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METHODS

Dry-Reagent Disposable Biosensor for Visual Genotyping of Single Nucleotide Polymorphisms by Oligonucleotide Ligation Reaction: Application to Pharmacogenetic Analysis

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Most genotyping methods for known single-nucleotide polymorphisms (SNPs) are based on hybridization with allele-specific probes, oligonucleotide ligation reaction (OLR), primer extension or invasive cleavage. OLR offers superior specificity because it involves two recognition events; namely, the hybridization of an allele-specific probe and a common probe to adjacent positions on target DNA. OLR products can be detected by microtiter well-based colorimetric, time-resolved fluorimetric or chemiluminometric assays, electrophoresis, microarrays, microspheres, and homogeneous fluorimetric or colorimetric assays. We have developed a simple, robust, and low-cost disposable biosensor in dry-reagent format, which allows visual genotyping with no need for instrumentation. The OLR mixture contains a biotinylated common probe and an allele-specific probe with a $(dA)_{20}$ segment at the 3′-end. OLR products are denatured and applied to the biosensor next to gold nanoparticles that are decorated with oligo(dT) strands. The sensor is immersed in the appropriate buffer and all components migrate by capillary action. The OLR product is captured by immobilized streptavidin at the test zone (TZ) of the sensor and hybridizes with the oligo(dT) strands of the nanoparticles. A characteristic red line is generated due to the accumulation of nanoparticles. The excess nanoparticles are captured by immobilized oligo(dA) at the control zone of the strip, giving a second red line. We have applied successfully the proposed OLR-dipstick assay to the genotyping of four SNPs in the drug-metabolizing enzyme genes CYP2D6 (*3 and *4) and CYP2C19 (*2 and *3). The results were in agreement with direct sequencing. Hum Mutat 29(8), 1071–1078, 2008.

KEY WORDS: oligonucleotide ligation reaction; OLR; biosensor; genotyping; single nucleotide polymorphisms; SNPs; dipstick; dry-reagent strip; nanoparticles

INTRODUCTION

Single nucleotide polymorphisms (SNPs) are emerging as a new generation of markers for genetic disorders, disease susceptibility, cancer predisposition, and variation in the response to medication [Hirschhorn and Daly, 2005]. As a consequence, there is a constantly increasing interest in the improvement of the available methods for SNP analysis [Kwok, 2001; Shi, 2001; Syvanen, 2001]. Amplification, usually by PCR, is a central step of most genotyping methods. Screening of unknown SNPs is performed by single-stranded conformation polymorphism analysis, denaturing gel electrophoresis, denaturing HPLC, chemical cleavage [Ellis et al., 1998], ligation-mediated PCR [Zhang et al., 2002], and permanganate oxidation [Tabone et al., 2006]. Allele discrimination methods for known SNPs rely on the following general approaches: hybridization of allele-specific oligonucleotide (ASO) probes, oligonucleotide ligation reaction (OLR), primer extension reaction, and invasive cleavage. Enzyme-based genotyping assays have proven to be more robust and specific than ASO hybridization.

In OLR-based genotyping [Landegren et al., 1988], a probe that is common for the two alleles and an allele-specific probe hybridize at adjoining positions on the target DNA. One end of the allele-specific probe is complementary to the interrogated nucleotide. DNA ligase discriminates the single-nucleotide mismatches and will join the two ends only if they are fully complementary to the target at the junction. Two OLRs are required, one for each allele,

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TABLE 1. Oligonucleotides Used as PCR Primers and Probes in Oligonucleotide Ligation Reaction (OLR)

Oligonucleotide	Name	Sequence	Size	Label
PCR				
CYP2C19*2 Upstream primer	C19M*2UP	AATTACAACCAGAGCTTGGC	20mer	–
CYP2C19*2 Downstream primer	C19M*2DN	TATCACTTTCCATAAAAGCAAG	22mer	–
CYP2C19*3 Upstream primer	C19M*3UP	AAATTGTTTCCAATCATTTAGCT	23mer	–
CYP2C19*3 Downstream primer	C19M*3DN	ACTTCAGGGCTTGGTCAATA	20mer	–
CYP2D6*3 Upstream primer	CYP2D6*3UP	GACCAGCTGGATGAGCTG	18mer	–
CYP2D6*3 Downstream primer	CYP2D6*3DN	GCCGAGAGCATACTCGGGAC	20mer	–
CYP2D6*4 Upstream primer	D6M*4UP	AAAGCGGGAACTGGGAAG	18mer	–
CYP2D6*4 Downstream primer	D6M*4DN	CACGGCTTTGTCCAAGAGA	19mer	–
OLR				
CYP2C19*2 Common probe	2CC19	ACTATCATTGATTATTTCCC	20mer	5′-Biotin
CYP2C19*2 Normal probe	2NC19	GGGAACCCATAACAATTACT$(A)_{20}$	40mer	5′-Phosphate
CYP2C19*2 Mutant probe	2MC19	AGGAACCCATAACAATTACT$(A)_{20}$	40mer	5′-Phosphate
CYP2C19*3 Common probe	3CC19	AGGATTGTAAGCACCCCCTG	20mer	5′-Biotin
CYP2C19*3 Normal probe	3NC19	GATCCAGGTAAGGCCAAGTT$(A)_{20}$	40mer	5′-Phosphate
CYP2C19*3 Mutant probe	3MC19	AATCCAGGTAAGGCCAAGTT$(A)_{20}$	40mer	5′-Phosphate
CYP2D6*3 Common probe	3CD6	ATGAGCTGCTAACTGAGCAC	20mer	5′-Biotin
CYP2D6*3 Normal probe	3ND6	AGGATGACCTGGGACCCA$(A)_{20}$	38mer	5′-Phosphate
CYP2D6*3 Mutant probe	3MD6	GGATGACCTGGGACCCA$(A)_{20}$	37mer	5′-Phosphate
CYP2D6*4 Common probe	4CD6	GCATCTCCCACCCCCA	16mer	5′-Biotin
CYP2D6*4 Normal probe	4ND6	GGACGCCCCTTTCG$(A)_{20}$	34mer	5′-Phosphate
CYP2D6*4 Mutant probe	4MD6	AGACGCCCCTTTCG$(A)_{20}$	34mer	5′-Phosphate

employing three probes per locus. OLR offers high specificity because it requires two recognition events.

Several methods have been reported for detection of ligation reaction products. These include: 1) heterogeneous assays that involve affinity capture of the product to a solid surface (e.g., microtiter wells) and detection by colorimetric, fluorimetric or bio(chemi)luminometric assays [Nickerson et al., 1990; Tobe et al., 1996; Samiotaki et al., 1994; Hansen et al., 1995; Tannous et al., 2003]; 2) electrophoretic methods [Baron et al., 1996; Eggerding et al., 1995; Sakthivel et al., 2001; Tobler et al., 2005; Nishida et al., 2005; Grossman et al., 1994]; 3) multiplex assays based on microarrays or spectrally encoded microspheres [Gerry et al., 1999; Consolandi et al., 2004; Li et al., 2006; Soper et al., 2005; Hashimoto et al., 2006; Iannone et al., 2000]; 5) homogeneous assays based on fluorescence resonance energy transfer [Chen et al., 1998; Lopez-Crapez et al., 2001], fluorescence coincidence analysis [Yeh et al., 2006], and nanoparticle reporters [Li et al., 2005].

In the present work, we have developed and evaluated a simple, robust, and low-cost disposable biosensor in dry-reagent format, which allows visual detection of OLR products with no need for instrumentation and without the multiple incubation and washing steps that are common to other currently-used methods. Specialized and costly equipment is essential for carrying out large-scale association studies that require high-throughput analysis. However, it is anticipated that in the clinical laboratory only a small number of SNPs will be analyzed routinely in each patient (except for countries with extensive genetic heterogeneity). During the last few years, dry-reagent strip tests have been introduced in SNP analysis but not in combination with the OLR. One approach (competitive allele-specific short oligonucleotide [ASO] hybridization [CASSOH]) was based on ASO hybridization in the presence of competitive oligonucleotides to enhance the specificity [Matsubara and Kure, 2003]. Another approach employed the primer extension reaction [Litos et al., 2007]. The proposed OLR-biosensor genotyping method was applied successfully to the analysis of four SNPs in two drug-metabolizing enzyme genes: 1) CYP2D6 (MIM# 124030; GenBank M33388.1)—CYP2D6*3 (dbSNP: rs4986774:delA, g.4168del>A) and CYP2D6*4 (g.3465G>A); and 2) CYP2C19 (MIM# 124020; GenBank M61854.1)—CYP2C19*2 (dbSNP: rs4244285, c.681G>A) and CYP2C19*3 (dbSNP: rs4986893, c.636G>A). The mutation nomenclature is indicated as recommended by the 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 AND METHODS

Instrumentation

PCR and OLR were performed in the PTC-0150 MiniCycler obtained from MJ Research (Watertown, MA). A digital camera, Kodak DC 120, and the Gel Analyzer software for DNA documentation were purchased from Kodak (New York, NY). The desktop scanner, ScanJet 4300C, was from Hewlett Packard (Palo Alto, CA).

Materials

Thermostable DNA ligase (Taq DNA ligase) was purchased from New England Biolabs (Ipswich, MA). Taq DNA polymerase was from Promega (Lyon, France). The QIAamp DNA blood mini kit were purchased from Qiagen (Hilden, Germany). Agarose, deoxynucleoside triphosphates (dNTPs), and DNA molecular weight markers (ϕX174 DNA, HaeIII digest) were from HT Biotechnology (Cambridge, UK). The dry-reagent biosensor was manufactured as described previously [Glynou et al., 2003] and was obtained from Medicon Hellas SA (Gerakas, Greece). NaOH pellets were purchased from Carlo Erba Analyticals (Milano, Italy). All other common reagents were from Sigma (St. Louis, MO). Oligonucleotide primers and probes were synthesized by MWG-Biotech (Ebersberg, Germany) and Invitrogen (Carlsbad, CA). The sequences of all oligonucleotides are shown in Table 1. For convenience, we refer to the wild-type nucleotide sequences as "normal" (N) and the variant nucleotide sequences as "mutant" (M). For each polymorphism we used a set of three probes: a common probe (probe C) that is biotinylated at the 5′-end; a probe specific for the normal allele (probe N); and a probe specific for the mutant allele (probe M). Both probes N and M carried a phosphate group at the 5′-end to allow for ligation and were synthesized with a $(dA)_{20}$ segment at the 3′-end to allow hybridization with oligo(dT) conjugated gold nanoparticles.

The developing solution contained 40 mL/L glycerol and 10 g/L 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 after informed consent. Genomic DNA was isolated with QIAamp DNA blood mini kit and stored at −20°C.

PCR

The PCR mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM of each primer, 2 μL genomic DNA, and 1.25 U Taq DNA polymerase in a total volume of 50 μL. The cycling conditions were as follows: 95°C for 1 min, followed by 35 cycles at 95°C for 20 s, 60°C for 20 s, and 72 °C for 30 s. After cycling, the mixture was incubated at 72°C for 10 min and cooled down to 4°C. Each series of PCRs included negative controls (containing water instead of DNA target) to confirm the absence of contamination. The PCR products were visualized by 2% agarose gel electrophoresis and ethidium bromide staining.

OLR

The OLR mixture consisted of 20 mM Tris-HCl, 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM dithiothreitol, 1 mM nicotinamide adenine dinucleotide (NAD), 0.1% Triton X-100 (pH 7.6), 1.5 U Taq DNA ligase, 2 pmol of each probe (probes C, N, and M) for CYP2C19*2 and CYP2C19*3 or 1 pmol of each probe for CYP2D6*3 and CYP2D6*4, and 0.2 pmol of amplified PCR products for CYP2C19*2 and CYP2C19*3 or 0.1 pmol of amplified PCR products for CYP2D6*3 and CYP2D6*4, respectively, in a total volume of 25 μL. The cycling conditions were as follows: 95°C for 5 min followed by 20 cycles each consisting of a denaturation step at 95°C for 30 s and an annealing/ligation step at 60°C for 90 s for CYP2C19*2 or 63°C, 90 s for CYP2C19*3 and CYP2D6*3, or 57°C, 90 s for CYP2D6*4. The mixture was cooled down to 4°C.

Detection of Ligation Products by a DNA Biosensor in Dry-Reagent Dipstick Format

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 in diameter) were deposited and dried on the conjugate pad. The laminated membrane contained a zone of immobilized streptavidin (test zone [TZ]) and a zone of immobilized oligo(dA) (control zone). Denaturation of each ligated product was carried out by mixing with freshly prepared dilutions of NaOH pellets in double-distilled water (ddH_2O). For each CYP, 5μL of OLR product was mixed with 5 μL of 1.5 M NaOH (or 0.75 M NaOH for CYP2C19*2) and the mixture was incubated for 10 min at room temperature. Subsequently, a 5-μL aliquot of the denaturation 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 developing solution. The visual detection of the ligation products was complete within 30 min.

RESULTS

Assay Principle

The principle of the proposed genotyping method is illustrated in Fig. 1. The genomic region that spans the locus of interest is first amplified by PCR. Subsequently, an OLR is performed, in which the target DNA is thermally denatured and allowed to hybridize to two oligonucleotide probes: a biotinylated common probe (C) that terminates one nucleotide upstream of the interrogated site; and a 5′ phosphorylated allele-specific probe (N or M). Probes N and M contain the allelic base at the 5′-end and a $(dA)_{20}$ segment at the 3′-end. If the probes are perfectly complementary to the target, then ligase catalyzes the formation of a phosphodiester bond. On the contrary, a mismatch at the junction prevents ligation. Thus, two different ligation products, C-N and/or C-M, are formed, depending on the alleles that are present in the sample. Both products have a biotinylated 5′-end and carry a $(dA)_{20}$ tail at the 3′-end.

For detection, the OLR products are first denatured, in order to ensure separation of nonligated probes from the target, and then applied to the conjugate pad of the dipstick-type biosensor, near the oligo(dT)-functionalized gold nanoparticles. Next, the sensor is immersed in the appropriate buffer. As the buffer migrates, via capillary action, the OLR products are captured from immobilized streptavidin at the TZ of the strip and the $(dA)_{20}$ segments hybridize with the oligo(dT) strands of the nanoparticles. This leads to accumulation of nanoparticles on the TZ and appearance of a characteristic red line. The excess nanoparticles continue their migration and bind to immobilized oligo(dA) strands at the control zone (CZ) of the strip. The red line at the control zone confirms the proper performance of the sensor. Free (nonligated) biotinylated common probe (C) is also captured on the TZ but not detected because it lacks the $(dA)_{20}$ segment. Free N or M probes hybridize with the oligo(dT) functionalized nanoparticles through their $(dA)_{20}$ sequence but are not detected since they lack biotin and can not be captured on the TZ. Consequently, the biosensor detects only ligation products. Two OLRs are performed per sample, one with C and N probes (N-reaction) and the other with probes C and M (M-reaction). The genotype is thus assigned by the results of two strips, one for each OLR. The presence of a red zone for N-reaction and absence of a red zone for M-reaction indicate a normal genotype (N/N). The mutant genotype (M/M) is characterized by a red zone for M-reaction only. A heterozygote (N/M) gives red zones for both reactions.

Optimization Studies

The proposed assay was applied to genotyping of the CYP2D6*4 [Gough et al., 1990], CYP2D6*3 [Marez et al., 1997], CYP2C19*2 [De Morais et al., 1994a], and CYP2C19*3 [De Morais et al., 1994b] polymorphisms. The relative positions of the polymorphic sites in the genes and the respective PCR products are shown in Fig. 1.

The CYP2D6*4 polymorphism was used as a model for optimization of various parameters of the ligation reaction. Amplified DNA from a normal individual (N/N) and a homozygote for the mutation (M/M) were used as target DNA fragments for the optimization studies we used.

DNA denaturation, after completion of the ligation reaction and prior to detection by the sensor, is a critical step of the method. If denaturation is not performed, then a signal is obtained even in the absence of ligase regardless of the genotype. This is because the target DNA holds together, through hybridization, the C probe and the N or M probes; and the hybrids that are formed contain both the biotin moiety and the $(dA)_{20}$ sequence, which are necessary for signal generation. Upon denaturation, the probes separate from the target and only ligated probes carry both biotin and $(dA)_{20}$.

We studied thermal denaturation (95°C for 5 min, then placed on ice) as well as denaturation with NaOH at various concentrations ranging from 0.5 to 5 M. A ligation product from a normal sample

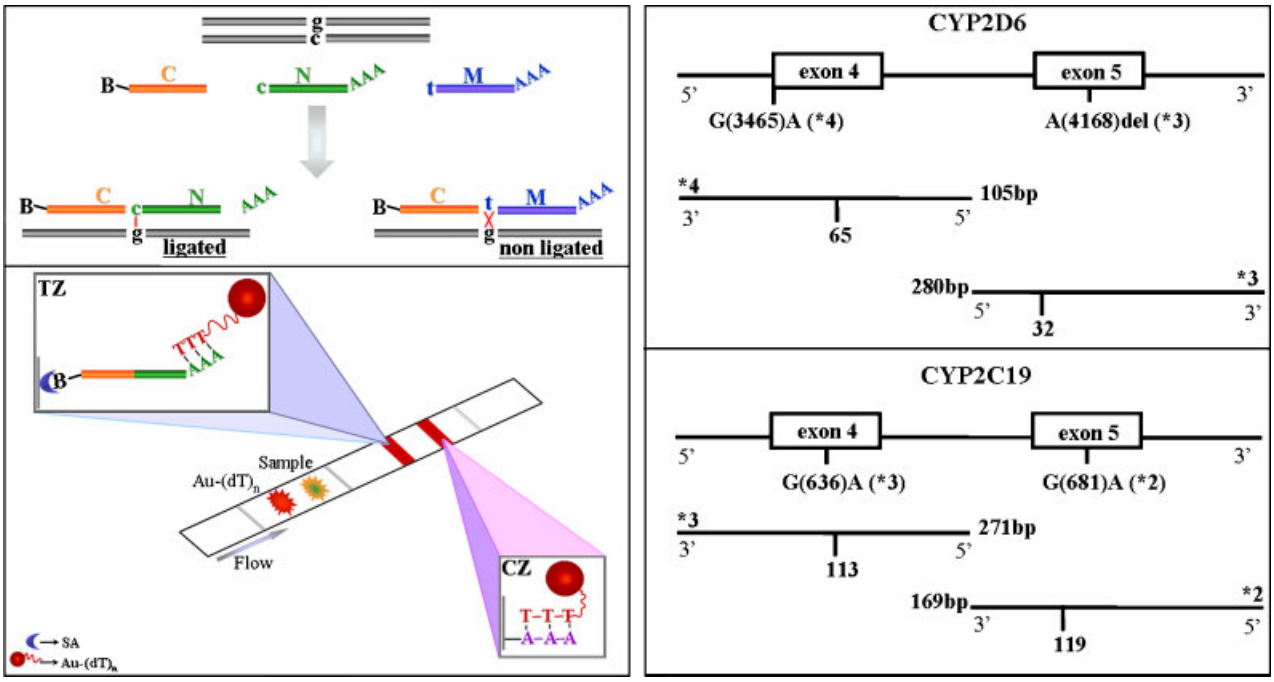

FIGURE 1. **Left panel: Schematic illustration of SNP genotyping by OLR in a dipstick format. PCR amplified DNA fragments that span the SNP of interest are subjected to two OLR using a biotinylated common probe (C) and an allele-specific probe, normal (N) or mutant (M). Both allele-specific probes contain a $(dA)_{20}$ segment at the 3′-end. A thermostable ligase joins two adjacent oligonucleotide probes only if they perfectly match the target sequence. The OLR product is denatured and applied to the DNA biosensor. As the developing solution migrates along the strip, the ligation products are captured from streptavidin at the TZ and hybridize with oligo(dT)-decorated gold nanoparticles. The accumulation of nanoparticles gives a characteristic red line. The excess nanoparticles are captured at the control zone (CZ) of the strip by hybridization to immobilized oligo(dA). Right panel: Schematic presentation of the relative positions of SNP in CYP2D6 and CYP2C19 genes and the generated PCR products flanking the polymorphic sites. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

and the respective product from a negative ligation reaction (a ligation reaction mixture containing all the components except ligase) were tested. Denaturation with NaOH gave a stronger signal and better discrimination of the alleles than heat denaturation. The optimum allele discrimination was achieved with 1.5 M NaOH. At higher NaOH concentrations the intensity of the TZ decreases considerably because the high pH interferes with the hybridization of $(dA)_{20}$ to oligo(dT) strands on the nanoparticles.

OLR was performed by thermal cycling using a thermostable DNA ligase. Each cycle consisted of target denaturation followed by annealing and ligation of the probes. The effect of the number of cycles on the yield of OLR was studied in the range of 14 to 24 cycles. The results are presented in Fig. 2. The intensity of the TZs increases with the cycle number both for normal and mutant alleles due to accumulation of ligation product. However, more than 20 cycles do not affect the signals of the strips. We chose 20 cycles because of the best discrimination of the alleles; i.e., the highest signal for the specific reaction and the lowest nonspecific signal. Nonspecific signal is the signal obtained when an M-reaction (M-probe) is performed on the N/N target or an N-reaction is carried out with an M/M target.

We optimized the annealing/ligation temperature in OLR, in the range of 51–63°C. The results are presented in Fig. 2. For temperatures ranging from 51°C to 57°C, we observe that the intensity of the TZs increases with the temperature both for normal and mutant alleles. This is due to the increase of ligase activity, which has an optimum at 60–65°C. Annealing temperatures higher than 57°C in OLR resulted in loss of the signals of the strips because of the decreased efficiency of probe hybridization to the target. Based on the above findings the optimum annealing/ligation temperature for CYP2D6*4 was 57°C. Similar studies for CYP2C19*2, CYP2C19*3, and CYP2D6*3 showed that the optimum temperatures were 60°C, 63°C, and 63°C, respectively.

The amount of amplified DNA in the OLR mixture was optimized in the range of 50–400 fmol (Fig. 2). The highest zone intensities both for normal and mutant alleles were obtained by using 100 or 200 fmol of amplified DNA. Both, the 50 and 400 fmol amounts gave faint signals, indicating either not enough DNA or a large excess of target DNA, which interferes with the ligation reaction due to competition of strand reannealing and probe hybridization. Based on similar studies, we chose 100–200 fmol of target DNA for all SNPs.

The molar ratio of target DNA to oligonucleotide probe was studied in the range of 1:2.5 to 1:40 in a series of ligation reactions containing a constant amount of amplified DNA (100 fmol) and varying amounts of probes. We observed (Fig. 2) that the ability of the method to discriminate the two alleles is practically the same throughout the range of probe to target ratios.

Genotyping of Clinical Specimens

The proposed assay was applied to the genotyping of blood samples for CYP2D6*4, CYP2D6*3, CYP2C19*2, and CYP2C19*3 polymorphisms. Extraction of genomic DNA, PCR, OLR, and detection by the biosensor were all performed as described in the experimental section. The genotype was assigned by two strips. The first strip detects the product of the ligation reaction with N probe (N-reaction) and the second strip shows the result of OLR with the M probe (M-reaction). After completion of the assay, the strips may be scanned using a common desktop

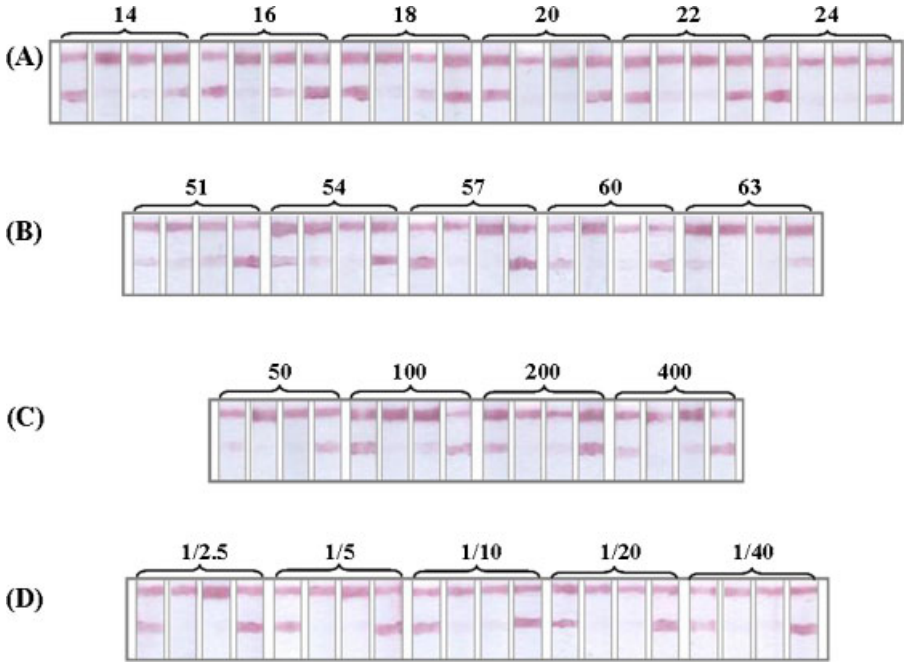

FIGURE 2. **A: Effect of the number of cycles in OLR. The number of cycles is indicated above each set of four strips. The strips represent: N/N sample with N probe (first strip), N/N sample with M probe (second strip), M/M sample with N probe (third strip), and M/M sample with M probe (fourth strip). B: Effect of ligation temperatures in OLR reaction. The ligation temperature (°C) is indicated above each set of four strips. C: Effect of the amount of amplified DNA in the OLR reaction. The amount of amplified DNA (fmol) is indicated above each set of four strips. D: Effect of the target DNA/probe molar ratio in OLR. The ratio is indicated above each set of four strips. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

scanner and the images analyzed by densitometry. The allelic fraction for the wild-type allele (AF_{wt}) is then calculated from the equation: $AF_{wt} = wt/(wt + mut)$, where wild type (wt) and mutant (mut) correspond to the densitometric signals obtained from the ligation reactions with N and M probes, respectively [Ahmadian et al., 2001]. Heterozygous samples are expected to have allelic fractions near 0.5 (a 1:1 ratio of the two alleles) whereas homozygotes for wt and mut are expected to give AF_{wt} values that are higher than 0.8 and lower than 0.2, respectively. Therefore, besides visual detection, the calculated allelic fraction indicates the genotype for each sample.

Fig. 3 shows the results (strips and allelic fractions) of genotyping of 20 samples (10 N/N, nine N/M, and one M/M) for CYP2D6*4. CYP2D6*3 genotyping data for 13 samples (nine N/N, three N/M, and one M/M) along with their respective allelic fractions are also presented in Fig. 3. Results pertaining to the genotyping of 15 samples (13 N/N, one N/M, and one M/M) for CYP2C19*3 are presented in Fig. 4A. Finally, Fig. 4B shows the results of genotyping of 15 samples (eight N/N, six N/M, and one M/M) for CYP2C19*2.

All sample genotypes were previously confirmed by sequencing. The results of the proposed method were in total agreement with sequencing.

Reproducibility

The reproducibility of OLR and biosensor detection was assessed by analyzing ligation products from normal (N/N) and mutant (M/M) genotypes of CYP2D6*4. The ligation reaction was performed on different days over a one-month period. The intensity of the control zone was estimated densitometrically. The results are presented in Fig. 5. For the normal genotype (N/N), the coefficients of variation (CVs) of the signals obtained from the N-reaction and M-reaction were 5.5% and 12.2%, respectively. For the mutant (M/M), the CVs of the signal obtained from the N-reaction and M-reaction were 11.4% and 5.3%, respectively.

DISCUSSION

We have developed a genotyping technique that exploits the high allele-discrimination ability of ligase reaction with the simplicity of a dipstick-type dry-reagent biosensor. OLR is considered to be the most specific genotyping reaction because it is based on two recognition events; i.e., two probes that bind to adjacent positions on the interrogated sequence, and the specificity of joining the probes by ligase only if they are perfectly complementary to the target. The biosensor allows visual detection of OLR products with no need for instrumentation after the DNA amplification step. In recent years it has been established that the variation in the response of patients to different drugs is closely related to the presence of SNPs in drug-metabolizing enzymes. This, in turn, paves the way for the introduction of personalized medicine. Therefore, it becomes important to develop simple, robust, and cost-effective methods for routine genotyping and particularly methods for point-of-care testing. Heterogeneous assays of OLR products, which are based on their capture to a solid surface (microtiter wells, microspheres, glass, etc.) and detection by colorimetric, fluorimetric, or bio(chemi)luminometric assays, require multiple incubation and washing steps along with specialized instrumentation. Homogeneous fluorimetric assays also require expensive reagents and specialized equipment. The proposed biosensor greatly simplifies OLR-based molecular tests. The key characteristic of the biosensor is that both the incubation step for

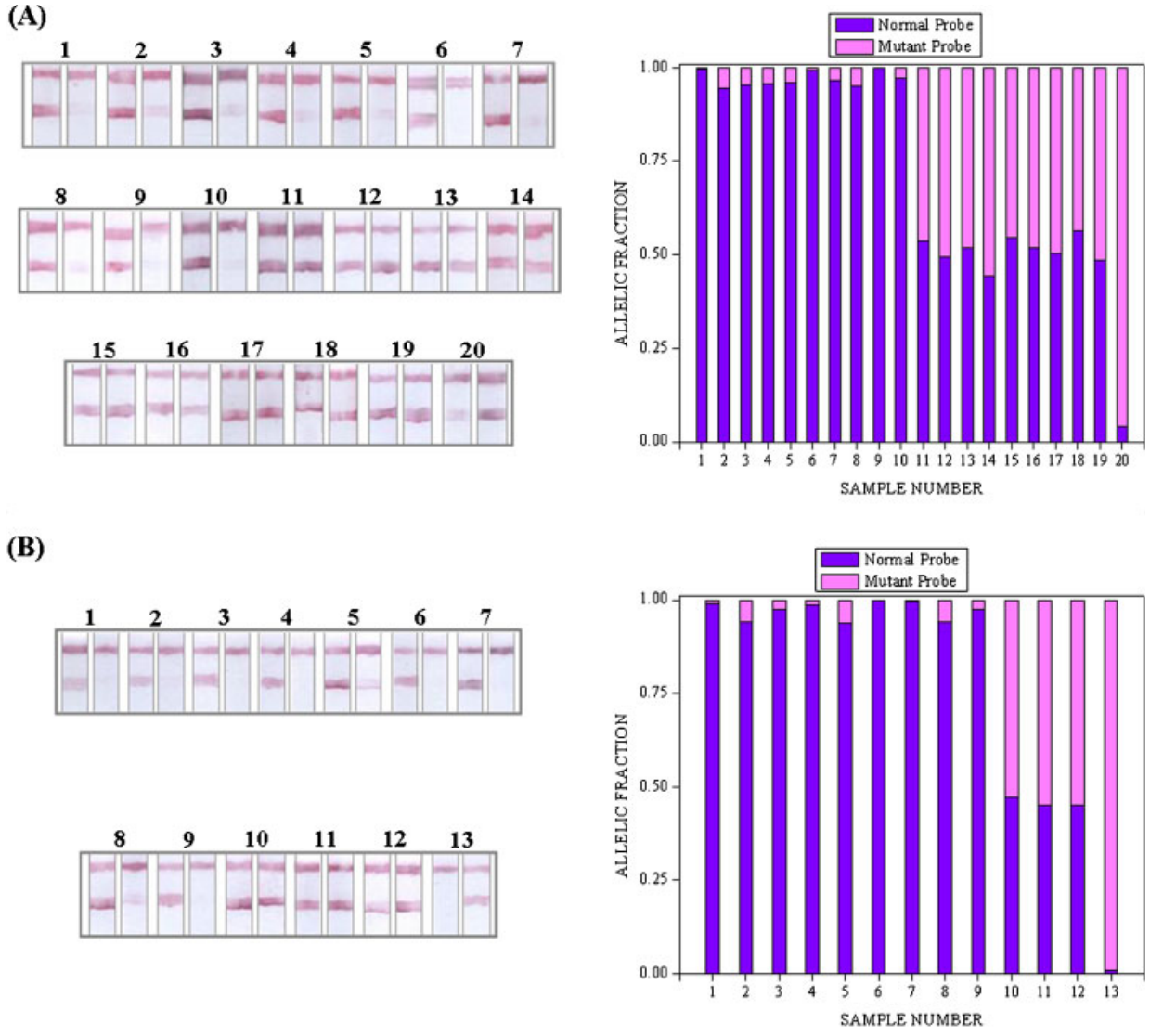

FIGURE 3. **Application of the OLR/dipstick assay to clinical samples, for CYP2D6 gene polymorphisms. A: Genotyping of SNPs at CYP2D6∗4. B: Genotyping of SNPs at CYP2D6∗3. Each pair of strips corresponds to OLR performed with the normal probe (left strip of the pair) and the mutant probe (right strip of the pair). The allelic fraction for each sample is also plotted versus the sample number. The dark bars correspond to the allelic fraction obtained with the normal probe and the light bars represent the allelic fraction obtained with the mutant probe. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

hybridization reaction and the capture of OLR products take place during migration of the solution along the strip by capillary action. Similarly, the removal (washing) of the excess of reagents, including the oligo(dT)-functionalized gold nanoparticle reporters, is accomplished by means of the continuous flow of fresh solution through the TZ and the control zone of the strip. As a result, this approach involves little hands-on manipulation and dramatically reduces the need for highly-qualified personnel. The cost of the assay in terms of reagents and biosensor is about 2 € per call. Although the performance of the assay was demonstrated in pharmacogenetic analysis, it is conceivable that the technique can also be applied to molecular diagnosis of disease (e.g., detection of mutations causing thalassemia or cystic fibrosis) by designing appropriate probes for OLR and using exactly the same biosensor. Indeed, the biosensor is universal because it detects DNA fragments that carry both a biotin moiety and a oligo(dA) segment regardless of the remaining sequence.

One limitation of the described assay format is that each dipstick only reports information about one allele of the SNP; i.e., as many reactions have to be set up and sensors loaded as the number of alleles scored. A method that is capable of detecting both alleles via a single OLR and a single biosensor strip is highly desirable. Work is currently in progress in our laboratory to develop a biosensor comprising two TZs, one for each allele. This sensor will be combined with a single OLR that generates allele-specific fragments carrying different affinity tags for capture on the TZs.

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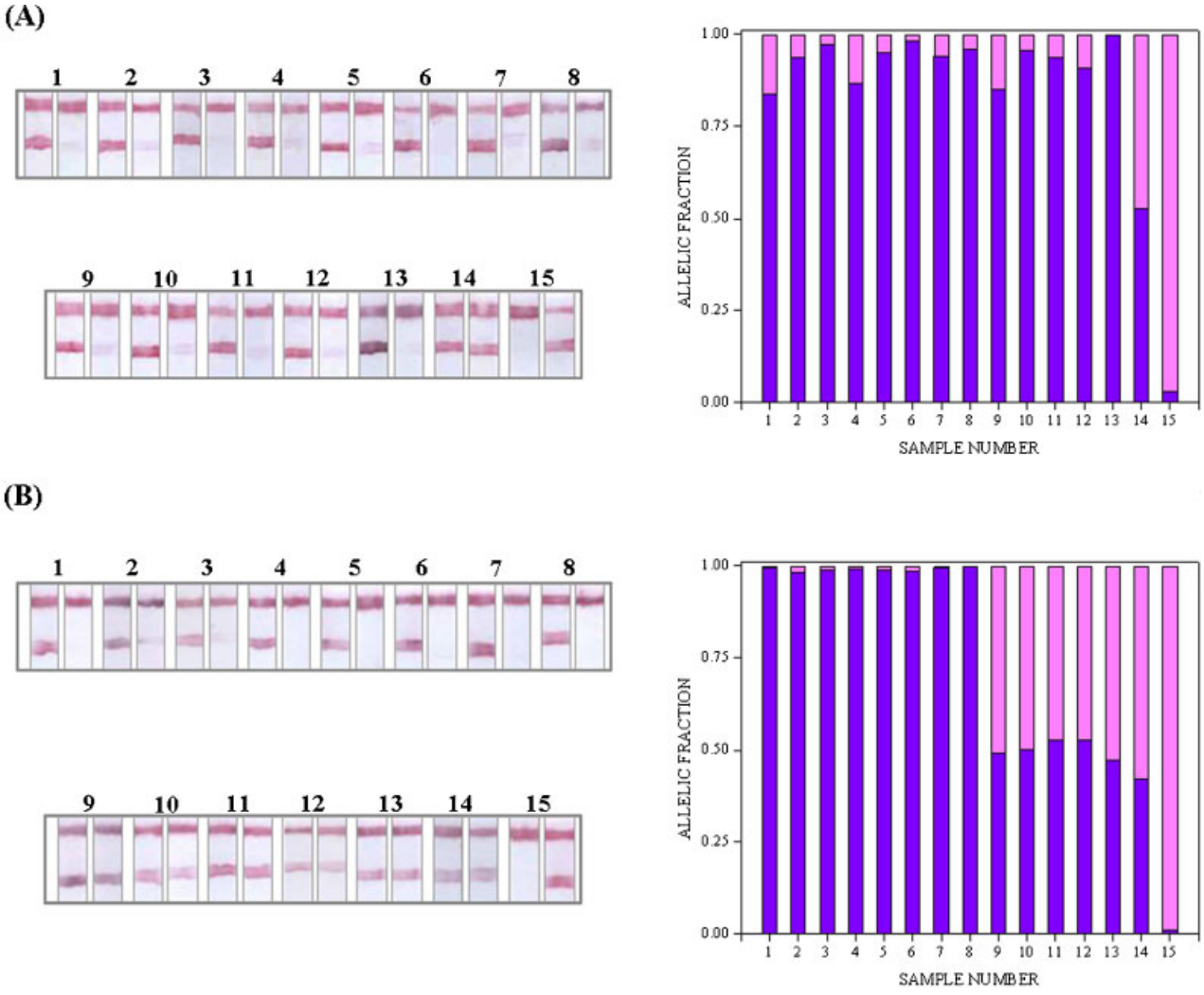

FIGURE 4. **Application of the proposed OLR/dipstick assay, for CYP2C19 gene polymorphisms. A: Genotyping of SNPs at CYP2C19∗3. B: Genotyping of SNPs at CYP2C19∗2. The strip pairs are presented as in Fig. 3. The allelic fraction of each sample is also plotted versus the sample number. Dark and light bars are as in Fig. 3. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

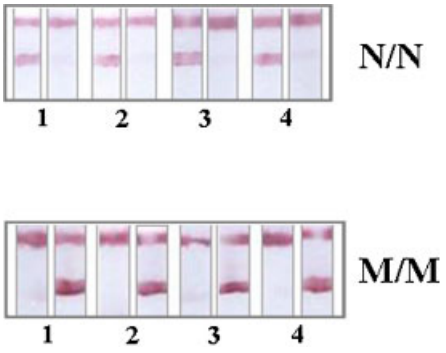

FIGURE 5. **Study of the reproducibility of the OLR/dipstick assay. Ligated products from normal (N/N) and mutant (M/M) samples for CYP2D6∗4 were subjected to OLR on different days over a one-month period with subsequent analysis on the dipstick biosensor. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]**

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