allow a higher degree of assay integration and lower reaction volumes (nanoliter scale). In exchange, the PLA multi-well chip presents easier manipulation, no fluidic control equipment requirements and a simpler fabrication process with a 16-fold lower cost.

This study can open up ways to test PLA-microfluidic devices, e.g., integration of RPA amplification and real-time detection, prior to their mass production in other thermoplastics, such as PC (e.g., injection molding).

Table 1. Characteristics of the tested amplification platforms

	 PLA chip	 PP vial	 PC chip
Response negative control	3 ± 2 a.u.	4 ± 1 a.u.	4 ± 1 a.u.
Response reference gene	37 ± 3 a.u.	41 ± 2 a.u.	39 ± 3 a.u.
Response target genes	34 ± 3 a.u.	37 ± 1 a.u.	35 ± 1 a.u.
Fabrication technique	3D printing	Molding	Molding + Milling
Platform dimensions	52 mm × 52 mm × 10 mm	100 × (20 mm, ϕ 7 mm)	30 mm × 30 mm × 12 mm
Material thermal conductivity	0.13 $W \cdot m^{-1} \cdot K^{-1}$	0.20 $W \cdot m^{-1} \cdot K^{-1}$	0.19 $W \cdot m^{-1} \cdot K^{-1}$
Number of simultaneous samples	100	1	9
Reaction volume per assay	4 μL	25 μL	4 μL
DNA amount per assay	2.56 ng	16 ng	2.56 ng

a.u.: arbitrary units. Data from three replicated assays

Analytical performance of the genotyping assay

Having demonstrated that AS-RPA can be used for SNP genotyping in a single format, the capabilities of a multiplex detection method were studied. Among the techniques currently available (i.e. optical, electrochemical, etc.), AS-RPA on PLA chips combined to a hybridization assay on PC chips was tested. This detection approach showed excellent performance to simultaneously identify several PCR products [10].

The AS-RPA products from the target genes related to the tobacco use disorder were simultaneously determined. Selectivity was estimated from cross-reactivity experiments by hybridizing products from single amplification assays on a chip that contained probes for the five studied genes (four target genes and a control gene) (Figure 3). Positive responses were obtained only for the specific probe, and were negative for the remaining ones. Sensitivity was determined by preparing heterozygous mixtures with increasing percentages of wild-type DNA

compared to the mutant type (Supplementary Material). Mismatched DNA was detected up to 5-10 %, which indicated that the system was capable of discriminating both genotypes selectively. Intra-day repeatability and inter-day reproducibility, expressed as the relative standard deviation of spot intensities for the replicated assays (five replicates), were 13 % and 17 %, respectively. The ANOVA test showed that the end-point responses were comparable for the four studied genes (p-value > 0.05).

Our detection method of AS-RPA products displayed comparable performance to others previously described for AS-PCR, such as capillary electrophoresis [8], real-time fluorescence [32], the fluorescent-based droplet technique [9] and hybridization to covalently immobilized probes in fluorescent magnetic beads [33].

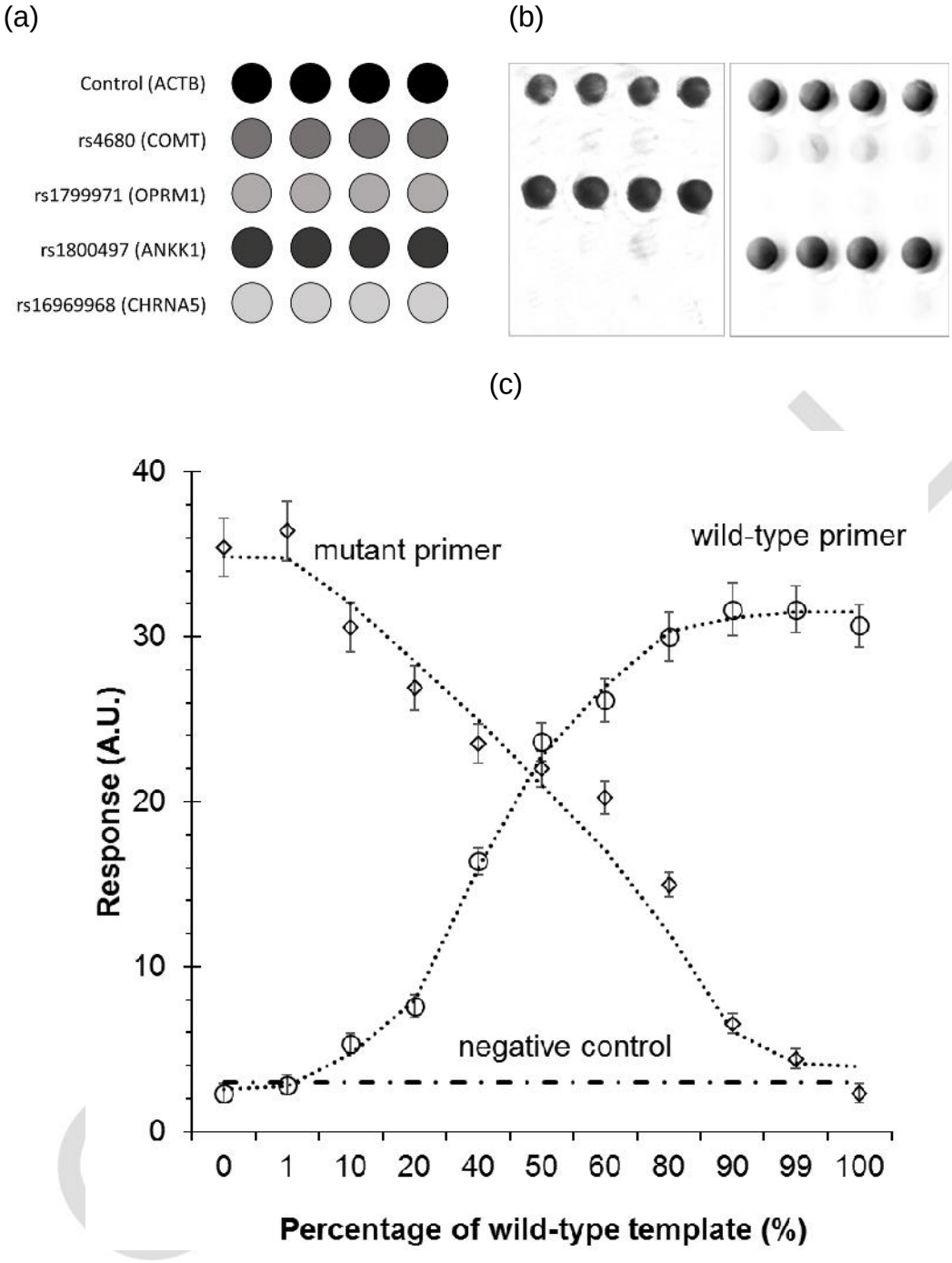

Figure 3. (a) Probe layout of a microarray chip (b) Microarray images obtained for the amplification products: rs1799971 (left) and rs1800497 (right).(c) Assay response depending on the percentage of wild-type template compared to the total template for both RPA mixtures (wild-type FP and mutant FP). Logistic regressions: y = 32 / (1+exp(2.43 – 0.07 t), R= 0.982 for the wild-type and y = 37 / (1+exp(-2.80 + 0.05 t), R= -0.935 for the mutant.

Analysis of patient samples

The applicability of chip-based AS-RPA for genotyping screening in tobacco cessation treatment, or for drugs used in nicotine dependence, was investigated. As a biological sample, buccal swab extracts were selected. In a clinical scenario, the use of buccal swabs is noninvasive, less stressful and a much easier technique to collect DNA samples. Moreover, sample storage does not require refrigeration and DNA extraction is a much simpler process than blood samples. As a detection strategy, a desktop scanner was used for chip reading. Then the procedure was performed using low-resource laboratory materials and equipment (i.e. a primary health center). The analysis time was 210 min (DNA extraction: 50 min, amplification: 60 min, hybridization-detection: 100 min).

The absorption measurements indicated that a sufficient amount of high quality amplifiable human DNA was isolated from all the tested samples. The reference (*ACTB* gene) and targeted regions were amplified and submitted to on-chip hybridization. Figure SI.3 illustrates some examples of the microarray images. Presence of mutated variants in rs4680 and rs1799971 was detected. A subsequent comparison of the acquired chip signals with patient stratification based on the reference method clearly demonstrated perfect matching. The clinical implications of the provided genotyping results are a review of drug treatment, including anti-depressives or nicotine replacement products (e.g. patch). Functional polymorphisms in dopamine pathways (rs4680) are associated with the use of bupropion to mitigate lapsing to smoking following a quit attempt [34]. Better prolonged abstinence rates have been reported after the nicotine replacement therapy tailored to each smoker, and based on either genotype in the opioid receptor (rs1799971) [26].

Presence of mutant alleles was detected for 70.6 % (rs4680), 52.9 % (rs1799971), 41.2 % (rs1800497), and 41.2 % (rs16969968) of the smoker patients. A discrimination factor for genotype assignment was calculated from the signal-to-noise ratios recorded in the microarray images (Figure 4). Each call type within each target polymorphism statistically and significantly differed from the others (p-values <0.0001). Homozygous genotypes led to

discrimination factors above 0.3 and under −0.3 for the wild-type and the mutant, respectively. An intermediate discrimination factor (between −0.3 and +0.3) was calculated for each heterozygous genotype. The genotype assignments, listed in Supplementary Material, agreed with those obtained by the reference method (100 % coincidence).

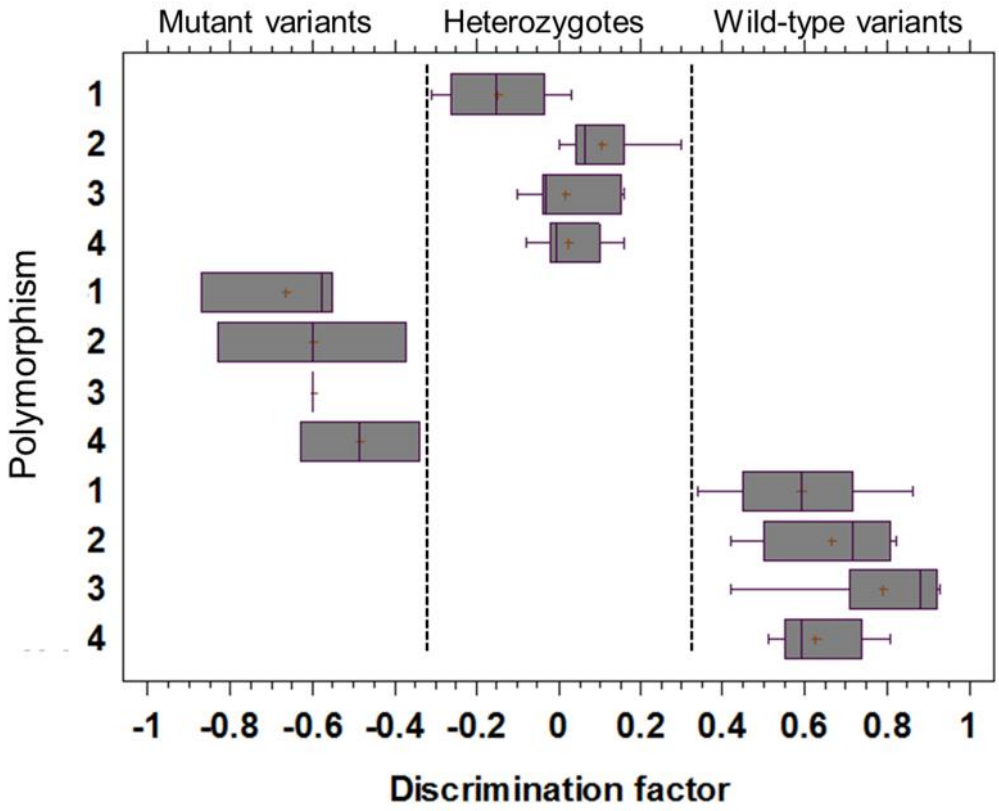

Figure 4. Boxplots of the discrimination factors classified according to the polymorphism and population group. 1: rs4680, 2: rs1799971, 3: rs1800497, and 4: rs16969968

CONCLUSIONS

In the last few decades, patient genome information has been proposed to select individual clinical care, particularly drug treatment decisions. However, the impact of personalized medicine is low compared to the research advances made. The results reported herein study demonstrate how pharmacogenomics knowledge combined with emerging analytical methodologies can benefit clinical practice more broadly. Although more in-depth research must be

conducted, the combination of two innovative solutions was a success. Firstly, the advantages of the isothermal amplification reaction were incorporated to acquire the demanded copy number for sensitive SNP loci detection. The best features were their fast-response (4-fold compared to the PCR), low temperature (37 °C) and few design restrictions. However, the reduced multiplexing capabilities forced single parallelized reactions. Secondly, we employed additive manufacturing based on using a 3D printer as the chosen technology to create a customer-tailored platform for high-throughput analyses. The 100-well PLA-chip design considerably reduced reagent consumption and avoided expensive manufacturing processes or complex pumping systems associated with some DNA detection instruments. In fact the assay can be performed with standard materials (i.e. pipettes, oven) found in clinical laboratories. The present work demonstrates that PLA is an adequate material for performing enzymatic reactions in a static format. The following step is to achieve better point-of-care performance and the next challenge is to develop microfluidic devices fabricated with this material that integrate all DNA assay steps, from extraction to detection. The advantages include the method's flexibility and accessibility compared to other micro-prototyping or micro-fabrication techniques.

Regarding the clinical impact, today pharmacogenomics is applied mainly to certain treatments in psychiatry, oncology and cardiology. One main reason is the cost-effectiveness of genotyping methods. In addition, only some diseases apply to this approach because their treatment generally involves expensive pharmaceutical products or drugs with highly probable adverse effects. With our approach, personalized therapies based on incorporating genetics into patient stratification can be offered, and even for relatively less-impact treatments. The methodology's cost-effectiveness, flexibility and portability will support the well-known genetic marker for predicting drug responses. In the particular case of tobacco addiction, genotyping information will help predict the degree of success in smoking cessation.

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REFERENCES

(1) Manolio TA, Chisholm RL, Ozenberger B, Roden DM, Williams MS, Wilson R, *et al.* (2013) Implementing genomic medicine in the clinic: the future is here. Genet Med 15:258-267.

(2) Scott SA (2013) Clinical pharmacogenomics: Opportunities and challenges at point-of-care. Clin Pharmacol Ther 93:33.

(3) Limaye N (2013) Pharmacogenomics, Theranostics and Personalized Medicine-the complexities of clinical trials: challenges in the developing world. Appl Transl Genomics 2:17-21.

(4) Abul-Husn NS, Owusu Obeng A, Sanderson SC, Gottesman O, Scott SA (2014) Implementation and utilization of genetic testing in personalized medicine. Pharmacogenomics Pers Med 7:227-40.

(5) Knez K, Spasic D, Janssen KP, Lammertyn J (2014) Emerging technologies for hybridization based single nucleotide polymorphism detection. Analyst 139:353-370.

(6) Shen W, Tian Y, Ran T, Gao Z (2015) Genotyping and quantification techniques for single-nucleotide polymorphisms. TrAC Trends Anal Chem 69:1-13.

(7) Milbury CA, Li J, Makrigiorgos GM (2009) PCR-based methods for the enrichment of minority alleles and mutations. Clin Chem 55:632-640.

(8) Asari M, Watanabe S, Matsubara K, Shiono H, Shimizu K. (2009) Single nucleotide polymorphism genotyping by mini-primer allele-specific amplification with universal reporter primers for identification of degraded DNA. Anal Biochem 386:85-90.

(9) Taira C, Matsuda K, Yamaguchi A, Sueki A, Koeda H, Takagi F, Kobayashi Y, Sugano M, Honda T. (2013) Novel high-speed droplet-allele specific-polymerase chain reaction: Application in the rapid genotyping of single nucleotide polymorphisms. Clin Chim Acta, 424:39-46.

(10) Tortajada-Genaro LA, Mena S, Niñoles R, Puigmule M, Viladevall L, Maquieira A (2016)Genotyping of single nucleotide polymorphisms related to attention-deficit hyperactivity disorder. Anal Bioanal Chem 408:2339-2345.

(11) Woolley CF, Hayes MA (2014) Emerging technologies for biomedical analysis. Analyst139:2277-2288.

(12) Craw P, Balachandran W (2012) Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review. Lab Chip, 12:2469-2486.

(13) Zhang L, Zhang Y, Wang C, Feng Q, Fan F, Zhang G, Kang X,Qin X, Sun J, Li Y, Jiang X (2014) Integrated microcapillary for sample-to-answer nucleic acid pretreatment, amplification, and detection. Anal Chem 86:10461-10466.

(14) Chen F, Zhao Y, Fan C, Zhao, Y (2015) Mismatch Extension of DNA Polymerases and High-Accuracy Single Nucleotide Polymorphism Diagnostics by Gold Nanoparticle-Improved Isothermal Amplification. Anal Chem 87:8718-8723.

(15) Li J, Macdonald J (2015) Advances in isothermal amplification: novel strategies inspired by biological processes. Biosens Bioelectron 64:196-211.

(16) Santiago-Felipe S, Tortajada-Genaro LA, Morais S, Puchades R, Maquieira A (2014)One-pot isothermal DNA amplification–Hybridisation and detection by a disc-based method. Sens Actuator B-Chem 204:273-281.

(17) Santiago-Felipe, S., Tortajada-Genaro, L. A., Puchades, R., Maquieira, Á. (2016) Parallel solid-phase isothermal amplification and detection of multiple DNA targets in microliter-sized wells of a digital versatile disc. Microchimica Acta, 183:1195-1202.

(18) Tortajada-Genaro LA, Santiago-Felipe S, Amasia M, Russom A, Maquieira A (2015) Isothermal solid-phase recombinase polymerase amplification on microfluidic digital versatile discs (DVDs). RSCAdv 5:29987-29995.

(19) Li Z, Liu Y, Wei Q, Liu Y, Liu W, Zhang X, Yu Y (2016) Picoliter Well Array Chip-Based Digital Recombinase Polymerase Amplification for Absolute Quantification of Nucleic Acids. PloS one, 11:e0153359.

(20) Daher RK, Stewart G, Boissinot M, Boudreau DK, Bergeron MG (2015) Influence of sequence mismatches on the specificity of recombinase polymerase amplification technology. Mol Cell Probes29:116-121.

(21) Shin Y, Perera AP, Kim KW, Park MK (2013) Real-time, label-free isothermal solid-phase amplification/detection (ISAD) device for rapid detection of genetic alteration in cancers. Lab Chip 13:2106-2114.

(22) NgePN, Rogers CI, Woolley AT (2013) Advances in microfluidic materials, functions, integration, and applications. Chem Rev 113:2550-2583.

(23) Bhattacharjee N, Urrios A, Kang S, Folch A (2016)The upcoming 3D-printing revolution in microfluidics. Lab Chip 16:1720-1742.

(24) Waheed S, Cabot JM, Macdonald NP, Lewis T, Guijt RM, Paull B, Breadmore MC (2016) 3D printed microfluidic devices: enablers and barriers. Lab Chip 16:1993-2013.

(25) Bierut LJ, Madden PA, Breslau N, Johnson EO, Hatsukami D, Pomerleau OF, Swan GE, Rutter J, Bertelsen S, Fox L, Fugman D, Goate AM, Hinrichs AL, Konvicka K, Martin NG, Montgomery GW, Saccone NL, Saccone SF, Wang JC, Chase GA, Rice JP Ballinger DG (2007) Novel genes identified in a high-density genome wide association study for nicotine dependence. Hum MolGen 16:24-35.

(26) Carpenter MJ, Jardin BF, Burris JL, Mathew AR, Schnoll RA, Rigotti NA, Cummings KM (2013) Clinical strategies to enhance the efficacy of nicotine replacement therapy for smoking cessation: a review of the literature. Drugs, 73:407-426.

(27) Moody C, Newell H, Viljoen H (2016) FA mathematical model of recombinase polymerase amplification under continuously stirred conditions. Biochem Eng J 112:193-201.

(28) Dimitrov RA, Zuker M (2004) Prediction of hybridization and melting for double-stranded nucleic acids. Biophys J 87:215-226.

(29) Zhang C, Xing D (2007) Miniaturized PCR chips for nucleic acid amplification and analysis: latest advances and future trends. Nucleic Acids Res 35:4223-4237.

(30) Liu B, Huang PJJ, Zhang X, Wang F, Pautler R, IpACF, Liu J (2013) Parts-per-million of polyethylene glycol as a non-interfering blocking agent for homogeneous biosensor development. Anal Chem 85:10045-10050.

(31) Wu J, Kodzius R, Cao W, Wen W (2014) Extraction, amplification and detection of DNA in microfluidic chip-based assays. Microchim Acta 181:1611-1631.

(32) Li J, Wang L, Mamon H, Kulke MH, Berbeco R, Makrigiorgos GM (2008) Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. Nat Med 14:579–84.

(33) Shen R, Fan JB, Campbell D, Chang W, Chen, J., Doucet, D., Yeakley J, Bibikova M, Garcia EW, McBride C, Steemers F, Garcia F, Kermani BG, Gunderson K, Oliphant A (2005) High-throughput SNP genotyping on universal bead arrays. Mut Res Fund Mol M 573:70-82.

(34) David SP, Strong DR, Leventhal AM, Lancaster MA, McGeary JE, Munafò MR, Bergen AW, Swan GE, Benowitz NL, Tyndale RF, Conti DV, Brown RA, Lerman C, Niaura R (2013) Influence of a dopamine pathway additive genetic efficacy score on smoking cessation: results from two randomized clinical trials of bupropion. Addiction 108:2202-2211.