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Digital versatile discs as platforms for multiplexed genotyping based on selective ligation and universal microarray detection

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Developing high-performance assays readout by integrated detectors is a current challenge to implement DNA tests into diagnostic laboratories, particularly for supporting pharmacogenetic tests. A method for allelic discrimination, associated with single nucleotide polymorphisms (SNPs), is presented. Genomic DNA is extracted from blood and buccal swab samples. The procedure comprises fast multiplex ligation-dependent probe amplification, PCR amplification using universal primers and subsequent barcode hybridization. In this last step, each product is recognized by the specific probes immobilized on the surface of an optical disc. Assay results can be collected with a disc reader. The optical sensing method in a DNA microarray format was optimized and evaluated for the simultaneous identification of 28 polymorphisms associated with psychiatry pharmacogenomics. The target biomarkers were located in the genes related to drug-metabolizing enzymes and drug transporters. The multiplexing capability and assay selectivity strongly depended on correct design (ligation probes, tails and barcodes). The discriminant analysis of reader outputs (spot intensities) led to patients being classified into different allelic populations. The obtained assignations correlated properly with the results provided by the reference technique (bead arrays), and the assay ended in an 8-fold shorter time using affordable equipment. The combination of high selective genotyping reaction as array-MLPA and the compact disc technology provide a reliable point-of-care approach. This genotyping tool is useful for selecting personalized drug therapies in decentralized clinical laboratories.

Introduction

Individuals often show different clinical evolutions for the same disease or treatment. In recent years, molecular technologies have identified genetic biomarkers associated with drug metabolism and transportation, where single-nucleotide polymorphisms (SNPs) are the most abundant and relevant. [1,2] These relationships between genetic populations and drug response have been successfully applied to decision making.[3] In fact pharmacogenomics is currently considered one of the most actionable areas of the personalized medicine paradigm.[4] As a result, clinical practice guidelines based on genetic variants of specific SNPs have been defined to increase treatment efficiency and to minimize adverse reactions.[5]

Genetic assays have been simultaneously developed for the accurate genotyping of SNPs in a given DNA sample.[6-8] However, the availability of high-throughput techniques (e.g. bead arrays, sequencers) in healthcare systems is poor given their costs for instrumental and personal resources.[9] Authors claim that the personalized medicine paradigm will be achieved when alternative approaches with a reasonable impact on current clinical activities can be applied.[10] Therefore, the research challenge involves developing technologies that integrate essential properties with functional properties such as low cost, flexibility, portability or simplicity. In particular, instruments designed for point-of-care (POC) applications offer rapid sample-to-result assays to be performed at or near the site of clinical care.[11,12] The goal involves integrating an allele-selective assay and a method to report the presence of the targeted variant or variants.

Multiplex ligation-dependent probe amplification (MLPA) is the most relevant technique, which was originally designed to detect copy number variations.[13] Further modifications have provided new applications, including methylation status determination, the relative quantification of mRNAs and SNP genotyping.[14-17] Typically, benchtop capillary electrophoresis equipment detects the fluorescent-labeled products of the allele-specific ligation, while the peak pattern determines sample genotyping. However, conventional MLPA introduces several constraints to generate specific products for SNP genotyping. For instance, the analysis of up to 50 target sequences simultaneously involves each ligated probe and gives rise to a PCR product of a specific length ranging from 90 to 500 bp. The consequences are complex probe design, restrictive ligation conditions and differences in amplification yield. There is an interesting approach, called array-MLPA, which focuses on detecting deletions and duplications,[18] pathogens,[19] and cancer

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† Footnotes relating to the title and/or authors should appear here.
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biomarkers.[20] In this method, the enzymatic reaction is combined with a universal microarray by including barcode sequences in ligation probes. Then each targeted region is identified according to the location on the chip where the unique barcode sequence is immobilized. Two detection principles have been proposed for array-MLPA: conventional fluorescent scanning of glass slides and pulse amperometric readout of gold electrode array.[18-20] These approaches imply major barriers for real integration into clinical practice.

Several research groups have demonstrated that consumer electronic devices, such as compact disc, smartphone and flatbed scanner, can be used as DNA diagnostic tools.[21-23] Their application to genotype genetic variants, based on allele-specific amplification or hybridization, suffer limited multiplexing capability (less than 5 SNPs simultaneously) due to restrictive assay conditions. The objective of the present study was to develop a modified array-MLPA methodology, combined with compact disc technology, for a fast, sensitive SNP genotyping analysis to fulfil POC test requirements. Since the analytical platform is a digital versatile disc (DVD), the method is called DVD-array-MLPA (Fig.1). We exploit the advantages of an easy, cheap, robust, user-friendly, portable, mass-produced technology that is compatible with biosensing.[24] Our research focused on modifying the ligation reaction by implementing a barcode microarray on the disc surface and colorimetrically reading the results using a disc drive as a multiplex genotyping tool.

Results and discussion

1. Improving the ligation process

The first step of the proposed approach (DVD-array-MLPA) relies on the hybridization and ligation of two adjacently situated oligonucleotides to a specific genomic DNA sequence (discrimination ligation probe and common ligation probe). Depending on the allele to be detected, the probe was designed to contain a perfect-match or mismatch nucleotide at the ligation site. All ligation oligonucleotides had identical tails for later amplification of all recognized hotspots with a single primer pair (universal amplification).

The assays performed under different experimental conditions (e.g. buffer composition, enzyme concentration, ligation cycles, incubation times, temperature) allowed us to identify the annealing time to be a key variable (Supplementary Material, Fig. SI.2). In conventional MLPA approaches, the time required before starting ligation is about 16 h. However, our study showed that this step can be drastically reduced by about 120-fold because comparable responses were obtained for a 7.5-minute annealing time (Fig. 2A). These results were interpreted after considering the kinetic profile for the homogenous hybridization of short probes. In the liquid phase, the perfect-match duplex formation quickly achieved maximum efficiency.[25] Conventional MLPA requires long reaction times to ensure that annealing processes are completed, and considers not only a wide range of hybrid sizes (e.g. copy number

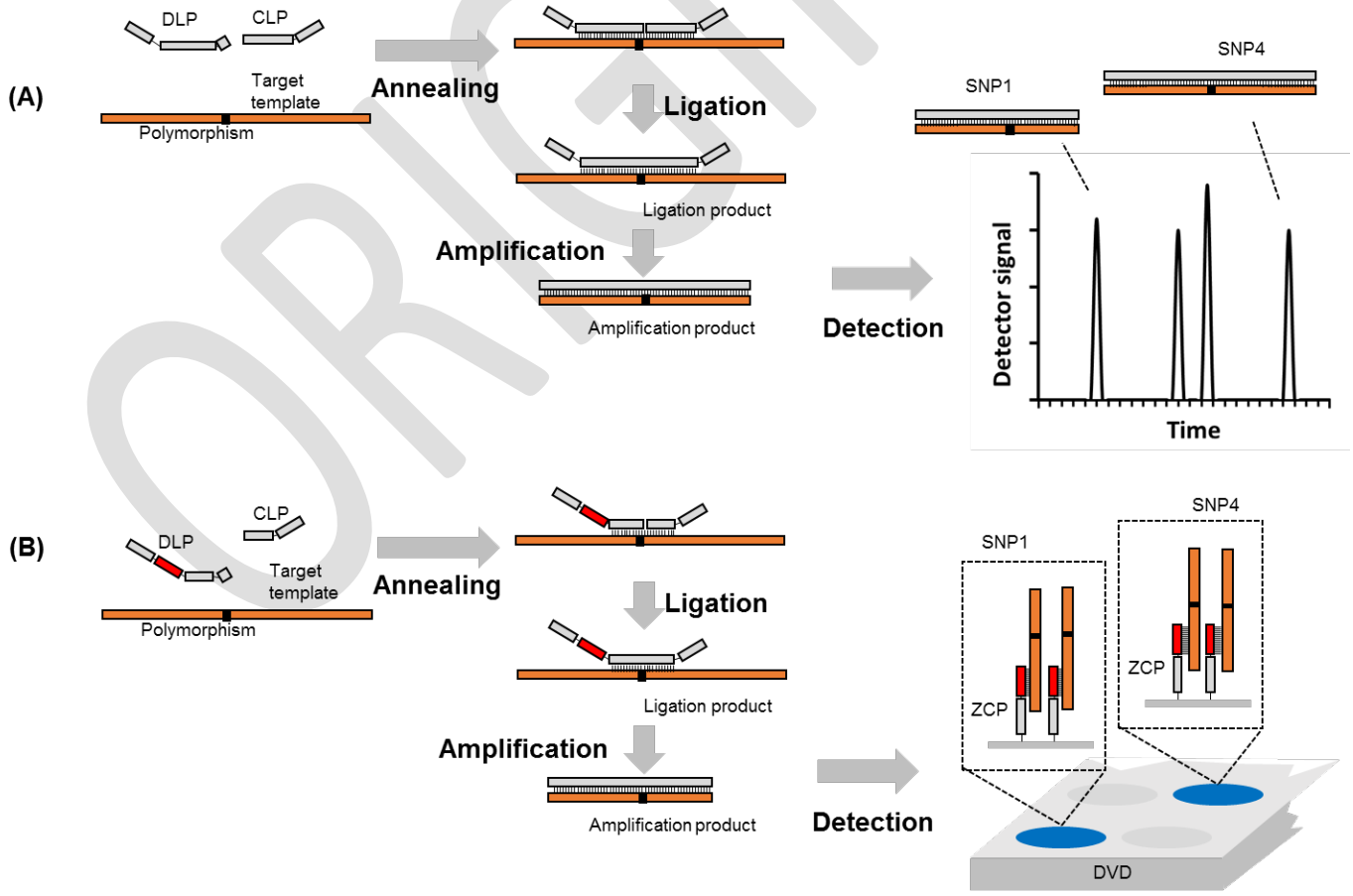

Fig. 1 Scheme of the SNP discrimination principle. (A) Conventional MLPA (B) Array-MLPA. DLP: discrimination ligation probe, CLP: common ligation probe, ZCP: barcode probes

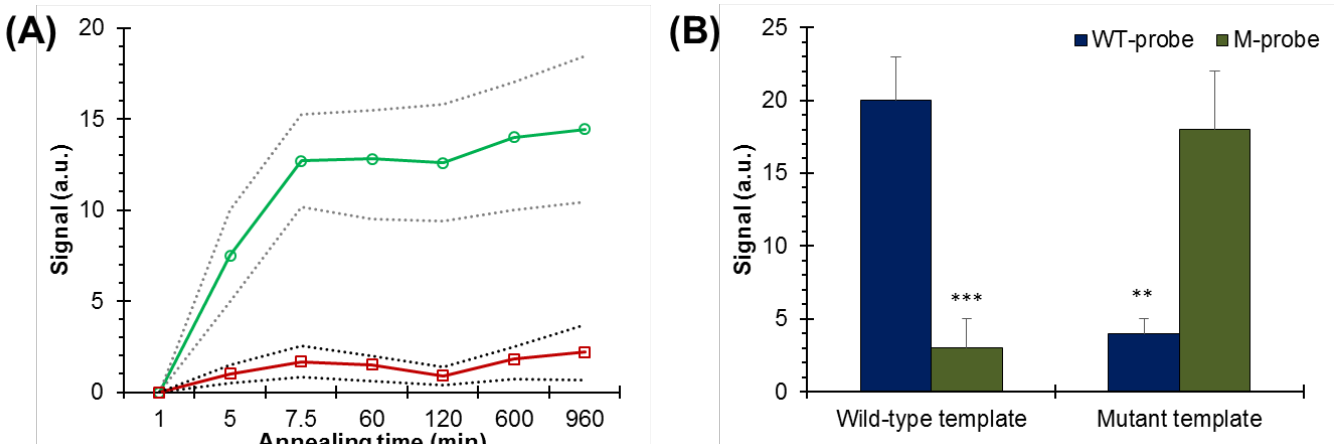

Fig. 2 Fluorescence measurements after ligation-universal PCR (A) Effect of annealing time on assay response. Green circles: DNA templates (1000 copies). Red square: blank solutions. Dashed lines: error range. (B) Ligation selectivity depending on the ligation probe sequence: wild-type (WT-probe) and mutant (M-probe). ***: test t, p-value<0.001; **: test t, p-value<0.01. Target SNP: rs4680 located in the *COMT* gene.

determination), but also the presence of stable intramolecular secondary structures and partially hybridized species. However, using ligation probes with similar thermodynamic properties (i.e. absence of loops and similar melting temperatures) can accelerate the process. Furthermore, ligation errors can be minimized if the genotyping is based on the relative responses between wild-type and mutant alleles.

The next experiments focused on evaluating assay selectivity using the DNA templates from different human genome regions (Supplementary Material, Table SI.2).

Ligation occurred selectively because no signal was recorded for the unmatched templates (t-test, $p<0.05$), while amplification took place for the expected complementary sequences. The results also confirmed that a mismatch at the 3'-end of the ligation probe completely prevented a non specific product from appearing (Fig. 2B). Therefore, the allelic variant discrimination for a specific polymorphism was possible because the generated signals of the perfect-match products were easy to distinguish from the background.

2. Array detection

The first modification for enabling the detection of ligation products was the structure of ligation oligonucleotides. In conventional MLPA, those contain a stuffer sequence to achieve the electrophoretic separation based on ligation product length. In array-MLPA, the discrimination ligation probe only contained a central region (hybridization barcode) between the amplification tail (5'-end) and the specific sequence (3'-end). Hence the sequences of these regions differed for each ligation probe, but length was the same. A second modification was related to detection through non coding barcodes. Estimated selectivity improved because their sequences had no homology to any human genome region. Thus each allele-specific product should hybridize to the specific barcode attached to the DVD surface.

Solid-phase hybridization in the barcode format was done to simultaneously detect each product yielded from the previous discrimination process. The challenge consists in a proper oligonucleotide design by minimizing the cross annealing between oligonucleotides and the formation of secondary structures (i.e. self-annealing).

A specific algorithm based on thermodynamic properties was used to choose the candidate sequences.[26] Outputs were sequences that showed similar annealing to the template hotspot (ΔG° = -28.1 ± 2.6 kcal mol^{-1}) and the absence of significant hybridization to erroneous regions (ΔG° < -8.8 kcal mol^{-1}). Regarding SNP discrimination, the alteration expected due to the presence of a mismatch was comparable for all cases. In fact the estimated variation in standard free energies, expressed as the difference between the single-base mismatch (ΔG°mm) and the perfect match (ΔG°pm), ranged between 3.2 and 4.6 kcal mol^{-1}.

For the immobilization of the barcode probes on the top DVD layer, indirect passive attaching (streptavidin-biotin interaction) was chosen. This option was versatile and provided an adequate immobilization yield (surface density of about 0.5 pmol/cm^2). The hydrophobic nature of the polycarbonate surface (contact angle 90º) rendered a blocking stage unnecessary (e.g. low background signal) and simplified the washing protocols compared to other solutions. The barcode array involved shorter incubations and less restrictive conditions (hybridization and washing) than the conventional formats of DNA microarrays.[27,28] After colorimetric staining, the hybridization assay between the probes and the ligation-amplification products yielded blue spots with a diameter of 500 ± 20 μm. These features enabled optical detection based on a DVD player/recorder (laser diode emission at 650 nm, numerical aperture 0.6) because its optical resolution was about 8.2 μm/pixel. In the absence of a solid deposit (reaction product), the reflection properties of the disc surface remained unchanged and the maximum intensity of the reflected beam was collected by the DVD drive (the background signal).

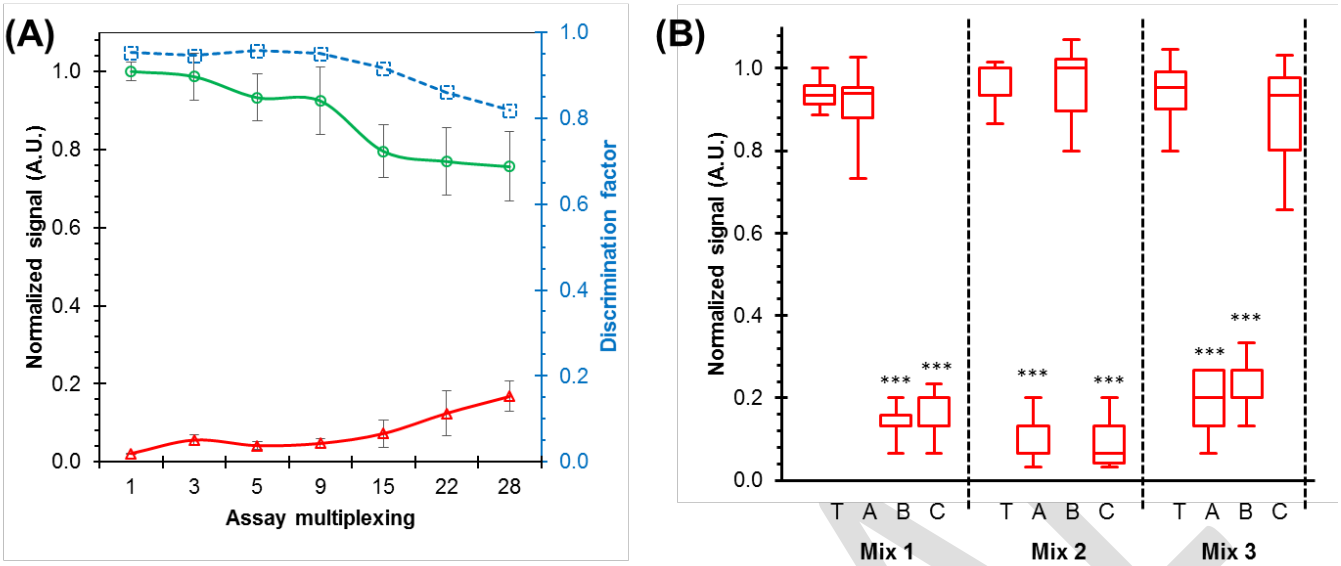

Fig. 3 (A) Effect of format multiplicity on discrimination capabilities for a specific SNP (rs2319398 located in the *GSK3B* gene). Green circles: signal of the wild-type mixture; red triangles: signal of the mutant mixture; blue squares: the discrimination factor. (B) Microarray spot intensities for different ligation reactions in the 10-plex format. T: Probes of the target SNP (rs2319398). A: Probes for Mix 1 (1-9 SNPs); B: Probes for Mix 2 (1, 10-18 SNPs); C: Probes for Mix 3 (1, 19-27 SNPs). ***: test t, p-value<0.001

However when the laser hit a microarray spot, the reflected laser beam attenuated. Consequently, the laser beam intensity that reached the photodiode of the DVD pickup diminished.

A set of experiments was performed to check the designed oligonucleotides in hybridization efficiency terms in a single format, as described in the Supplementary Material (Fig. SI.3). The method output was an individual hybridization image, and the signal of each spot correlated with the recognition process (probe-allele-specific product). The signal-to-noise ratios, calculated from the mean spot signal and chip background, went up to 50. Unlike conventional MLPA, the detected signals did not change between products because the global number of nucleotides added per time unit during amplification (polymerase processability) was similar (test t, $p<0.05$). Therefore, the results demonstrated the capabilities for reliable detection in a DVD microarray format of MLPA products.

3. Multiplexing capability

In the DVD-array-MLPA method, the analytical performances associated with a particular variant could depend on the nature and number of hotspots that had been simultaneously assayed. As the studied technique exploited the high-throughput capabilities of the universal PCR and the barcode array, a set of experiments was run to evaluate the effect of multiplexing on genotyping a target SNP. The tested assay formats went from the single option (1-plex) to an approach for 28 polymorphisms (28-plex), as described in the Supplementary Material (Fig. SI.4 and Fig. SI.5).

The experiments indicated that a higher multiplexing capability was limited mainly for ligation yield, which agrees with previous studies.[15,29]

Specific responses were observed in all the integrated formats (Fig. 3A). Thus ligation, universal PCR amplification and chip hybridization were achieved regardless of the number of products present in the reaction solutions. However, when assay complexity increased, the mismatch responses rose (up to 8-fold) and the perfect-match responses and discrimination factor decreased by up to 25 % and 15 %, respectively. In order to understand these variations, alternative ligation reactions run in a 10-plex format were studied by changing nine targeted SNPs and keeping one constant (Fig. 3B). The results showed that the signal recorded for the specific SNP was the same, independently of mixture composition (ANOVA test, p-value 0.3-0.7). These experiments demonstrated that the yield reduction was associated with weak cross-reactivity due to inherent enzymatic activities rather than to oligonucleotide incompatibilities. The ANOVA test showed that the spot intensities for all the polymorphisms were similar, irrespectively of assay format (mismatch complexes: p-value = 0.44 and perfect-match complexes: p-value = 0.86). All the genotypes were also correctly assigned, which indicates the success of the simultaneous allele-specific assays. In conclusion, the results demonstrated that the developed method was a useful medium-scale genotyping tool.

4. Sample analysis

The DVD-array-MLPA method performances were evaluated by genotyping clinically relevant SNPs from human samples (buccal swap and blood). Fig. 4 illustrates an example of the microarray images generated from the DVD detector outputs (assay for 28 SNPs).

The discrimination capability between the patient populations was studied from the training set samples. First, a scatterplot per studied SNP was created from the signal-to-noise responses of the wild-type probe against those of a mutant probe (Fig. 5A). Although different patients scored distinct optical intensities, probably caused by differences in process yields, data clouds were observed depending on the SNP population. Discriminant

functions were constructed as linear combinations of probe intensities. Here the objective was to automatically classify new observations as belonging to one population group or another. The discriminating functions obtained by the multiple regression model were statistically significant at the 95 % confidence level (p-value < 0.05).

(A)

gene	SNP	1	2	gene	SNP	1	2
ABCB1	rs2235048	A	G	*C+*			
CYP1A2	rs762551	A	C	*COMT*	rs4680	A	G
GSK3B	rs2319398	A	C	*DRD3*	rs963468	A	G
*CYP2C19*17*	rs12248560	T	C	*HTR2A*	rs6313	A	G
LPHN3	rs6551665	A	G	*CYP2C9*2*	rs1799853	A	G
C-				*CYP2C19*3*	rs4986893	A	G
CACNG2	rs2284017	T	C	*C+*			
GRIA3	rs4825476	A	G	*ANKK1*	rs1800497	T	C
CYP2D6	rs16947	A	G	*CYP2C9*3*	rs1057910	T	G
*CYP2D6*41/69*	rs28371725	T	C	*CYP2D6*	rs1135840	G	C
C-				*RGS4*	rs2661319	A	G
DRD2	rs6277	A	G	*C+*			
GNB3	rs5443	T	C	*DRD4*	rs11246226	T	G
GSK3B	rs13321783	T	C	*GRIK2*	rs2518224	A	C
LOC729622	rs4675690	T	C	*HTR1A*	rs10042486	T	C
NR3C1	rs852977	T	C	*LPHN3*	rs2345039	C	G
C-				*NR3C1*	rs10482633	T	G

(B)

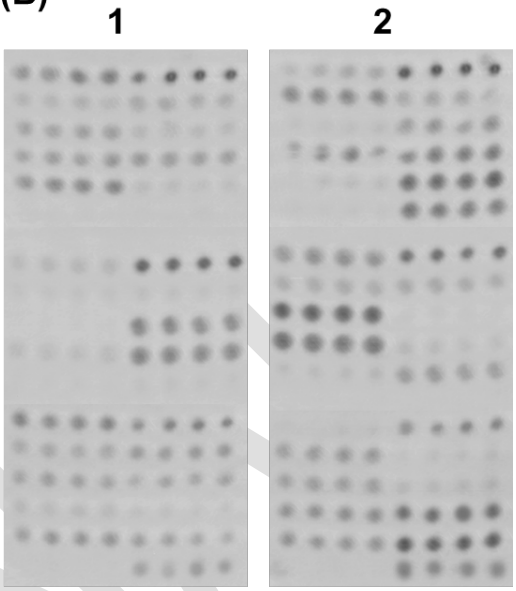

Fig. 4 Patient analysis. (A) Microarray layout indicating the printed probes. (B) Images obtained from a blood sample. Patient genotype: rs2235048 AA, rs762551 CC, rs2345039 AA, rs12248560 CT, rs6551665 AA, rs4680 AG, rs963468 GG, rs6313 AG, rs1799853 GG, rs4986893 GG, rs2284017 CC, rs4825476 GG, rs1800497 CC, rs16947 GG, rs1057910 AA, rs28371725 CC, rs1135840 GG, rs2661319 GG, rs6277 AA, rs5443 CT, rs11246226 TT, rs13321783 CT, rs2518224 AA, rs4675690 CC, rs10042486 CC, rs852977 CT, rs2345039 CG, and rs10482633 GT.

Although slight variations were observed, the mean coefficients of the discriminant functions were 0.10±0.03 (function 1) and 0.05±0.02 (function 2). These results confirmed that the method yielded similar responses for both alleles. The model's goodness was confirmed because the percentage of correctly classified cases was higher than 98%, except for rs4680 and rs2319398, with 91 %. Fig. 5B shows that the differences among the three clusters gave us an unequivocal genotype classification.

In a blind study, these discriminant functions, calculated from the training set, were used to classify patients' answers from the validation set. The corresponding discriminant scores were calculated for each possible group (wild-type homozygote, mutant homozygote and heterozygote) and the genotype for each studied polymorphism was assigned (Supplementary Material, Table SI.6). The accuracy of the genotype calls by the microarray-based method was verified by analyzing the same genomic DNA extracts using the Illumina Goldengate platform. This high-throughput platform combines primer extension and ligation to generate allele-specific products, followed by a PCR for the amplification and hybridization to the codified beads for individual readout. The mean coincidence percentage was 96±4 % (28 SNPs), with comparable values to other low-throughput simple methods.[13,25,29-31]

The other analytical performances of the DVD-array MLPA method were also estimated for point-of-care applications.[3]

Assay reproducibility was expressed as the relative standard deviation of the spot intensities for triplicate assays, and was lower than 15 %. The multiplexing results confirmed the method capability for the simultaneous genotyping of up to 28 SNPs in a single DVD (8 samples per disc). In the developed method, both enzymatic steps and array-based detection lasted less than 2 h and 1.5 h, respectively, but was "hands off" for most of the time (2.6 h). Thus the estimated analysis time was under 5 h, which contrasts with the 2 days spent in conventional MLPA. As a smart sensing detector was used for DVD technology, the method is also portable, low-cost and requires very little specialized laboratory equipment. For instance, the prospective costs (<3 € disc and <500 € reader) are lower than current platforms.[6,8,33]

Experimental

Patients

Volunteers, and the patients diagnosed by specialist psychiatrists, were recruited for the present study according to ethics after giving informed consent. Subjects were divided for method optimization (n=15) and validation (n=35). Buccal swabs and blood samples were collected.

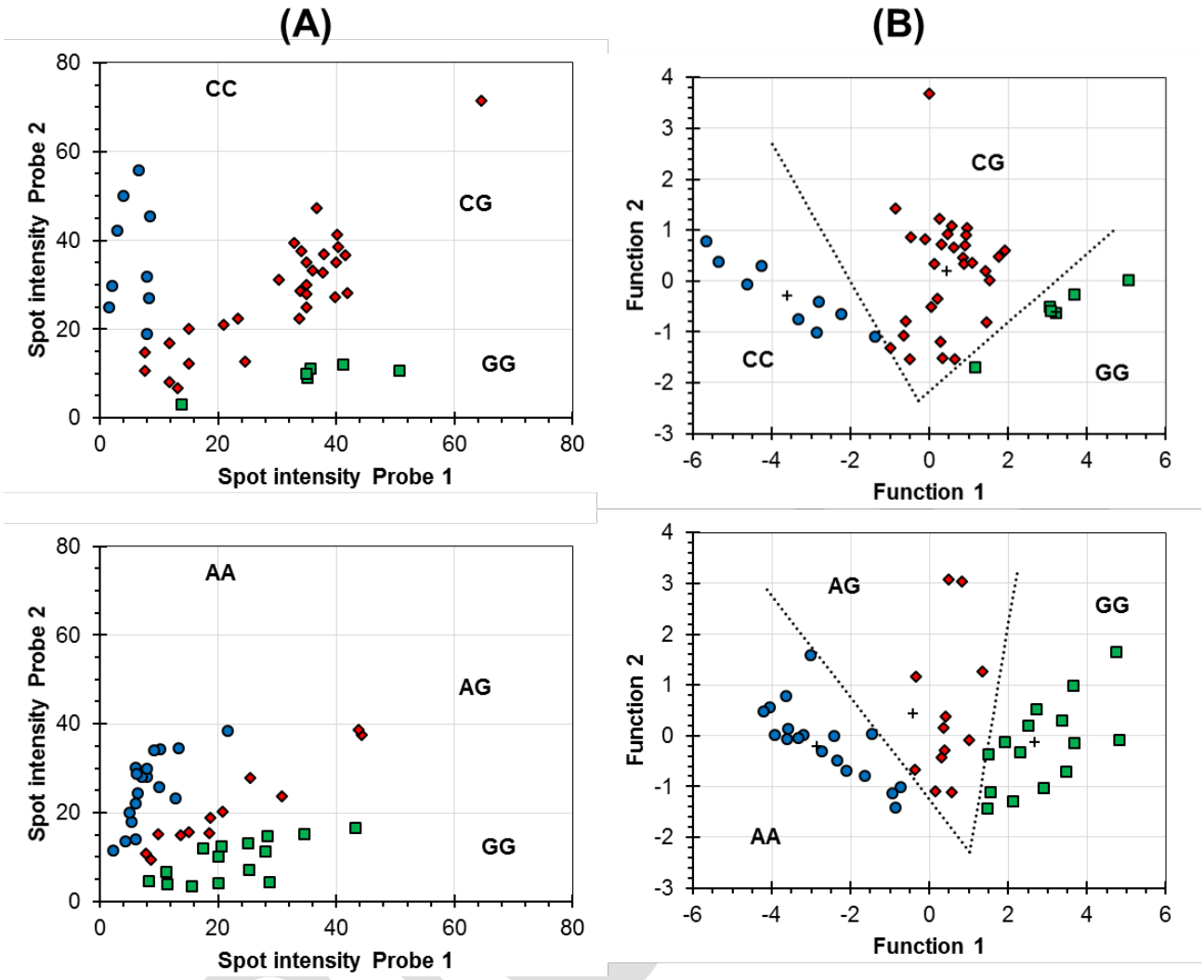

Fig 5. (A) The XY scatter plots created from the spot intensities of the wild-type probe and mutant probes: rs2345039 (top) and rs2661319 (bottom). (B) Plots of discrimination function: rs2345039 (top) and rs2661319 (bottom). Cross symbols indicate the group centroids. Data: 15 human samples of the training set (3 replicates).

Ligation-based discrimination reaction

Two probe mixtures (alleles A and B) were prepared, and comprised 28 oligonucleotide sets (Supplementary Material). Each set consisted of two oligonucleotides: the left probe oligonucleotide (LPO) and the right probe oligonucleotide (RPO). LPO is allele-specific at the 3'-end and has a 5'-tail for amplification. RPO is a 5'-phosphorylated oligonucleotide that hybridizes to the directly adjacent target sequences.

RPO also contains a 3'-tail with a specific barcode for each targeted polymorphism and a common sequence for amplification. The concentration of each LPO and RPO was 1.3 nM in Tris-EDTA buffer (TEB: Tris-HCl 10 mM pH 8, EDTA 0.1 mM). Two reaction solutions (4 µL) were prepared. They contained genomic DNA from the patients' sample (30 ng) and an aliquot of each probe mixture (0.75 µL). After DNA denaturation (98°C, 5 min) and probe annealing (60ºC, 7.5 min), the ligation mixture was added, which contained 0.5 µL of Salsa Ligase-65 (MRC-Holland) in a final 20-µL volume. Then ligation was performed (60 °C, 7.5 min), followed by enzyme deactivation (98 °C, 5 min).

Universal amplification

Two PCR reaction mixtures were prepared. They contained Tris-HCl at 75 mM pH 9.0, KCl at 50 M, (NH4)2SO4 at 20 mM, MgCl2 at 3 mM and dNTPs at 100 µM. The first mixture contained a type-A forward primer (digoxigenin-ACTTCGTCAGTAACGGAC) and a reverse primer (GTCTGCCTATAGTGAGTC) at 300 nM. The second mixture contained a type-B forward primer (digoxigenin-GAGTCGAGGTCATATCGT) and the same reverse primer. To each mixture (10 µL), the ligation product solution was added. The cycling conditions were 35 denaturation cycles (95 °C, 30 s), primer annealing (64 °C, 30 s), elongation (72 °C, 30 s), and a final elongation (72 °C, 10 min). For optimization, process efficiency was monitored by a fluorescence plate reader (EX485 and EM535 nm, Victor 3TM V1420, Perking Elmer) after SYBR Safe-staining in a 96-well black polystyrene plate.

Universal hybridization

For probe immobilization, each biotinylated probe (50 nM) in printing buffer (50 mM carbonate buffer, 1% glycerol (v/v) and

streptavidin (10 mg/L) at pH 9.6) was transferred to the DVD disc surface (50 nL) with a non contact printer (relative humidity 90%, AD 1500 BioDot Inc.). The microarray layout consisted of eight arrays of 17×6 dots each, with a 1-mm track pitch corresponding to the targeted genes, positive controls and negative controls (immobilization and hybridization). For hybridization, 2 µL of purified PCR product were mixed with 38 µL of the hybridization solution composed of SSC buffer 1× (sodium citrate 15 mM, NaCl 150 mM, pH 7), 35% formamide, and 1.5× Denhardt's reagent. The solution was denatured by heating (95 °C, 5 min), transferred to the slide surface and incubated (37 °C, 1 h). Discs were gently washed for 1 min with diminishing dilutions of SSC (SSC 1×, SSC 0.5×, SSC 0.05×).

Colorimetric staining consisted in an immunoreaction. Subsequently, the 1-mL mix of 1:2500 sheep antidigoxigenin-antibody (Abcam) and 1:300 antisheep-antibody labelled with horse radish peroxidase (Abcam) in phosphate-buffered saline solution containing 0.05 % detergent Tween-20 (PBST) was dispensed to the DVD and was incubated (room temperature, 30 min). After washing with PBST and water, 1 ml of TMB (3,3',5,5'-tetramethylbenzidine) was spread on the slide surface, incubated (room temperature for 8 min) and washed with water. The disc was placed in the DVD-drive and was scanned by a 650 nm-laser. The signals from the detection areas were processed for digitization and deconvoluted into a gray-scale image (Tagged Image File Format, colour depth 16 bit, scale 0-65535). The optical intensity signals of each spot were quantified by in-home software.

Discrimination criteria

The Statgraphics Centurion statistical package for Windows v.16 was used for the data analysis. A genotype decision rule was constructed based on the discriminant functions calculated from the spot intensities of the wild-type and mutant probes (multiple regression). The functions for polymorphism i were: $D_i = c_{i0} + c_{iA} X_{iA} + c_{iB} X_{iB}$, where ci are the regression coefficients and Xi are the spot intensities for each allele. By substituting the data for a new sample (X_{iA} and X_{iB}), a score can be calculated and a patient can be classified and placed in a population group. The squared Mahalanobis distances from the group centroids were calculated in the space of the discriminant functions for each observation.

Conclusions

Quantitative PCR, used as a genotyping technique, is limited by its low-multiplexing capabilities, while sequencing techniques pose major technological barriers for a wide range of clinical scenarios. Our study developed a DVD-array-MLPA approach that overcomes some of these restrictions. This technique is able to perform the simultaneous detection of several nucleic acid sequences (low-middle scale). Key elements are based on ligation-mediated discrimination, amplification employs universal primers and the latter hybridization uses barcode probes. Furthermore, the integration with consumer electronic technology, such as a compact disc, provides additional advantages that derive from their low-cost materials and user-friendly optical read-outs. The result is a cheap, versatile, fast, portable and parallel format that provides a commercially affordable analytical system. Nevertheless, more efforts are required towards an automated assay (i.e. microfluidic DVD-based disc). Since the genotyping method includes PCR for universal amplification, the next step could be directed to isothermal techniques in order to simplify the required auxiliary equipment.

Regarding clinical applications, the discrimination of DNA variants (polymorphisms or point-mutations) is basic to advance in pharmacogenomics and genetic diagnoses. The reported results demonstrate that this biosensing methodology is extremely powerful for analysing high-identity sequences, as required in personalized medicine. In short, this research contributes to the important scientific challenge of developing affordable analytical tools for mass access to specific genetic information.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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