Visual genotyping of SNPs of drug-metabolizing enzymes by tetra-primer PCR coupled with a dry-reagent DNA biosensor

Background: SNP-typing strategies involve an exponential amplification step, an allele discrimination reaction and detection of the products. Usually, allele discrimination is performed after amplification. Tetra-primer PCR allows allele discrimination during the amplification step, thereby avoiding additional genotyping reactions. However, to date, electrophoresis is the only method used for detection of tetra-primer PCR products. We report a dipstick test that enables visual detection of tetra-primer PCR products within minutes without instruments. The method is applied to the genotyping of *CYP2C19*2* (c.681G>A) and *CYP2D6*4* (g.3465G>A). **Materials & methods:** A pair of external primers amplifies a segment encompassing the SNPs. Biotinylated inner primers have a 3′-mismatch and pair off with the external primers to guide a bidirectional amplification that generates allele-specific fragments. The products are hybridized briefly with poly(dA)-tailed probes and applied to the DNA biosensor, which is then immersed in the appropriate buffer. As the buffer migrates along the biosensor, the hybrids are captured from streptavidin at the test zone and interact with oligo(dT)-functionalized gold nanoparticles leading to the formation of a red line. Another red line is formed at the control zone to indicate proper function of the sensor. **Results:** We genotyped 55 samples for *CYP2C19*2* and 49 samples for *CYP2D6*4*. The accuracy of this method was confirmed by sequencing and electrophoresis. **Conclusions:** The unique advantages of the proposed method are its simplicity and low cost. Contrary to electrophoresis, hybridization provides sequence confirmation of amplified fragments. The dry-reagent dipstick format minimizes the requirements for highly qualified personnel.

KEYWORDS: *CYP2C19* ▪ *CYP2D6* ▪ DNA biosensor ▪ nanoparticles ▪ SNP genotyping ▪ tetra-primer PCR

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SNPs are emerging as a new generation of markers for genetic disorders, susceptibility to various diseases and response to medication [1,2]. Over the past years, a large number of different SNP-typing technologies have been developed [3–5]. SNP-typing strategies involve exponential amplification of the target sequence, usually by a PCR allele discrimination reaction and detection of the products. Allele discrimination is generally performed after PCR amplification by a number of methods including sequencing, allele-specific oligonucleotide hybridization, primer extension, oligonucleotide ligation, invasive cleavage and melting-curve analysis [3–6]. Detection of genotyping products is accomplished by heterogeneous hybridization assays, homogeneous fluorometric assays, mass-spectrometry and microarrays [7–13].

The present work focuses on methods that allow allele discrimination during the amplification step, thereby avoiding additional genotyping reactions after completion of PCR. Allele discrimination during PCR relies on the fact that, for full activity, DNA polymerase requires perfect complementarity between the 3′-end of the primer and the DNA template. Methods such as the amplification refractory mutation system (ARMS) [14], allele specific PCR (ASPCR) [15], allele-specific amplification (ASA) [16] and PCR amplification of specific alleles (PASA) [17], are all based on two allele-specific oligonucleotide primers and a common primer. The primer with a wild-type base at the 3′-end amplifies mainly the wild-type allele whereas the primer with a 3′-mutant base amplifies the mutant allele. Deliberate mismatches at bases near the 3′-end may be introduced for better discrimination of the alleles [14]. However, each amplification reaction provides information on the presence of one allele only. Two separate PCRs are required for the genotyping of a biallelic SNP.

A method named PCR amplification of multiple specific alleles (PAMSA) [18] enables genotyping in a single PCR. PAMSA utilizes three primers and generates two allele-specific fragments that differ in size. However, differences in the amplification efficiency of the two fragments cause problems with the application of PAMSA. Tetra-primer PCR was introduced in order to overcome these problems [19]. The

technique relies on the amplification of allele-specific products simultaneously and in opposite directions in a single PCR using four primers, for example, a pair of outer (external) primers and two inner primers that are allele-specific (wild-type and mutant). The inner primers anneal to opposite strands of the interrogated DNA. The outer primers have melting temperatures (T_m) that are at least 10°C higher than those of the inner primers. A first set of cycles is performed at a high annealing temperature, producing only a long PCR product defined by the outer primers and spanning the polymorphic site. The annealing temperature then drops and a second set of cycles is performed in which the long PCR product acts as a template for the inner primers that amplify short DNA fragments specific for the allele(s) present in the sample. Detection is based on the size of the short products. Modifications of the original tetra-primer PCR have been reported. The bidirectional PASA (Bi-PASA) [20] differs in the mismatch position (in the middle of the primer for the original tetra-primer PCR versus mismatch at or near the 3′-end for Bi-PASA), the annealing step (two different temperatures for tetra-primer versus constant temperature for Bi-PASA), the inner-to-outer primer molar ratio as well as the presence of a tail at the 5′-end of the inner primers in Bi-PASA. The tetra-primer ARMS-PCR [21] employs inner primers with an allele-specific mismatch at the 3′-end and an additional deliberate mismatch at position -2 from the 3′-end, uses a constant annealing temperature (or touchdown) and an inner-to-outer primer molar ratio of 1:10.

To date, electrophoresis is the only method used for detection of tetra-primer PCR products. Agarose-gel electrophoresis, polyacrylamide-gel electrophoresis and capillary electrophoresis have been applied depending on the size differences of the allele-specific PCR products and the availability of specialized equipment. In this work, we propose a dipstick test that enables visual detection of the tetra-primer PCR products within minutes without instruments. Detection of the allele-specific short product is accomplished by hybridization, thus providing sequence confirmation as opposed to electrophoresis that gives only the size of the amplified fragments. As a model, we applied the proposed method to the genotyping of two SNPs in cytochrome P450 genes *CYP2C19* (Mendelian inheritance in Man [MIM] 124020; GenBank M61854.1 – *CYP2C19*2* [dbSNP: rs4244285, c.681G>A] and *CYP2D6* [MIM] 124030; GenBank M33388.1 – *CYP2D6*4* [dbSNP: rs3892097, g.3465G>A]). The mutation nomenclature is indicated as recommended by the Human Genome Variation Society (HGVS), with numbering according to indicated genomic reference sequence (g.) or cDNA numbering (c.) from reference sequence listed (based on +1 as A of ATG initiation codon).

Materials & methods

Materials

DyNAzyme II hot start DNA polymerase and DyNAzyme II DNA polymerase were purchased from Finnzymes (Espoo, Finland). AmpliTaq® gold DNA polymerase was from Applied Biosystems (CA, USA). Terminal deoxynucleotidyl transferase (TdT) was obtained from New England Biolabs (MA, USA). The QIAamp DNA blood mini kit and QIAquick gel extraction kit were purchased from Qiagen (Hilden, Germany). Agarose, deoxynucleoside triphosphates (dNTPs) and DNA molecular weight markers (φ*X*174 DNA, *Hae*III digest) were from HT Biotechnology (Cambridge, UK). The dry-reagent dipstick biosensor was manufactured as described previously [22] and obtained from Medicon Hellas (Gerakas, Greece). All common reagents were from Sigma (MO, USA). The primers and probes were synthesized by Invitrogen (CA, USA). For convenience we refer to the wild-type oligonucleotide sequences as 'normal' (N) and the variant nucleotide sequences as 'mutant' (M). The following oligonucleotides [23] were used as primers for tetra-primer PCR of *CYP2C19*2* (the bold nucleotide corresponds to the polymorphic site): 5′-CAGAGCTTGGCATATTGTATC-3′ (external upstream primer), 5′-GTAAACA-CAAAACTAGTCAATG-3′ (external downstream primer), 5′-biotin-ATCATTGATT-ATTTCCCA-3′ (inner upstream primer), 5′-biotin-AATTTGTTATGGGTTCCC-3′ (inner downstream primer). For *CYP2D6*4*, the primer sequences were: 5′-TCCCAGCT-GGAATCCGGTGTCG-3′ (external upstream primer), 5′-GGAGCTCGCCCTGCAG-AGACTCCT-3′ (external downstream primer), 5′-biotin-TCTCCCACCCCCAA-3′ (inner upstream primer), 5′-biotin-CGAAAGGGG-CGTCC-3′ (inner downstream primer) [24]. The oligonucleotide probes used for detection of the normal alleles (N probe) and mutant alleles (M probe) in *CYP2C19*2* were: 5′-AATTATTGTTTTCTCTTAGATA-TGC-3′ and 5′-GGGTTGTTGATGTCCATC-GATTCTT-3′, respectively. Oligos 5′- AAG-AAGTCGCTGGAGCAGTGGGTGA-3′

and 5′-CAGAAAGCCCGACTCCTCCTTC AGT-3′ were used as the N probe and M probe, respectively, for *CYP2D6*4* genotyping.

The developing solution contained 4% glycerol and 1% sodium dodecyl sulfate polyacrylamide (SDS) in phosphate-buffered saline (PBS;0.14 M NaCl, 2.7 mM KCl, 10 mM sodium phosphate and 1.7 mM potassium phosphate, pH 7.4).

Extraction of genomic DNA from whole blood

Whole-blood specimens were obtained from the University Hospital of Crete, Greece, after informed consent. Genomic DNA was isolated with the QIAamp DNA blood mini kit and stored at -20°C. The genomic DNA from blood samples was previously isolated for another study.

Tetra-primer PCR

Polymerase chain reactions were performed in the PTC-150 MiniCycler (MJ Research, MA, USA). Negative controls (containing water instead of DNA target) were included in each series of PCRs to confirm the absence of contamination. For *CYP2C19*2*, the PCR mixture contained 1× standard Gold reaction buffer, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 0.16 µM and 0.2 µM of upstream and downstream external primers, respectively, 0.375 µM each of the inner primers, 2 µl of genomic DNA and 2 U AmpliTaq Gold DNA polymerase in a total volume of 50 µl. The cycling conditions were as follows: 94°C for 10 min followed by 10 cycles (first phase of amplification) of 94°C for 30 s, 60°C for 30 s, 72°C for 60 s and 30 cycles (second phase of PCR) at 94°C for 30 s, 50°C for 30 s and 72°C for 60 s. After completion of the cycles, the mixture was incubated at 72°C for 7 min and cooled down to 4°C.

For *CYP2D6*4*, the PCR mixture (50 µl) consisted of 15 mM Tris-HCl (pH 8.2), 30 mM KCl, 5 mM $(NH_4)_2SO_4$, 0.02% bovine serum albumin, 2.5 mM $MgCl_2$, 200 µM of each dNTP, 0.2 µM of external primers, 0.3 µM and 0.2 µM of inner upstream and downstream primers, respectively, 2 µl genomic DNA and 2 U of DyNAzyme II Hot Start DNA polymerase. The cycling conditions were: 94°C for 10 min, followed by 15 cycles (first phase) at 94°C for 30 s, 63°C for 30 s, 72°C for 60 s and 27 cycles (second phase) at 94°C for 30 s, 53°C for 30 s and 72°C for 60 s. After completion of the cycles, the mixture was incubated at 72°C for 7 min and cooled to 4°C.

Tailing of probes with dATP

Oligonucleotide probes specific to tetra-primer amplification products were tailed enzymically with dATP. The tailing reaction mixture (20 µl) consisted of 20 mM tris-acetate, 50 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (DTT), pH 7.2, 0.25 mM $CoCl_2$, 2 mM dATP, 400 pmol oligonucleotide and 10 U terminal deoxynucleotidyl transferase. The mixture was incubated at 37°C for 1 h and the reaction was stopped by adding 2 µl of 0.5 M EDTA at pH 8.0.

Visual detection of tetra-primer PCR products by dry-reagent disposable DNA biosensor

The DNA biosensor consisted of an immersion pad, a conjugation pad, a laminated membrane and an absorbent pad assembled on a plastic adhesive backing. The four parts were positioned in such a way that their ends overlapped in order to ensure continuous flow of the developing solution from the immersion pad up to the absorbent pad. Oligo(dT)-conjugated gold nanoparticles (40 nm diameter) were prepared as described previously [22] and placed on the conjugate pad. The laminated membrane contained a zone of immobilized streptavidin (test zone) and a zone of immobilized oligo(dA) (control zone).

For the detection of tetra-primer PCR products, a 3-µl aliquot of PCR solution was mixed with 1 µl of 0.9 M NaCl, 1 pmol of dATP-tailed specific probe and ddH_2O in a total volume of 10 µl. The mixture was heated at 95°C for 2 min and then hybridization was allowed to proceed for 5 min at 25°C. Both the denaturation and hybridization steps were performed in the thermal cycler. The above mixture was applied to the conjugation pad next to the gold nanoparticles. The immersion pad of the biosensor was then dipped into 250 µl of the developing solution. The visual detection of the tetra-primer products was completed within 10 minutes.

Results & discussion

Assay principle

The principle of tetra-primer PCR dipstick assay is illustrated diagrammatically in Figure 1. Following genomic DNA isolation from whole blood, a single PCR is performed with two sets of primers. During the initial phase of PCR, the external primers amplify a segment that spans the SNPs of interest. The second phase of PCR takes place at a lower annealing temperature at which the inner primers (allele-specific primers) anneal to opposite strands. The inner primers pair off

with the external primers to guide a bidirectional amplification that uses the long PCR product as a template and mostly generates short allele-specific fragments, although amplification of the long product continues to some degree. The inner primers were designed with a mismatch at the 3′-end and a biotin moiety at the 5′-end, thus the short products are biotinylated. The amplified

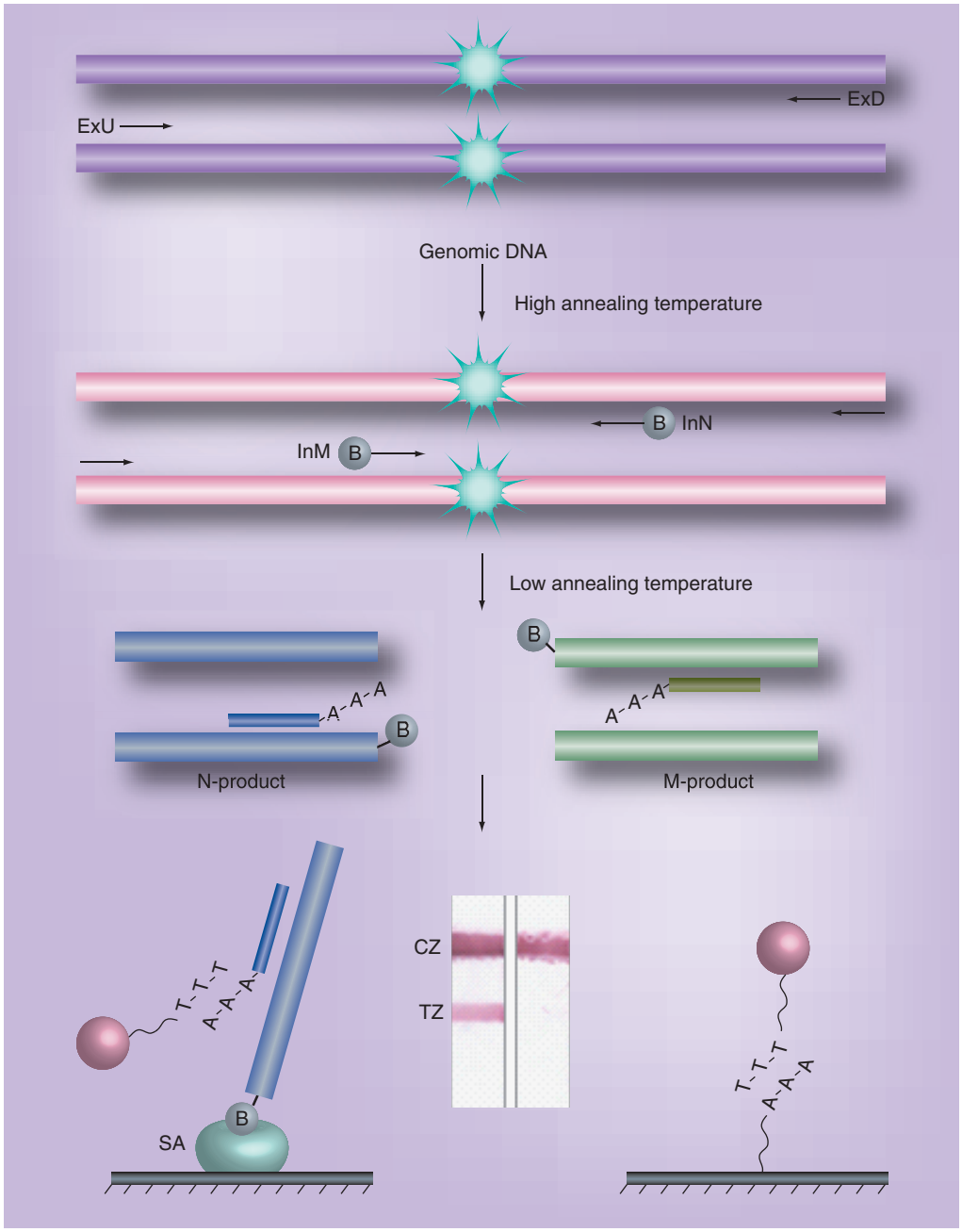

Figure 1. Visual genotyping of SNPs by tetra-primer PCR coupled with a dry-reagent dipstick-type biosensor. Genomic DNA is amplified by PCR using four primers, two external and two 5′ B inner primers. The external primers (ExU and ExD) generate a fragment that spans the SNP of interest. Following a decrease of the annealing temperature, inner primers specific for the InN and InM allele pair off with ExU and ExD, respectively, to produce two short products (N-product and/or M-product) depending on the allele(s) present in the sample. The products hybridize, briefly, with specific probes carrying a poly(dA) tail at the 3′ end. The solution is applied to the biosensor which is immersed in the appropriate buffer. As the buffer migrates upwards by capillary action, it rehydrates oligo(dT)-conjugated gold nanoparticles. The hybrids are captured from immobilized SA at the TZ of the biosensor producing a red zone. The excess of nanoparticles are captured from immobilized poly(dA) strands at the CZ of the sensor generating a second red line.
B: Biotinylated; CZ: Control zone; InM: Mutant; InN: Normal; SA: Steptavidin; TZ: Test zone.

DNA hybridizes briefly in solution (in the thermal cycler) with probes N and M comprising a segment complementary to the target and a poly(dA) tail. Probes N and M are complementary to normal (N-product) and mutant product (M-product), respectively. The mixture is applied to the conjugate pad of the biosensor, which is then immersed into the developing solution. The solution migrates along the strip by capillary action and rehydrates the oligo(dT)-conjugated gold nanoparticles. The hybrids are captured from immobilized streptavidin at the test zone of the biosensor and hybridize with the oligo(dT) strands through the poly(dA) tail of the probe. As a result, there is an accumulation of gold nanoparticles and generation of a characteristic red line at the test zone of the biosensor, since gold nanoparticles have an absorbance peak at 520 nm due to plasmon resonance. The excess nanoparticles are captured from immobilized oligo(dA) strands at the control zone of the strip generating a second red line that confirms the proper function of the biosensor. The biosensor detects only the short, allele-specific PCR products and not the long product. The latter hybridizes to both N and M probes but is not captured at the test zone because it lacks a biotinylated end (the outer primers are not biotinylated). The genotype is assigned by the results of the two strips. The presence of a red zone for N probe and absence of a red zone for M probe indicate a normal genotype (N/N). The mutant genotype (M/M) is characterized by a red zone for M probe only. A heterozygote (N/M) gives red zones for both probes.

Genotyping profiles obtained by agarose gel electrophoresis and by the dry-reagent dipstick-type biosensor are shown in Figure 2. For each SNP (*CYP2D6*4* and *CYP2C19*2*), a normal, a heterozygous and a mutant sample is presented. One strip (left) gives the signal obtained by hybridization of PCR product with the N probe whereas the other strip (right) indicates hybridization with the M probe. The nonspecific amplification products may lead to misgenotyping when electrophoresis is used as a detection method. The dipstick assay, however, detects only the specific fragments. The genotypes of the clinical samples used throughout this study were confirmed by sequencing.

Optimization studies

The proposed assay was applied to the genotyping of *CYP2D6*4* and *CYP2C19*2*. The *CYP2D6*4* polymorphism was used as a model for optimization studies. Amplified DNA from a normal individual (N/N) and a homozygote for the mutation (M/M) were used as targets.

In order to study the effect of the amount of allele-specific short product on the density of the test-zone of the biosensor, we used tetra-primer

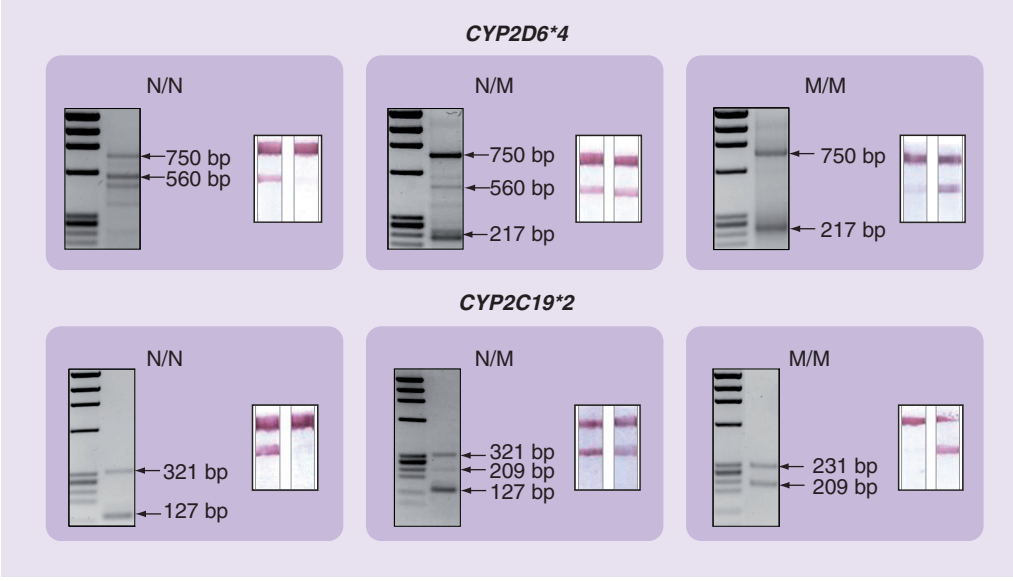

Figure 2. Genotyping profiles obtained by agarose gel electrophoresis compared with the dry-reagent dipstick-type biosensor. For each SNP (*CYP2D6*4* and *CYP2C19*2*) a normal (N/N), a heterozygous (N/M) and a mutant (M/M) sample were subjected to tetra-primer PCR/biosensor assay. The sizes of long and allele-specific PCR products of interest are indicated next to the electropherogram. The respective biosensor assay includes two strips: the left strip indicates hybridization of PCR product with a N probe specific for the wild-type (normal) product whereas the right strip indicates hybridization of the PCR product with M probe that is specific for mutant product. M: Mutant; N: Normal.

PCR mixtures from a normal (N/N) and a mutant (M/M) sample. The concentration of allele-specific product in each mixture was determined by agarose gel electrophoresis, ethidium bromide staining and densitometry with φ*X*174 DNA and *Hae*III digest DNA fragments as calibrators. The concentrations were found to be 13.2 and 36.2 nM for the N and M product, respectively. Various aliquots of the tetra-primer PCR mixture were then hybridized with the N and M probes and 10 µl applied to the strip. The results (data not shown) indicate that 10 and 27 fmol of the N- and M-specific fragments, respectively, are readily detectable. These quantities correspond to 0.75 µl of PCR mixture. The zone density increases with the amount of target up to 40 and 109 fmol, respectively, corresponding to 3 µl of PCR mixture. Larger amounts of target DNA result in no improvement of the signal. Based on the above findings we chose to use 3 µl of PCR mixture for all clinical samples without prior quantification of the amplified DNA. The effect of hybridization temperature was studied in the range of 25–55°C. The results are presented in Figure 3A. The density of the test zone remains constant up to 42°C and decreases at 55°C. Therefore, all subsequent hybridizations were performed at 25°C. The influence of the amount of oligonucleotide probe in the hybridization mixture was studied in the range of 0.25–4 pmol (Figure 3B). The density of the test zone increases with the amount of the probe up to 1–2 pmol and then remains constant.

Reproducibility

The reproducibility of the biosensor was assessed by analyzing tetra-primer PCR products from normal (N/N), heterozygote (N/M) and mutant (M/M) alleles. These tetra-primer PCR products were the result of four parallel reactions of the respective genomic DNA for each genotype (n = 4). For a normal sample, the coefficients of variation (CVs) of the signals obtained with the N probe and M probe were 6.8% and 10%, respectively (by densitometric analysis). For a heterozygote, the CVs were 11% (N probe) and 3.5% (M probe). For a mutant sample, the CVs were 15% (N probe) and 4.6% (M probe).

Genotyping of clinical specimens

The biosensor was applied to the genotyping of *CYP2C19*2* and *CYP2D6*4* using genomic DNA isolated from whole blood. Each sample is represented by two strips. The first one provides the signal obtained from the hybridization of the tetra-primer PCR product with the N probe and the second strip gives the signal from the hybridization of the tetra-primer PCR product with the M probe. Figure 4 presents the results of genotyping of 55 samples for *CYP2C19*2* and 49 samples for *CYP2D6*4*, that is, a total of 208 alleles.

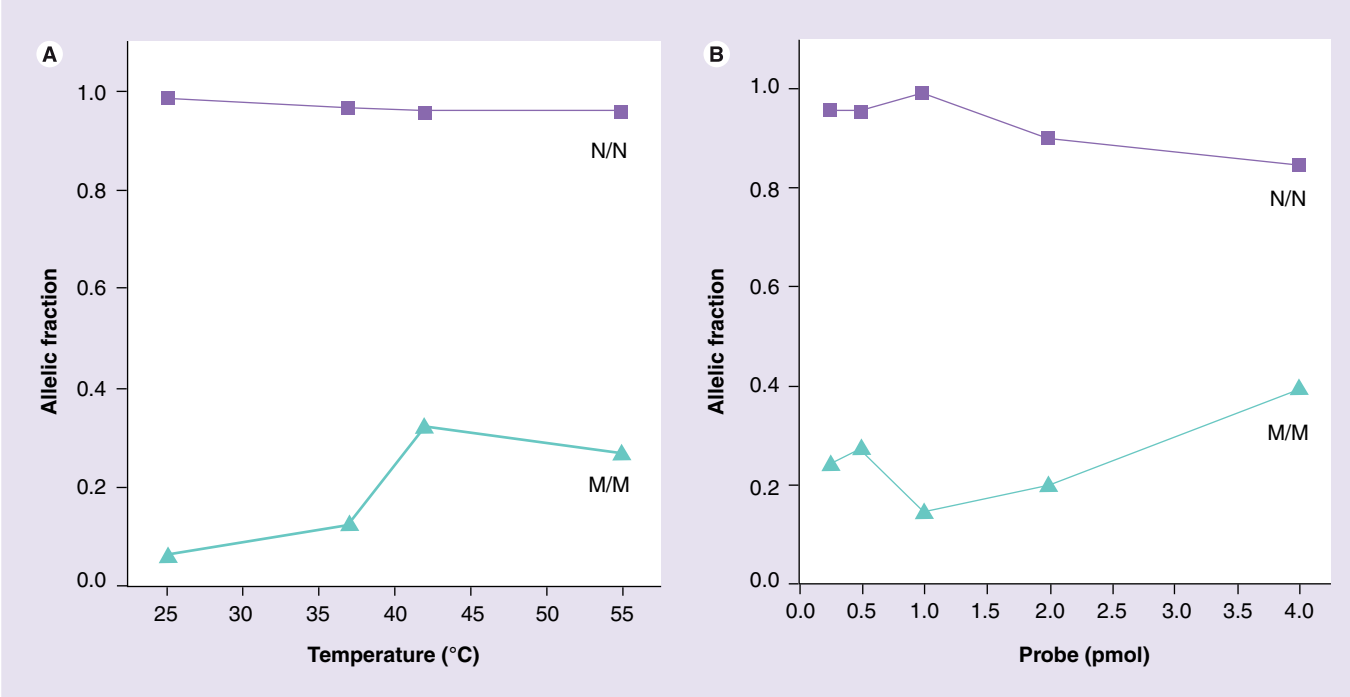

Figure 3. Dependence of signal density on the hybridization temperature (A) and the amount of specific probe (B). N/N and M/M are the normal and mutant homozygote genotypes, respectively.

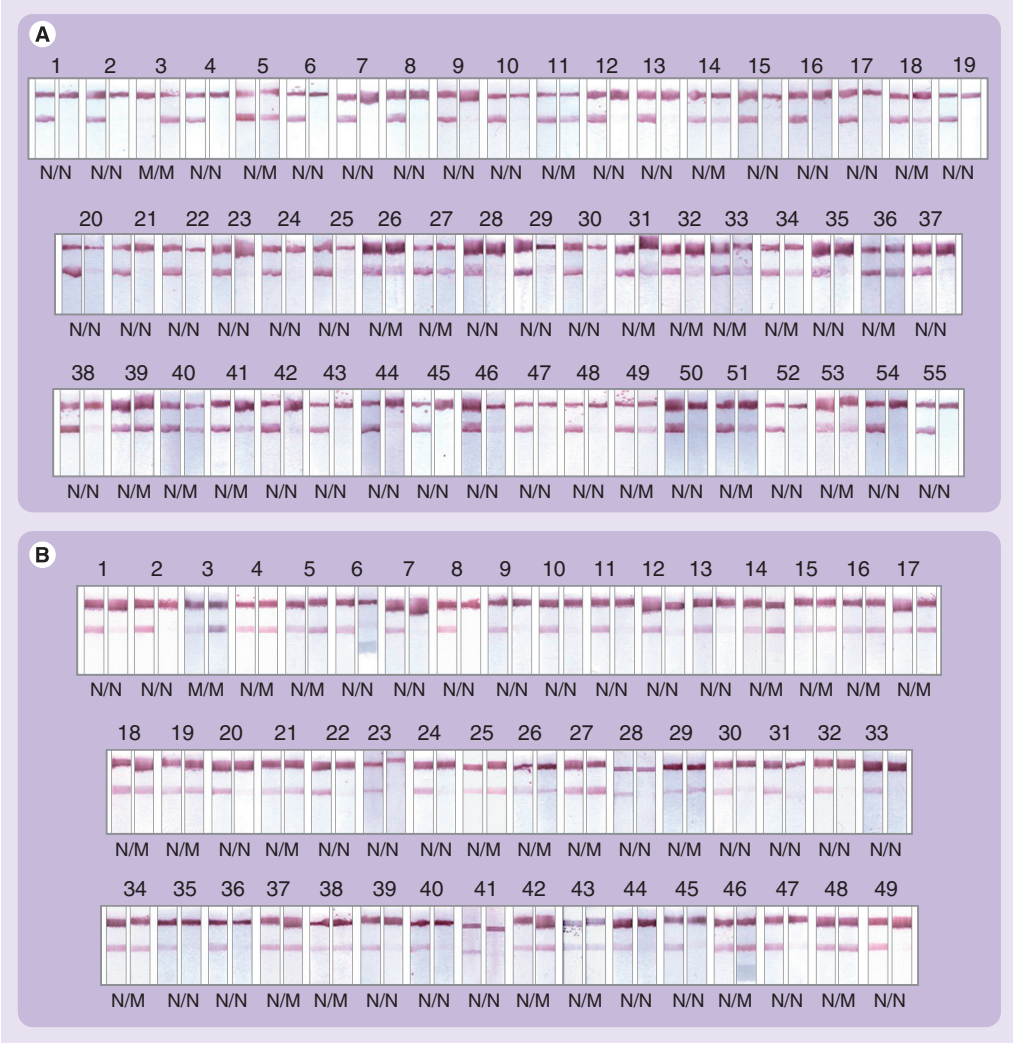

Figure 4. Visual genotyping of clinical samples for (A) *CYP2C19*2* and (B) *CYP2D6*4* by tetra-primer PCR/biosensor assay. A total of 55 samples (110 alleles) were genotyped for *CYP2D6*4* and 49 samples (98 alleles) were analyzed for *CYP2C19*2*. Each pair of strips represents the signal obtained from hybridization with the normal probe (left strip) and mutant probe (right strip). Each genotype is indicated as N/N (normal), N/M (heterozygous) and M/M (mutant).

All sample genotypes were confirmed by sequencing (the sequencing analysis was performed for previous studies by an independent laboratory) and 2% agarose gel electrophoresis with staining by ethidium bromide. The results of the proposed method were in total agreement with electrophoresis and sequencing, as well as with the results of other genotyping methods that were developed by our research group and used the same blood samples [10,25].

The strips were scanned using a common desktop scanner (ScanJet 4300C, from Hewlett Packard, CA, USA), and the images were analyzed by optical densitometry (the software for densitometry was developed by Dr C Efstathiou, Laboratory of Analytical Chemistry, Department of Chemistry, University of Athens, Greece). The allelic fraction for the wild-type allele (AF_{wt}) was then calculated from the equation: AF_{wt} = wt/(wt + mut), where wild-type (wt) and mutant (mut) correspond to the densitometric signals obtained from hybridizations with the N and M probes, respectively. The results for all samples and both polymorphisms are presented in Figure 5. Therefore, besides visual detection, the calculated allelic fraction provides the genotype for each sample. Sample-to-sample variation in the efficiency of tetra-primer PCR may occasionally lead to faint zones on the strip. In these cases, scanning densitometry is particularly useful in the assignment of a genotype thus avoiding the repetition of the amplification step with a higher amount of genomic DNA.

Data pertaining to the sensitivity and specificity of the dipstick assay were obtained from the genotyping of 104 clinical samples (55 samples

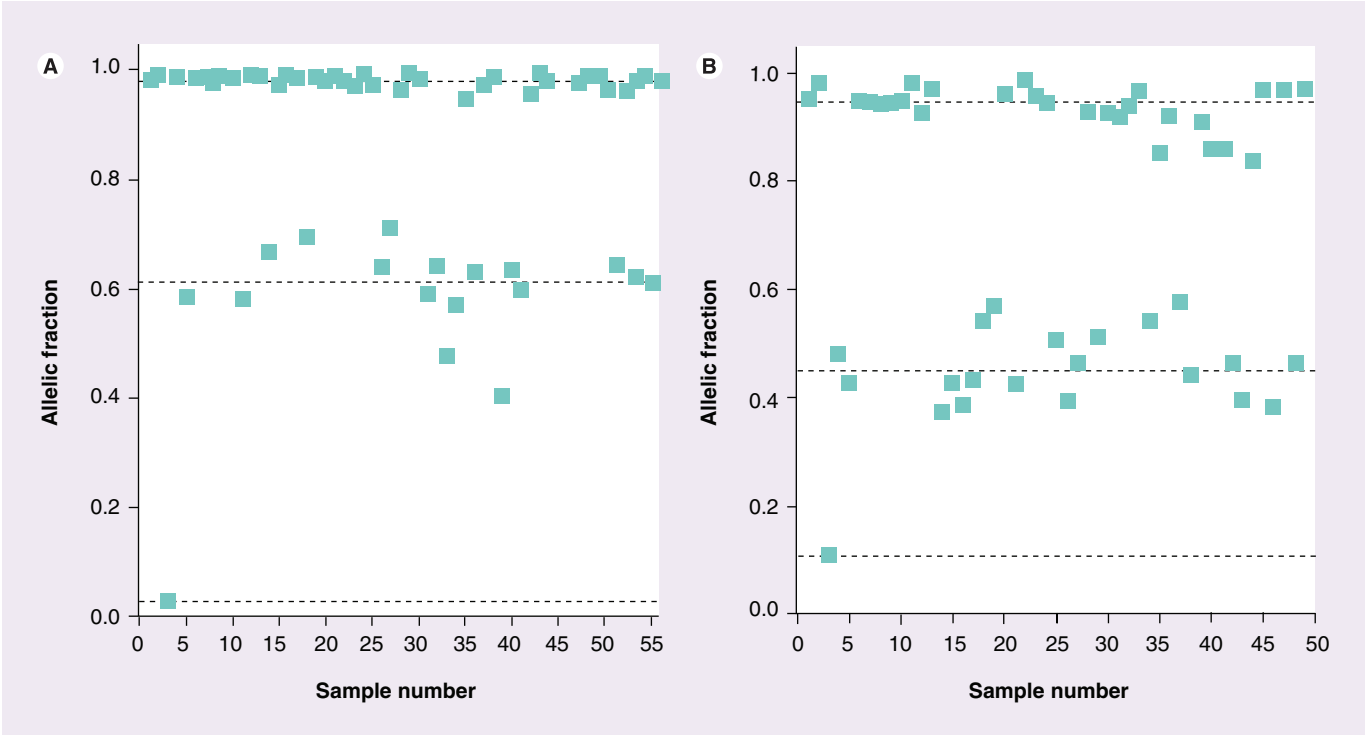

Figure 5. Quantification of the allelic fraction of clinical samples assayed in the tetra-primer PCR/biosensor assay. The allelic fraction is plotted against the sample number for genotyping of **(A)** 55 samples for *CYP2C19*2* and **(B)** 49 samples for *CYP2D6*4*.

for *CYP2C19*2* and 49 for *CYP2D6*4*), which were mentioned above (Figure 4). The products of the tetra-primer PCR for each sample were analyzed both by the proposed dipstick assay and agarose gel electrophoresis. The amplified fragments from normal and mutant alleles were then quantified by densitometry of the pictures taken from the ethidium bromide-stained gels. From these experiments it was estimated that as low as 3 fmol of amplified DNA was detectable by the biosensor. Moreover, quantification of the amplified products from heterozygous samples, which presented different N:M product ratios, showed that the dipstick assay had specificity for N:M product ratios in a wide range of N:M ratios (amplified products) extending from 1/0.03 to 1/144 (these data represent ratios of amplified products and not ratios of the two alleles in the sample prior to amplification).

In a recent publication we reported a dipstick-type SNP-genotyping assay based on the oligonucleotide reaction (OLR) [25]. However, the two methods are based on totally different principles. In OLR, allele discrimination relies on the ability of DNA ligase to join two oligonucleotides, which hybridize to adjacent positions, only if they are perfectly complementary to the target sequence. In tetra-primer PCR, allele discrimination is based on the fact that DNA polymerase extends the primer only if its 3′-end is fully complementary to the template. Moreover, the OLR method [25] requires two primers for PCR and three oligos for the ligation reaction (five oligonucleotides). The tetra-primer PCR requires four oligos. A careful design of the primers is required for tetra-primer PCR but this is done only once at the setting up of the method. The OLR method requires a PCR amplification step and a separate allele discrimination step whereas the proposed tetra-primer method combines these two steps in one. In regards to the accuracy of genotyping, the two methods perform equally well. The same clinical samples were genotyped (for both *CYP2C19*2* and *CYP2D6*4* SNPs) using both methods and the genotypes were identical (see Figure 4 of the present study and [25]).

In previously reported genotyping methods that are based on primer-extension or oligonucleotide ligation reactions [10,25] both the biotin and oligo(dA) moieties were in the same fragments (products of the genotyping reaction). On the contrary, in the proposed method the detection involves two biotinylated allele-specific PCR products and dA-tailed oligonucleotide probes that hybridize to unique sequences of the products. This configuration enhances the specificity because two events are required for genotyping, namely, tetra-primer PCR amplification and confirmation of the amplified sequences by hybridization.

Conclusion

The unique advantages of the proposed method are its simplicity and low cost. The cost of the assay in terms of reagents and the biosensor is approximately 2€ per call (the cost of enzyme, primers and probes is approximately 0.5€ per call and the strip costs approximately 1.5€). Tetra-primer PCR is performed in a standard thermocycler and enables discrimination of both alleles during a single amplification reaction. Up until now the only reported detection method for tetra-primer PCR is electrophoresis. The biosensor allows visual detection of tetra-primer PCR products within minutes without the need of any instruments. Detection of the allele-specific products is accomplished by hybridization, thus providing sequence confirmation as opposed to electrophoresis that gives only the size of amplified fragments. This is particularly important because quite often the identification of specific bands on the electropherogram is hindered by the presence of nonspecific amplification products of similar size. Finally, the dry-reagent dipstick format minimizes the requirements for highly qualified personnel. The test is complete in 2–2.5 h (including tetraprimer amplification and detection).

A PCR amplification step is required for the proposed method. However, the vast majority of methods that are currently used for routine genotyping do require amplification of the target DNA by PCR (or other exponential amplification techniques), followed by a separate genotyping reaction for allele discrimination. The advantage of the proposed method is that it combines two different steps, that is, the amplification and genotyping reactions, into a single step, thereby requiring less time for the preparation of the reactions and avoiding contaminations. The advantage of the proposed method compared with homogeneous genotyping methods (e.g., melting curve analysis and light cycler) is that the dipstick format alleviates the need for specialized and costly equipment and reagents.

The performance of the proposed method was demonstrated with the genotyping of drug-metabolizing enzymes but it can be applied to genotyping of other pharmacogenetic targets or for detection of mutations causing diseases, by designing appropriate probes and using the same biosensor. The method is not quantitative and thus is not suitable for determining the percentage of mutant alleles in a complex mixture (e.g., study of somatic mutations). The cytochrome P450 mutations are inherited and, therefore, would not be expected to have a variable ratio of the alleles [24].

In our opinion this dipstick-type of genotyping assays are extremely useful for the small clinical laboratory and constitute a major step towards point-of-care testing.

Future perspective

We envisage developing a whole-dry SNP genotyping assay that includes a dry-reagent tetra-primer PCR amplification followed by the dipstick assay of the products by the DNA biosensor.

Financial & competing interests disclosure

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Executive summary

- The unique advantages of the proposed SNP genotyping method are simplicity and its low cost.
- Tetra-primer PCR is performed in a standard thermocycler and enables discrimination of both alleles during a single amplification reaction.
- The biosensor allows visual detection of tetra-primer PCR products within minutes without the need of any instruments.
- Hybridization provides sequence confirmation as opposed to electrophoresis that gives only the size of amplified fragments.
- The dry-reagent dipstick format minimizes the requirements for highly qualified personnel.

Bibliography

1 Hirschhorn JN, Daly MJ: Genome-wide association studies for common diseases and complex traits. *Nat. Rev. Genet*. 6, 95–108 (2005).

2 Daly AK: Pharmacogenetics of the major polymorphic metabolizing enzymes. *Fundam. Clin. Pharmacol.* 17, 27–41 (2003).

3 Kwok PY: Methods for genotyping single nucleotide polymorphisms. *Annu. Rev. Genomics Hum. Genet.* 2, 235–258 (2001).

4 Shi MM: Enabling large-scale pharmacogenetic studies by high-throughput mutation detection and genotyping technologies. *Clin. Chem.* 47, 164–172 (2001).

5 Syvanen AC: Accessing genetic variation: genotyping single nucleotide polymorphisms. *Nat. Rev. Genet.* 2, 930–942 (2001).

6 Liew M, Pryor R, Palais R *et al.*: Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin. Chem.* 50, 1156–1164 (2004).

7 Glynou K, Kastanis P, Boukouvala S *et al.*: High-throughput microtiter well-based chemiluminometric genotyping of 15 *HBB* gene mutations in a dry-reagent format. *Clin. Chem.* 53, 384–391 (2007).

8 Tannous B, Verhaegen M, Christopoulos TK, Kourakli A: Combined flash- and glow-type chemiluminescent reactions for high-throughput genotyping of biallelic polymorphisms. *Anal. Biochem.* 320, 266–272 (2003).

9 Zerefos PG, Ioannou PC, Traeger-Synodinos J, Dimissianos G, Kanavakis E, Christopoulos TK: Photoprotein aequorin as a novel reporter for SNP genotyping by primer extension-application to the variants of mannose-binding lectin gene. *Hum. Mutat.* 27, 279–285 (2006).

10 Litos I, Emmanouilidou E, Glynou K *et al.*: Rapid genotyping of *CYP2D6*, *CYP2C19* and *TPMT* polymorphisms by primer extension reaction in a dipstick format. *Anal. Bioanal. Chem.* 389, 1849–1857 (2007).

11 Kwok PY: SNP genotyping with fluorescence polarization detection. *Hum. Mutat.* 19, 315–323 (2002).

12 Tost J, Gut IG: Genotyping single nucleotide polymorphisms by MALDI mass spectrometry in clinical applications. *Clin. Biochem.* 38, 335–350 (2005).

13 Ng JK, Liu WT: Miniaturized platforms for the detection of single-nucleotide polymorphisms. *Anal. Bioanal. Chem.* 386, 427–434 (2006).

14 Newton CR, Graham A, Heptinstall LE *et al.*: Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS). *Nucleic Acids Res.* 17, 2503–2516 (1989).

15 Wu DY, Ugozzoli L, Pal BK, Wallace RB: Allele-specific enzymatic amplification of β-globin genomic DNA for diagnosis of sickle cell anemia. *Proc. Natl Acad. Sci. USA* 86, 2757–2760 (1989).

16 Okayama H, Curiel DT, Brantly ML, Holmes MD, Crystal RG: Rapid, nonradioactive detection of mutations in the human genome by allele-specific amplification. *J. Lab. Clin. Med.* 114, 105–113 (1989).

17 Sommer SS, Cassady JD, Sobell JL, Bottema CD: A novel method for detecting point mutations or polymorphisms and its application to population screening for carriers of phenylketonuria. *Mayo Clin. Proc.* 64, 1361–1372 (1989).

18 Dutton C, Sommer SS: Simultaneous detection of multiple single-base alleles at a polymorphic site. *Biotechniques* 11, 700–702 (1991).

19 Ye S, Humphries S, Green F: Allele specific amplification by tetra-primer PCR. *Nucleic Acids Res.* 20, 1152 (1992).

20 Liu Q, Thorland EC, Heit JA, Sommer SS: Overlapping PCR for bidirectional PCR amplification of specific alleles: A rapid one-tube method for simultaneously differentiating homozygotes and heterozygotes. *Genome Res.* 7, 389–398 (1997).

21 Ye S, Dhillon S, Ke X, Collins AR, Day IN: An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res.* 29, E88 (2001).

22 Glynou K, Ioannou PC, Christopoulos TK, Syriopoulou V: Oligonucleotide-functionalized gold nanoparticles as probes in a dry-reagent strip biosensor for DNA analysis by hybridization. *Anal. Chem.* 75, 4155–4160 (2003).

23 Hersberger M, Marti-Jaun J, Rentsch K, Hanseler E: Two single-tube tetra-primer assays to detect the *CYP2C19*2* and **3* alleles of S-mephenytoin hydroxylase. *Clin. Chem.* 47, 772–774 (2001).

24 Hersberger M, Marti-Jaun J, Rentsch K, Hanseler E: Rapid detection of the *CYP2D6*3*, *CYP2D6*4*, and *CYP2D6*6* alleles by tetra-primer PCR and of the *CYP2D6*5* allele by multiplex long PCR. *Clin. Chem.* 46, 1072–1077 (2000).

25 Toubanaki DK, Christopoulos TK, Ioannou PC, Gravanis A: Dry-reagent disposable biosensor for visual genotyping of single nucleotide polymorphisms by oligonucleotide ligation reaction: Application to pharmacogenetic analysis. *Hum. Mutat.* 29, 1071–1078 (2008).