PRIMER DESIGN FOR SNP GENOTYPING BASED ON ALLELE-SPECIFIC AMPLIFICATION. APPLICATION TO ORGAN TRANSPLANTATION PHARMACOGENOMICS

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ABSTRACT

Diagnostic methods based on single nucleotide polymorphism (SNP) biomarkers are essential for the real adoption of personalized medicine. Allele specific amplification in a homogeneous format or combined to microarray hybridization are powerful approaches for SNP genotyping. However, primers must be properly selected to minimize cross-reactivity, dimer formation and nonspecific hybridization. This study presents a design workflow diagram for the selection of required oligonucleotides for multiplex assays. Based on thermodynamic restrictions, the oligonucleotide sets are chosen for a specific amplification of wild- and mutant-type templates. Design constraints include the structural stability of primer-template duplexes, template-probe duplexes and self-annealing complexes or hairpins for each targeted gene. The performance of the design algorithm was evaluated for the simultaneous genotyping of three SNPs related to immunosuppressive drugs administered after solid organ transplantation. The assayed polymorphisms were rs1045642 (*ABCB1* gene), rs1801133 (*MTHFR* gene) and rs776746 (*CYP3A5* gene). Candidates were confirmed by discriminating homozygote and heterozygote populations using a fluorescence solution method and two colorimetric microarray methods on polycarbonate chips. The analysis of patient samples provided excellent genotyping results compared to those obtained by a reference method. The study demonstrates that the development of the allele-specific methods as pharmacogenetic tools can be simplified.

Keywords: primer design; SNP genotyping; microarray; pharmacogenomics; organ transplantation

INTRODUCTION

Single nucleotide polymorphisms (SNPs) have been proposed as the next generation of markers to identify loci associated with complex diseases and their therapeutic treatment [1]. Technological tools and pharmagenomics principles that support personalized medicine are required to identify of effective drugs in advance (nature and doses), cut costs and improve patient quality of life. Unfortunately, the incorporation of genomic findings into health care systems is limited [2,3]. In particular, low-cost genotyping tools are absolutely necessary for effective personalized medicine.

The allele-specific (AS) amplification of a particular nucleotide variant has a high potential to be developed for massive implementation into clinical laboratories [4]. The genotyping principle is based on an effective primer extension by the polymerase when the 3' terminal base of the primer matches its target, whereas extension is inefficient or nonexistent when the terminal base is mismatched. AS-PCR methods are quick, excellent and inexpensive strategies which require minimal instruments that are found in most laboratories. The main limitation is the low-throughput capability (small number of SNP simultaneously analyzed). However, there are hundreds of pharmaceutical applications given the relatively few SNPs clinically validated for a given disease [5]. A simple AS-PCR approach is to use primer mixtures combined to genotyping determination based on product size or amplification kinetics [6-8]. Microarray hybridization-based methods show important additional advantages, such as cost-effectiveness and increased multiplexing [9,10]. The allele discrimination reaction is carried out in solution by multiplex allele-specific PCR and a solid phase array (conventional or zip-code format) displays the amplification results.

AS-PCR methods involve up to five oligonucleotides per polymorphism. The oligonucleotide design is crucial in optimizing assays because a poorly selected primer or probe can result in little or no target product and, consequently, in erroneous discriminations. Many kinds of software tools are available for primer design for conventional PCR, including presence of a polymorphism in the input sequence or in primers [11-13]. Thanks to its capabilities and free accessibility, Primer3 Plus is the most popular non commercial primer design software [14]. In the genotyping field, some research teams have proposed tools and criteria for designing AS primers. The WASP tool is a primer design program applied specifically to AS-PCR [15]. However, a limited quantity of primers is provided, which might somewhat influence method performance. One remarkable program is BatchPrimer3, based on the Primer3 algorithm, which incorporates a specific module to choose the best primer pairs for AS-

amplification [16]. Nevertheless, these solutions were not developed to design hybridization probes or to include zip-codes.

In the present study, a novel design workflow is developed for generating the oligonucleotides employed in SNP genotyping methods based on AS-amplification. The innovative algorithm supports the simplest approach (single reaction per variant) to the most complex alternative (combination of multiplex AS-PCR in solution and solid phase array in the zip-code format). The work includes the study of design variables, the experimental confirmation of *in-silico* parameters, and the determination of the analytical performances of the selected oligonucleotide sets.

The design tool was applied to genotype the SNPs related to immunosuppressive drugs administered after organ transplantation (both initial and maintenance periods). Underexposure to calcineurin inhibitors, such as tacrolimus and cyclosporine, may result in acute rejection, while overexposure places patients at risk given its considerable toxicity. Nowadays, therapeutic drug monitoring helps determine suitable immunosuppressant dose adjustments per patient, but the work is usually done by an assay-error method. Several research studies have demonstrated that genetic factors (SNPs related in transport, metabolism and drug action) explain why patient groups respond differently to the administrated drug dose [17]. Therefore, their genotyping by simple diagnostic techniques will aid the fragile equilibrium there is between the risk and benefit of immunosuppression therapy, before administration.

MATERIAL AND METHODS

Target polymorphism. Different SNPs have been described to be related in transport, metabolism and drug action, but the clinical community accepts only a few of them [18]. The SNP catalogued as rs1045642 (C3435T polymorphism) is located in the *ABCB1* gene, also known as the multidrug resistance gene. In organ transplantation, a lower immunosuppressor dose is required in those individuals who are TT homozygotes than CC homozygotes [19]. The rs776746 polymorphism (A698G) is located in the *CYP3A5* gene, and the change of base produces a splicing defect that results in a nonfunctional cytochrome P450 protein found in the liver, small intestine and kidney. Homozygote AA, heterozygote AG and homozygote GG patients are normal, intermediate and slow metabolizers of immunosuppressants, respectively [20]. Another interesting polymorphism is rs1801133 (C677T), located in the *MTHFR* gene. Homozygote TT patients show diminished protein activity, which may lead to the risk of higher methotrexate toxicity [21]. As the prevalence of the TT genotype is high, these results may have implications for drug dosage.

Oligonucleotide design tool. The nucleotide sequences for the studied polymorphisms were retrieved from the SNP database of the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov). The multi-SNP program, supported in the Visual Basic language, was used to select oligonucleotides. The input data were the FASTA sequence of the gene regions, SNP alleles and design constraints. Alleles were indicated by IUPAC codes (G/C: S, A/T: W, G/A: R, T/C: Y, G/T: K, A/C: M). The design constraints and the program algorithm are described in Supplementary Material. The design output is the compatible primer sequences and amplification product sequences. Information is provided about oligonucleotides, such as size, melting temperature among others. The program also estimates the recommended annealing temperature under thermocycling conditions (*in-silico* PCR conditions). The expected value is calculated as $T_a = T_m + ln\ L$, where T_m is the lowest melting temperature of the primers and $ln\ L$ the natural logarithm of PCR fragment length. The generation of candidate sets for organ transplantation pharmacogenenomics is also described.

Reference design tools. Primer sets were also calculated from the Primer 3Plus software (http://www.bioinformatics.nl) and the WASP design tool (http://bioinfo.biotec.or.th/WASP). The primer constraints of the Primer 3Plus software

were 20 (18-27) mer, GC 20-80 %, melting temperature 60 ºC (57-63), product size 110-175 bp, self-complementarity ≤ 8, 3'-complementarity ≤ 3. The primer constraints of WASP design tool were 20 mer (18-36), GC 50 % (20-85), melting temperature 60 ºC (40-65), maximum temperature difference: 100, self-complementarity ≤ 8, 3'-complementarity ≤ 4, polyX in primer ≤ 3, product size 110-175 bp. The selected sets are listed in the Supplementary Material.

Assays for checking designed oligonucleotides. Experiments were performed to test the selected oligonucleotide sets by considering three assay formats: single assay, multiplex assay based on common probe hybridization and multiplex assay based on universal hybridization.

*Single assay***.** Six amplification reactions (12.5 μL) were performed per sample (two vials per SNP). Each reaction mixture contained 1×DNA polymerase buffer, 3 mM of $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 20 μM of digoxigenin-11-2'-deoxyuridine 5'-triphosphate, 0.2 μM of forward primer (wild-type or mutant), 0.2 μM of reverse primer, 1 unit of DNA polymerase (Biotools, Madrid, Spain), and 4 ng of genomic DNA. To improve the differences between wild-type and mutant variants, locked nucleotide acids (LNA) were used as primers. Then the *in-silico* annealing temperatures reported by the developed program were corrected by considering this chemical modification. The cycling conditions were: initial denaturation at 95 °C for 5 min followed by 38 cycles of denaturation at 95 °C for 30 s, primer annealing at 64 °C for 30 s, elongation at 72 °C for 30 s, and a final elongation at 72 °C for 5 min. Amplified DNA was quantified from the fluorescence measurements with a cyanine dye (SYBR-Safe, Invitrogen, Spain) at 0.01% (v/v) in a microtiter plate reader (Wallac, model Victor 1420 multilabel counter, Finland). Amplification yields were calculated in reference to the initial DNA (ng of amplified DNA/ng of initial DNA). Amplified products were also checked by electrophoresis on a 3% (w/v) agarose gel at 110 V and room temperature. Gels were stained for 30 min with 0.5× Tris/Borate/EDTA buffer that contained the fluorophore Real-Safe (Real Laboratories, Spain) at 0.01% (v/v). Bands were visualized with a UV transilluminator.

*Multiplex assay based on common probe hybridization***.** Raw polycarbonate slides were used as hybridization platforms. Each mixture of streptavidin (10 mg/L) and biotinylated probe (50 nM) in printing buffer (50 mM carbonate buffer, pH 9.6 and 1% glycerol (v/v)) was transferred to the slide surface (50 nL) with a non contact printer (AD 1500 BioDot Inc., CA, USA). A tail of 10 thymines was appended to the probe at the 5'-end to

reduce surface interactions. Working temperature and relative humidity were controlled at 25 °C and 90%, respectively. The amount immobilized probe was about 0.03 fmol/mm^2. Amplification procedures were based on two triplex PCR reactions (12.5 µL), which contained the three WT-FPs (WT-vial) or the three M-FPs (M-vial), respectively. The composition of the reaction mixture and the cycling conditions were the same as in the single assay, except for primer concentrations (0.3 µM). Amplification products were purified by silica-gel membrane adsorption (PCR Purification Kit, Jena Bioscience) and eluted in low-salt buffer. The hybridization protocol on the polycarbonate slide and chip scanning was performed as described in Tortajada-Genaro *et al.* [10].

*Multiplex assay based on universal hybridization***.** A hexaplex PCR reaction (12.5 µL) that contained the zip-code FPs and RPs for the three studied SNPs was performed. The composition of the reaction mixture and the cycling conditions were as in the previous assay, except for the primer concentrations (0.3 µM). Amplification products were purified using silica-gel membranes prior to hybridization on the polycarbonate slide. The microarray layout consisted of 12 arrays of 8×6 dots each, with a 1-mm track pitch that corresponded to targeted genes, positive controls and negative controls (immobilization and hybridization). For hybridization, 2 µl of purified PCR product were mixed with 38 µl of 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 at 95 °C for 5 min and transferred to the slide surface. Slides were incubated at 37 °C for 1 h in a conventional oven. Discs were gently washed for 1 min with diminishing dilutions of SSC (SSC 1×, SSC 0.5×, SSC 0.05×). Subsequently, the 1-mL mix of 1:2500 sheep antidigoxigenin-antibody (Abcam, Cambridge, UK) and 1:300 antisheep-antibody labeled with horse radish peroxidase (Abcam) in phosphate buffered saline solution, which contained 0.05 % detergent Tween-20 (PBST), was dispensed to the DVD and incubated for 30 min at room temperature in the dark. After washing with PBST and water, 1 ml of TMB (3,3′,5,5′-tetramethylbenzidine) was spread on the slide surface and incubated for 8 min at room temperature. The arrays were then washed with water. Chips were directly scanned (Epson Perfection 1640SU office scanner) to generate gray-scale images (Tagged Image File Format, color depth 16 bit, scale 0-65535). The optical intensity signals of each spot were quantified using in-house software for image analysis. Briefly, the spatial arrangement (or array addressing) was performed using a feature gridding reproducing the printing layout. Later, the image segmentation classified each pixel as foreground or as background using 250 µm-circle masks. A quality control of image

was applied checking variability in pixel values within each spot mask and measuring circularity of spots. The spot intensity and the local background were calculated from the mean of pixel intensities within the spot and from the regions surrounding the spot, respectively. This image analysis was automatically performed in less than 2 min.

Patient sample analysis. Thirty subjects (8 organ-transplantation patients and 22 controls) were recruited for method optimization (n=10) and validation (n=20) according to ethics and with informed consent. Buccal swab and blood samples were collected. DNA extraction was performed using the PureLink Genomic DNA Mini Kit (Invitrogen), according to the manufacturer's instructions. DNA concentration was determined using NanoDrop 2000/2000c (Thermo Scientific).

For single assays, discrimination was based on the detection of either the fluorescence signal or the electrophoretic band. For the microarray formats, the genotype decision rule was constructed based on a discrimination index using the spot intensities of a wild-type probe (WT) and a mutant probe (MUT). Intensities were normalized from the corresponding average signals of the validation set. The expression of the discrimination index was $(WT_{normalized} - MUT_{normalized})/(WT_{normalized} + MUT_{normalized})$ [10,22]. This ratio is used to discriminate between a perfect and a mismatched target sequence, which differ by only one nucleotide (polymorphic nucleotide).

Genotyping was also performed by means of reference techniques: MALDI-TOF by the MassArray platform (Sequenom) and Sanger sequencing by an ABI PRISM 3100 capillary sequencer (Applied Biosystems).

RESULTS

Design workflow

The theoretical pairs of the forward primer (FP) and reverse primer (RPs) to be used in a single AS-PCR assay are tens or hundreds. The number of possible combinations in a multiplex assay (genotyping of several loci at time) can achieve thousands. Methods based on universal hybridization chips (primers containing a Zip-code region complementary to a specific probe) are *a priori* compatible with an extremely large number of oligonucleotide sets. However, the performances of method are sensitive to the selected set from the possible candidates. A workflow for the simultaneous genotyping of several SNPs was developed based on the choice of the oligonucleotide sets available to satisfactorily amplify/detect the given variants. Table 1 shows the

different steps of the selection process. The design activities per single SNP included the calculation of the candidate primer properties (e.g. position, length, hybridization and secondary structures) and product properties (e.g. secondary structures). These calculations were similar to those considered in the algorithms previously designed for conventional PCR primers [11,14].

Specific design restrictions were incorporated by considering a multiplexed assay: similar melting temperatures of primers, absence of significant hybridization to the erroneous template, and variation in amplicon length. Melting calculations were made using available models and assumptions that describe the thermodynamic stability of DNA duplexes [23]. The sequence homology search and the measurement of their stability considering the ionic strength effect allowed cross-hybridization to be estimated. The objective was to effectively amplify all the targets by minimizing the risk of unbalanced amplification or cross-reactivity. Therefore, the design workflow eliminated the sets that did not fulfill restrictions. These restrictions have not been considered in previous algorithms for AS amplifications [12,15,16,24].

For the automated generation of pre-candidate oligonucleotides and thermodynamic calculations, a computational program was developed in Visual Basic language (described as Supplementary Material). The program, called multi-SNP, selected the oligonucleotides for the simultaneous genotyping of up to 5 SNPs. Table 1 shows the relationship between the program modules and the task list based on design restrictions. The algorithm was designed to support methods based on individual or multiple allelic detections. The category of individual allelic methods includes end-point AS-PCR, single channel fluorescence qPCR, and most biosensing methods. The second category comprises methods based on multiple channel fluorescence qPCR and AS-PCR combined to capillary electrophoresis or DNA microarrays. If a specific module is activated, the output is useful universal microarray detection, including the zip-code for each primer and probe sequences for each targeted SNP.

In-silico **selection of oligonucleotide sets**

As proof of concept, the design algorithm was applied to support the administration of drugs related to organ transplantation. This pharmaceutical problem is an interesting example of applications that a low throughput genotyping method is required because the number of clinically relevant SNPs is low. Particularly, the target polymorphism were rs1045642, rs776746, and rs1801133. A discussion about the specific effect of design restrictions is included in the Supplementary Material file. Table 2 shows the

values selected for each restriction parameter according to the optimization protocol. Oligonucleotide sets were chosen to discriminate wild-type variants (C, C, and A alleles, respectively) and mutant variants (T, T, and G alleles, respectively) using different AS-PCR detection formats.

The algorithm output for the conventional single approach (i.e. fluorescent detection of a specific genetic variant) was a list of the primer sets (wild-type FP, mutant-FP, common RP) selected for each targeted region. The candidate pairs of FP (direct/reverse strands) were 2/2, 2/8, and 0/2 and those of candidates RP were 33/27, 24/47, and 47/39 for rs1045642, rs776746, and rs1801133. The computing results (direct/reverse strands) were the 66/54, 48/141, and 0/39 sets for rs1045642, rs776746, and rs1801133, respectively. The list of single assays is included as Supplementary Material (Table SI.1).

For the multiplex assay, additional constraints collected in Multiplex module, were applied to these primer sets, such as cross-hybridization and product length differences. For direct strands, no set of rs1801133 was compatible. For reverse strands, the cross-annealing evaluation showed 28 sets had a primer that was theoretically hybridized with some templates of other genes (17 % of total cases). Of the 296,946 analyzed combinations, most sets were eliminated because the length differences between products were smaller than 10 bp (96 %). The output was a list of 12,784 combinations (Table SI.2).

The algorithm output for the advanced approach (Zip-code module) was a list of the primers, including zip-codes and corresponding probes. Zip-codes, also called tags, were sequences with no homology to either the target sequences or other sequences in the genome, which minimizes mismatch hybridizations. So primers contained the specific zip-code at the 5’ ends and the products hybridized to the specific zip-code probes were attached to the array. The algorithm evaluated possible combinations and eliminated the zip-codes for which template cross-annealing or zip-code-primer annealing was expected (melting temperature > 45 ºC). The selected flanking zip-codes were 16 of the 40 candidates (40 %). The list of sets is included in Table SI.3.

Primer sets were also calculated from the Primer 3Plus software and the WASP design tool. The first is a general program applied for allele specific amplifications by fixing the FP. The second tool was specifically applied to select the allelic primers per polymorphism. In both cases, the primer pairs were individually designed to support methods that discriminate a single SNP per assay. Table 3 compares the performances of the programs used. The two previous programs are not prepared for multiple amplifications, nor for assays that use primers with zip-codes. The computed

sets are listed in Tables SI.4 and SI.5. Most oligonucleotides agreed with those obtained by the developed workflow. Discrepancies were related to minor differences in the thermodynamic calculations (i.e. reference melting temperature). Nevertheless, some sets were ruled out by the Multi-SNP program due to cross-hybridization between targeted genes.

Checking the designed oligonucleotides for single assays

Sets of primers selected by the developed workflow (Table SI.6) were tested by a conventional genotyping methods based on one reaction mixture per allele and SNP. The selected methods were end-point fluorescence measurement and agarose gel electrophoresis. In this approach, discrimination of allelic variants was optimized mainly by adjusting the thermocycling temperatures.

The first AS-PCR experiments focused on confirming the best working temperatures that induced a selective perfect-match primer-template duplex. Wild-type and mutant templates were added to the reaction mixtures that contained the proposed sets and were amplified in an annealing temperature gradient (Figure 1). An erroneous amplification occurred at low annealing temperatures because both the forward primers hybridized and were extended. By increasing the temperature, a perfectly matched primer was more stable than a mismatched primer, and allele-specific amplification was preferentially produced. By applying a high temperature, the amplification yield lowered because a forward/reverse primer did not hybridize with target genes. A t-test was applied to establish the annealing temperature that produced a signal for the unspecific allele amplification comparable to the background signal. The selected annealing temperature was 64 ºC since the amplifications of wild-type template using mutant-FP or mutant template using wild-type FP were negligible (test-t, wild-type vs. background $p=0.06$ and mutant vs. background $p=0.07$). Thus the experimental results agreed with the predicted annealing temperature calculated from the *in-silico* parameters (63.5 - 66.1 ºC).

The following experiments studied the discrimination capability between primers sets for the same SNP. Single assays were performed for the specific amplification of each target allele using the tested primers. PCR product formation was determined from the end-point fluorescence measurements of PCR products and confirmed by gel electrophoresis. All the proposed sets showed correct discrimination between the wild-type and mutant templates for both reaction mixtures. A multi-factor variance analysis was applied to the fluorescence intensities measured by different sets selected for the

same SNP. The signal variability between reactions using different primers/probes was comparable to the signal variability between reaction replicates (p-value = 0.82 for rs1045642, p-value = 0.22 for rs776746, and p-value = 0.61 for rs1801133). These experimental results supported the equivalence between the sets reported for SNP genotyping.

Checking designed oligonucleotides for multiplex assays based on common probe hybridization

The method based on AS-PCR combined to common probe hybridization (perfect-match region that did not contain the genetic variant) was applied [10]. Although the throughput is not high, this approach based on chip hybridization shows several advantages against other multiplex methods. For instance, the equivalent assay real time PCR involves an expensive thermocycler with a detection system based on four fluorophores (3 SNPs and internal control). The list of tested oligonucleotides is included in Table SI.7. Wild-type and mutant genotypes were amplified in two triplex reactions that contained WT-primers or M-primers. PCR products were incubated on microarrays with a specific immobilized probe per gene. Low-stringent conditions (low salt content and presence of denaturing agent) were chosen to achieve hybridization in short incubation times (60 min) and at low temperature (37 ºC). Therefore, the hybridization temperature was 30-31 ºC below the calculated melting temperatures of the corresponding probes. In all cases, positive signals were observed according to the specific polymorphism in the perfectly matched probes (Figure 2). No cross-amplification or cross-hybridization problems were observed. Therefore, specific products for both variants of each SNP locus (rs1045642, rs776746 and rs1801133) were obtained using the proposed oligonucleotide sets.

Checking designed oligonucleotides for multiplex assays based on universal hybridization

An improved variation of the method reported by Li et al. 2008 [9] was applied. A description of the method principle and the list of tested oligonucleotides (primers and probes) are included in Table SI.8. The advantages compared to conventional hybridization approaches are related to the Zip-codes included in primers and as probes. The hybridization conditions can be easily selected improving the working range and yielding better assay selectivity and sensitivity. Hexaplex PCR was done to simultaneously amplify the specific alleles of the targeted regions (genes *ABCB1*,

MTHFR and *CYP3A5*). Then the amplification mixture contained the perfectly matched and mismatched forward primers with different zip-code tails. The products hybridized with the specific zip-code probes were immobilized on the slide surface (polycarbonate chip). SNP genotyping was achieved because the hybridization profiles differed according to the allelic profile. Under these conditions, the amplification factor was $(5.0 \pm 0.3) \times 10^8$, which is comparable to a single approach (test-t, $p>0.05$). The high yield, without losing discrimination capability, demonstrated that the selected primer sets were compatible for the simultaneous amplification of targeted SNPs. Hybridization assays were also performed from the PCR products of the homozygote mutant, the homozygote wild-type or the heterozygote individuals for three studied SNPs. First, the cross-hybridization between the single PCR products and mismatched probes was not observed because positive results were obtained for specific probes and negative results for the other probes.

Figure 3 shows an example of the optical signals recorded for a patient with a known genotype. As we can see, positive and negative controls provided detectable and background responses, respectively. High-intensity positive spots were observed in the corresponding probes according to the specific polymorphism.

The following experiments focused on evaluating the zip-codes provided by the proposed workflow under the optimized experimental conditions. Genotyping was achieved independently of the used zip-codes because the statistical analysis concluded that array signals were comparable (analysis of variance, $p>0.5$). Therefore, the results demonstrated that the oligonucleotides sets with similar design parameters displayed a similar behavior in the allele-specific amplification and hybridization on a solid support.

Patient sample analysis

The multiplex approach based on universal hybridization was applied to the genotyping of the targeted SNPs in patient samples to demonstrate the design workflow and the method capabilities as a pharmacogenetic tool. The genotyping of three clinically relevant SNPs will support administering related drugs, such as cyclosporine, tacrolimus or methotrexate.

The robustness of population assignation was checked evaluating the assay sensitivity and the variability in the registered signals. The sensitivity was calculated from mixtures of DNA from mutant homozygotes and wild-type homozygotes (4 ng of total genomic extract). The percentage of mutant DNA capable to be detected was 2 %.

Intra-assay reproducibility, expressed as the relative standard deviation of three replicates performed in the same assay, was between 5% (rs180113, wild-type allele) and 20% (rs1045642, mutant allele). Inter-assay reproducibility, expressed as the relative standard deviation of three replicates performed in different assays, was 15-25%. In all cases, the discrimination indexes, calculated from signals of wild-type and mutant probes, were enough consistent to score the same genotype. Hence the obtained analytical performances demonstrated that the selected set of oligonucleotides was suitable for a robust genotyping of the targeted SNPs.

The patient genotype was assigned according to the discrimination indexes calculated for each studied SNP, and three populations were perfectly distinguished. Figure 4 shows the values calculated for the different patient groups (wild-type homozygotes, mutant homozygotes and heterozygotes). The homozygous genotypes led to high positive (wild-type) and high negative (mutant) discrimination index. Intermediate indexes were calculated for the heterozygous genotypes. The results indicated that primers and probes could reliably distinguish between the wild- and the mutant-type PCR product and, consequently, different alleles of SNPs could be easily discriminated. The individual detected genotypes are summarized in Table SI.9. Assigned genotypes were validated by analyzing samples with reference techniques (MassArray and Sanger sequencing).

CONCLUSIONS

One challenge in pharmacogenomics and pharmacogenetics is to implement genotyping technologies that integrate conventional properties (i.e. accuracy, robustness, with functional properties), such as low-cost or simplicity. One key point is adequate oligonucleotide selection. Easy-to-use computational tools allow the adaptation of current or innovative technologies to genotype clinically relevant SNPs. So the proposed workflow, comprising simple calculations (few minutes analysis), allows the design of allele-specific primers to be employed by the end-user. The main advantages of the developed algorithm compared to previous software are multiplicity and flexibility by incorporating certain restrictions based on stability calculations. Moreover, this tool can support different reported AS-PCR methods including detection formats from agarose gel electrophoresis to chip hybridization. Particularly, the study demonstrates its effectiveness for setting up a versatile method based on the universal array format. This strategy is powerful in terms of SNP genotyping capabilities, but is a complex approach in oligonucleotide selection terms.

The experimental confirmation of *in-silico* parameters and the genotypes of patients reported by a gold-standard platform reinforce the reliability of the developed design workflow and the genotyping method to support organ transplantation as a model of relevant application of pharmaceutical analysis. The testing assays were performed on polycarbonate chips that it is the bulk material used in many low-cost integrated devices. This study can support the development of innovative bioanalytical devices (static or microfluidics) based on plastic polymers. Furthermore, the method can be extended to other target genes, particularly for the third approach (multiplex assays based on universal hybridization). The hybridization conditions make substituting or incorporating new SNPs simpler as the experimental efforts of the personalized platform to include continuous pharmacogenomics advances in clinical practice are reduced.

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Table 1

STEP DESCRIPTION	MODULE IN MULTI-SNP PROGRAM
Step 1: Define input variables FASTA sequence (5'-3') including SNP in IUPAC code Primer restrictions Multiplex restrictions Optional: zip-code database	**INPUT** **Objective:** Input interface to user
Step 2: Generation of pre-candidate primers List of all possible sequences for both strands based on primer length and SNP distance restrictions **Step 3: Application of single primer restrictions** Elimination of sets according to the values of %GC, melting temperature, self-annealing and folding **Step 4: Application of product restrictions** Elimination of sets according to hairpin formation compared to duplex structure (double-strand product)	**SINGLE SELECTION** **Objective:** Selection of FP-allele 1, FP-allele 2, and RP fulfilling primer restrictions
Step 5: Application of multiplex primer restrictions Elimination of sets with cross-hybridization (primers to other templates) Elimination of sets of inadequate product size	**MULTIPLEX SELECTION** **Objective:** Selection of sets for the simultaneous amplification of several genes
Step 6: Search of zip-codes (optional) Obtain all combinations of FP-zip-codes Elimination of sets according to self-annealing and product/primer annealing values	**ZIP-CODES SELECTION** **Objective:** Selection of zip-codes probes fulfilling restrictions
Step 7: Estimation of *in-silico* PCR conditions Calculation of annealing temperature for PCR thermocycling	**OUTPUT** **Objective:** Output interface to user

FP: forward primer, RP: reverse primer

Table 2

	Parameter	Studied values	Selected values
Forward primer	Length interval	17 – 25 mer	17 – 25 mer
	%GC interval	35 – 65 %	35 – 65 %
	T_m interval	50 – 70 ºC	58 – 62 ºC
	Self-annealing	Up to 10 nt	≤ 8 nt
	T_f threshold	Up to 65 ºC	≤ 50 ºC
Reverse primer	Length interval	17 – 25 mer	19 – 22 mer
	Distance to SNP	Up to 150 bp	70 – 125 bp
	%GC interval	35 – 65 %	40 – 60 %
	T_m interval	50 – 70 ºC	58 – 62 ºC
	Self-annealing	Up to 10 nt	≤ 8 nt
	T_f threshold	Up to 65 ºC	≤ 50 ºC
Products	T_m differences	Up to 30 ºC	≤ 20 ºC
	T_f threshold	Up to 65 ºC	≤ 50 ºC
	Cross-annealing	Up to 60 ºC	≤ 45 ºC
	Size differences (WT and M products)	Up to 3 bp	≤ 1 bp
	Size differences (target products)	Up to 20 bp	≥ 10 bp
	Zip-code length	20 – 30 mer	20 mer

T_m: melting temperature, T_f: folding temperature calculated for $[Na^+]$ = 50 mM

Table 3

MODULE	Primer 3 Plus	WASP	Multi-SNP
Objective	General	ASA	ASA
Support	Web pages	Web pages	Excel
Allelic primers	No	Yes	Yes
Primer constraints	Flexible	Flexible	Flexible
Output: set number	Low	Low	High
Multiplex capability	No	No	Yes
Array module	No	No	Yes

Figure 1

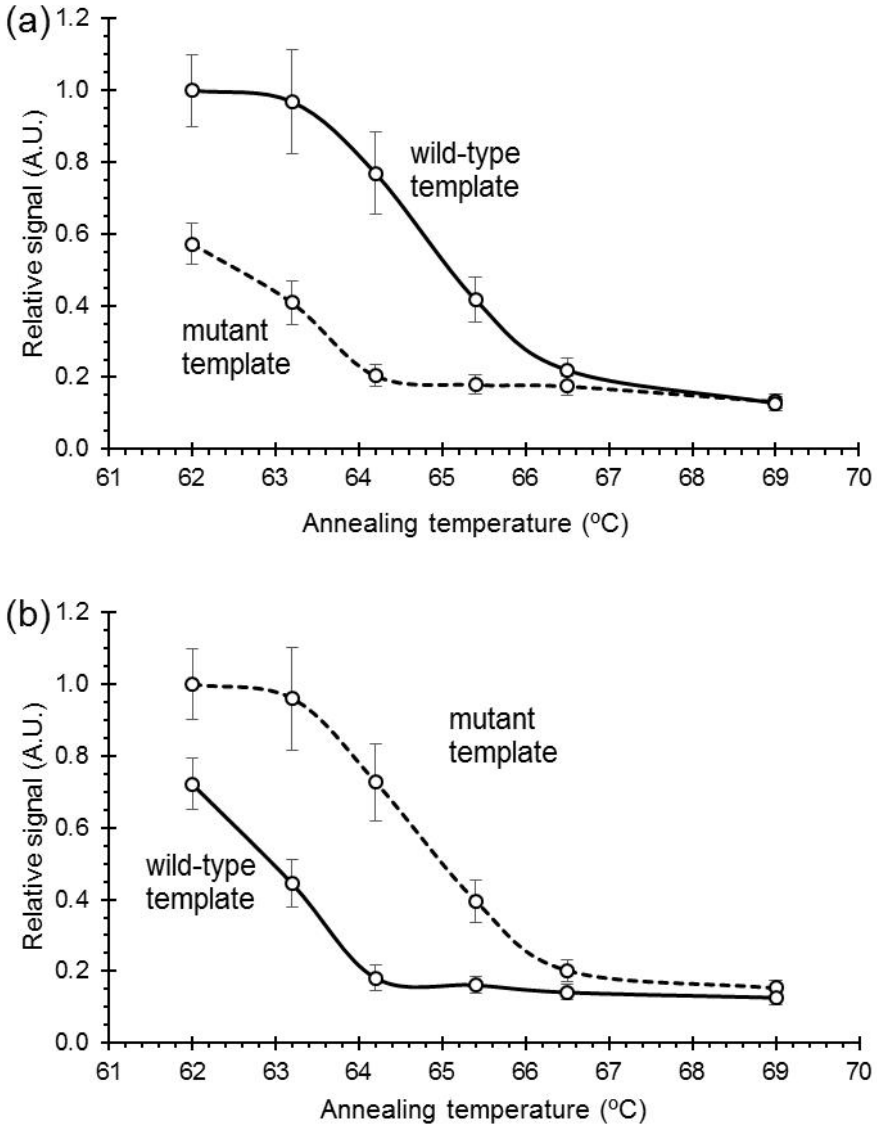

(a)
Relative signal (A.U.)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
wild-type
template
mutant
template
61
62
63
64
65
66
67
68
69
70
Annealing temperature (°C)
(b)
Relative signal (A.U.)
mutant
template
wild-type
template
Annealing temperature (°C)

Figure 2

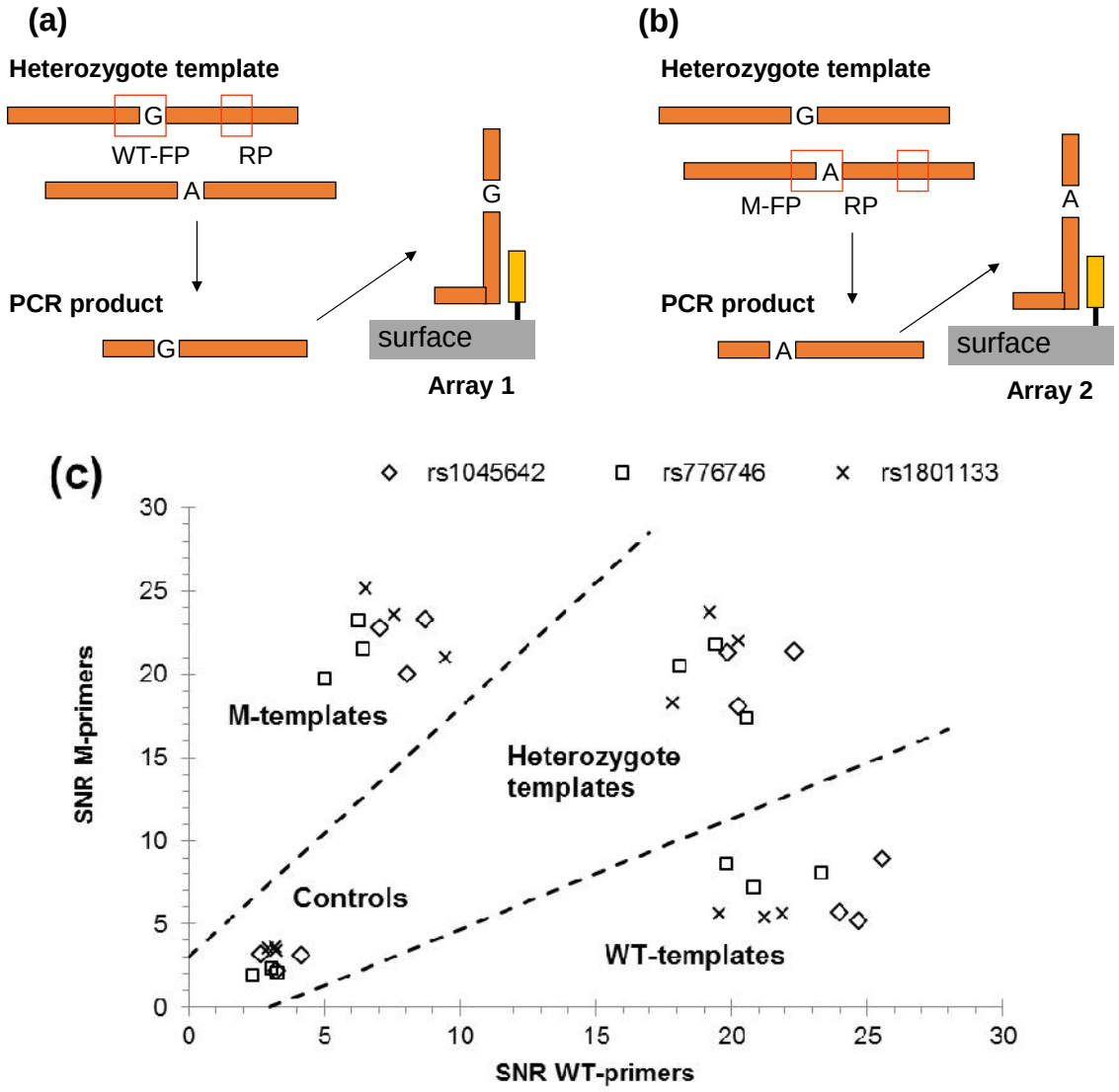

(a)
Heterozygote template
G
WT-FP
RP
A
PCR product
G
G
surface
Array 1
(b)
Heterozygote template
G
A
M-FP
RP
PCR product
A
A
surface
Array 2
(c)
rs1045642
rs776746
rs1801133
SNR M-primers
SNR WT-primers
M-templates
Heterozygote templates
Controls
WT-templates
0
5
10
15
20
25
30

Figure 3

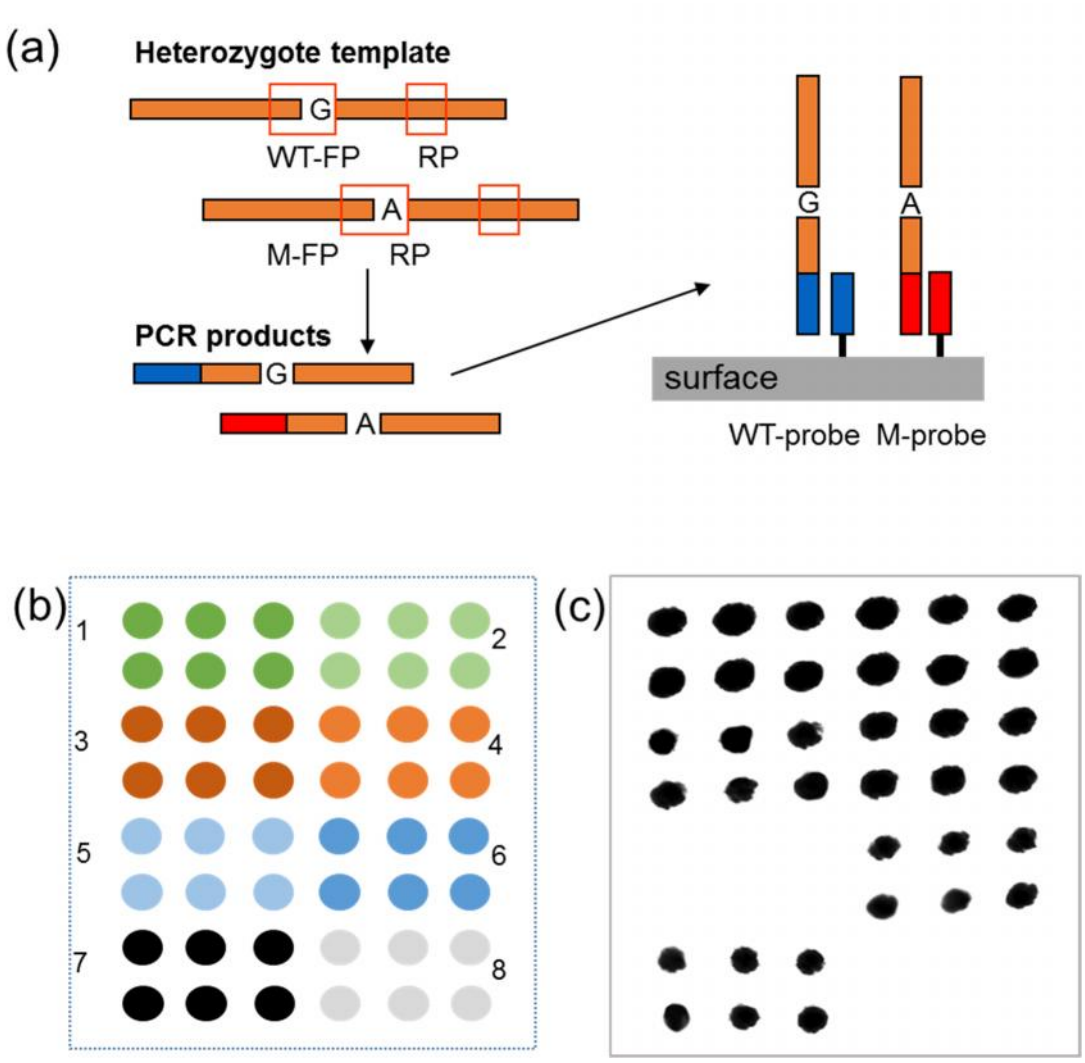
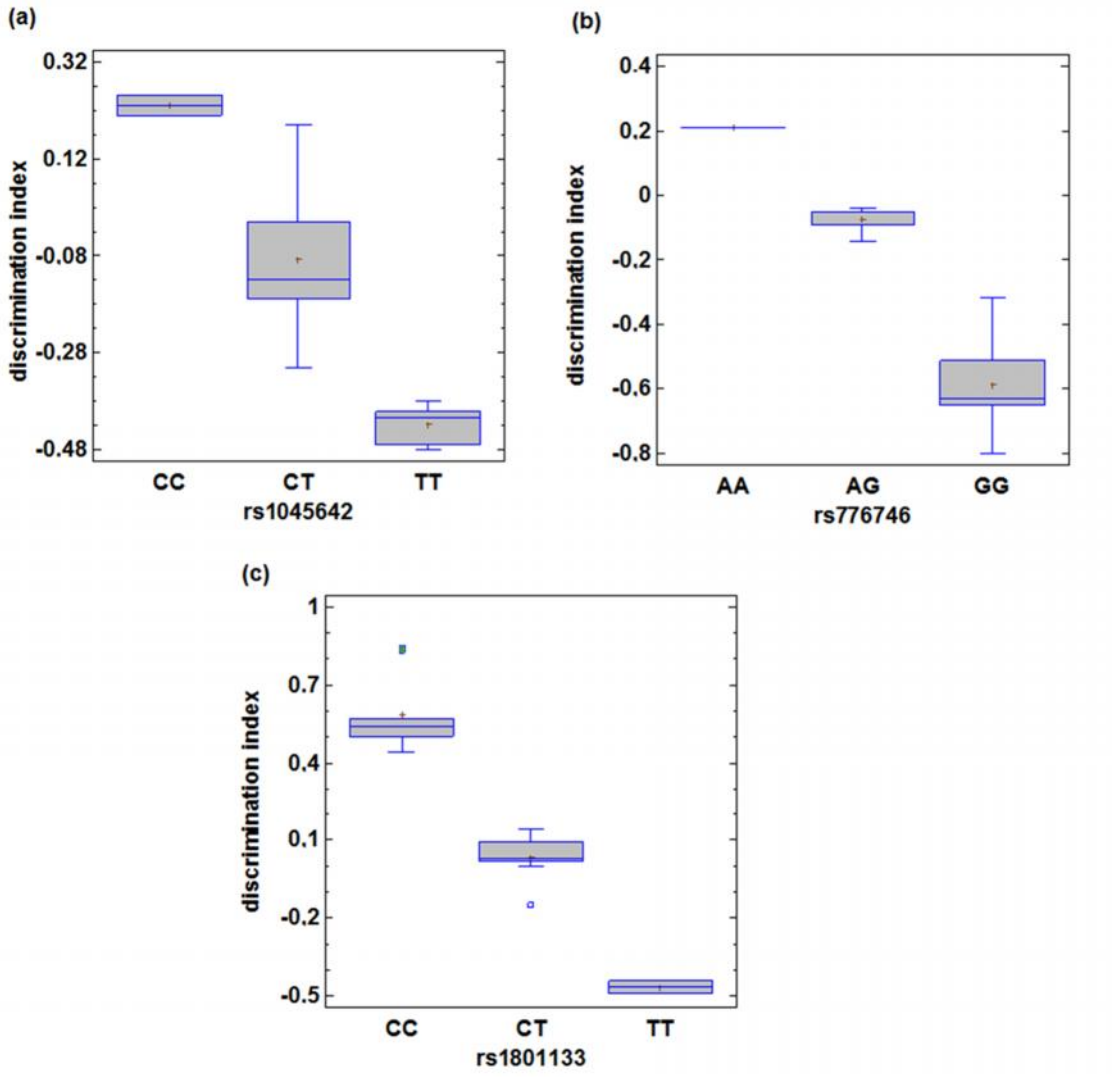

(a)
Heterozygote template
G
WT-FP
RP
A
M-FP
RP
PCR products
G
A
G
A
surface
WT-probe
M-probe
(b)
1
2
3
4
5
6
7
8
(c)

Figure 4


(a)
discrimination index
0.32
0.12
-0.08
-0.28
-0.48
CC
CT
TT
rs1045642
(b)
discrimination index
0.4
0.2
0
-0.2
-0.4
-0.6
-0.8
AA
AG
GG
rs776746
(c)
discrimination index
1
0.7
0.4
0.1
-0.2
-0.5
CC
CT
TT
rs1801133