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Identification of Single-Nucleotide Polymorphisms by the Oligonucleotide Ligation Reaction: A DNA Biosensor for Simultaneous Visual Detection of Both Alleles

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Although single nucleotide polymorphisms (SNPs) can be identified by direct hybridization with allele-specific oligonucleotide probes, enzyme-based genotyping methods offer much higher specificity and robustness. Among enzymatic methods, the oligonucleotide ligation reaction (OLR) offers the highest specificity for allele discrimination because two hybridization events are required for ligation. We report the development of a DNA biosensor that offers significant advantages over currently available methods for detection of OLR products: It allows simultaneous visual discrimination of both alleles using a single ligation reaction. Detection is complete within minutes without the need for any specialized instruments. It does not involve multiple cycles of incubation and washing. The dry-reagent format minimizes the pipetting steps. The need for qualified personnel is much lower than current methods. The principle of the assay is as follows: Following PCR amplification, a single OLR is performed using a biotinylated common probe and two allele-specific probes labeled with the haptens digoxigenin and fluorescein. Ligation products corresponding to the normal and mutant allele are double-labeled with biotin and either digoxigenin or fluorescein, respectively. The products are captured by antidigoxigenin or antifluorescein antibodies, or both, that are immobilized at the two test zones of the biosensor and react with antibiotin-functionalized gold nanoparticle reporters. The excess nanoparticles bind to biotinylated albumin that is immobilized at the control zone of the biosensor. The genotype is assigned by the characteristic red lines that appear at the two test zones. The proposed DNA biosensor constitutes a significant step toward point-of-care SNP genotyping.

In recent years, there has been a continuously increasing interest in the development of methods for genotyping of single nucleotide polymorphisms (SNPs), since it has become apparent that SNPs constitute a new generation of biomarkers for assessing disease predisposition and predicting the patients' response to medication.[1,2] Various approaches to SNP genotyping have been reported.[3−5] Typically, they comprise three steps: exponential amplification of the target sequence, genotyping reaction for the discrimination of the two alleles, and detection of the products. Hybridization with allele-specific oligonucleotides (ASO), primer extension reaction, oligonucleotide ligation reaction (OLR), and invasive cleavage have been exploited for the genotyping step. Detection of the genotyping products is based on fluorescence,[6,7] bio(chemi)luminescence,[8−10] surface plasmon resonance,[11] and quartz crystal microbalance[12] and electrochemical[13,14] methods.

Up to now, there has been no consensus for the best genotyping method in terms of SNP throughput, sample throughput, simplicity, robustness, and cost. For instance, the microarray format combined with fluorometric detection[7,15] allows genotyping

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of thousands of SNPs in one or a few samples and is most suitable for large-scale association studies. However, it is anticipated that eventually, a small number of SNPs per patient will be assessed routinely in the molecular diagnosis laboratory. Microtiter well-based assays[8−10] or spectrally encoded microspheres[16] offer high sample throughput for a relatively small number of SNPs. On the other hand, electrochemical, optical, or gravimetric biosensors[11−14] offer simplicity and low cost and are more suitable for a small laboratory or for field testing. In particular, the dry-reagent dipstick-type biosensors allow visual detection of the genotyping products without the need for instruments.[17−20] ASO hybridization is commonly used for allele discrimination but requires careful adjustment of the stringency conditions. Enzyme-based allele discrimination has proven to be much more specific and robust than ASO hybridization.[3−5]

In OLR-based genotyping, DNA ligase catalyzes the formation of a phosphodiester bond between two oligonucleotides hybridized to adjacent positions of the interrogated sequence only if there is perfect complementarity with the target sequence at the junction.[21,22] OLR offers high specificity because it requires two recognition events. Usually, two separate OLRs are required for genotyping, one ligation reaction per allele. Various methods have been reported for simultaneous detection of both alleles in a single assay. In electrophoretic methods, a tail of characteristic length is attached to one probe, whereas the other probe is labeled with a fluorophore.[23] Microtiter well-based assays entail capture of both ligated products via an affinity tag and detection by using two reporters.[24−27] In a surface plasmon resonance imaging method, gold nanoparticles are used as reporters.[28] Homogeneous OLR assays are based on fluorescence resonance energy transfer[29] or two-color fluorescence coincidence analysis.[30]

Recently, we reported a dipstick-type biosensor for the visual detection of OLR products without the use of special instruments.[20] However, the method required the performance of two separate oligonucleotide ligation reactions and use of two biosensors, one for detection of each allele. In the present work, we have developed a fundamentally different biosensor format that allows the simultaneous visual detection of both alleles, thereby enabling the performance of a single ligation reaction and detection by a single biosensor. The proposed biosensor was applied successfully to the genotyping of a single nucleotide polymorphism (T387C) in beta3 adrenergic receptor (ADRB3) gene (8p12-p11.2, MIM# 109691, GenBank NM_000025) in 22 samples. Adrenergic receptors mediate the actions of epinephrine and related compounds and the ADRB3 gene has been associated with a variety of human diseases. The T387C SNP has been correlated with diabetes,[31,32] obesity,[33] breast cancer,[34] and clozapine metabolism.[35]

In our opinion, the development of this biosensor constitutes an important step toward point-of-care testing of SNPs.

MATERIALS AND METHODS

Instrumentation. PCR and OLR were performed in a MJ Research PTC-0150 miniCycler (Watertown, MA). The digital camera, Dynax 5D, was from Konica Minolta (Tokyo, Japan), and Gel Analyzer software for DNA documentation was purchased from Kodak (New York, NY). The TLC applicator Linomat 5 and WinCats software were from Camag (Muttenz, Switzerland). The desktop scanner, ScanJet 4300C, was from Hewlett-Packard (Palo Alto, CA).

Reagents. Thermostable DNA ligase, Ampligase, was purchased from Epicentre (Madison, WI). DyNAzyme II DNA polymerase was from Finnzymes (Espoo, Finland). The Nucleospin Blood L kit was purchased from Macherey-Nagel (Duren, Germany). Agarose, deoxyribonucleoside triphosphates (dNTPs), and DNA molecular weight markers ($\varphi X174$ DNA, *Hae*III digest) were from HT Biotechnology (Cambridge, UK). NaN_3 was purchased from Merck (Darmstadt, Germany). Antibiotin, antidigoxigenin antibody (anti-D, Fab fragments), and antifluorescein antibody (anti-F) were purchased from Sigma (St. Louis, MO), Roche Molecular Biochemicals (Mannheim, Germany), and Biodesign International (Saco, ME), respectively. Bovine serum albumin (BSA) was obtained from Sigma (St. Louis, MO). Biotinylated BSA (B-BSA) was a gift from Dr. Kalogianni (Dept. of Chemistry, University of Patras). Gold nanoparticles (Au NP, 40 nm diameter, 9×10^{10} particles/mL) were purchased from British Biocell BBInternational (Cardiff, UK). Nitrocellulose membrane, Immunopore FP Whatman, wicking pad, glass-fiber conjugate pad, and absorbent pad were purchased from Schleicher & Schuell (Dassel, Germany). All other common reagents were from Sigma (St. Louis, MO). Oligonucleotide primers and probes were synthesized by MWG-Biotech (Ebersberg, Germany). The upstream and

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downstream primers for PCR were 5′-CGCCCAATACCGCCAACAC-3′ and 5′-CCACCAGGAGTCCCATCACC-3′, respectively. For convenience, we refer to the wild-type nucleotide sequences as "normal" (N) and the variant nucleotide sequences as "mutant" (M). For genotyping the ADRB3 polymorphism, we used a set of three probes: a 5′ biotinylated (B) common probe (probe C), 5′-B-TGGTCATCGTGGCCATCGCC-3′; a probe specific for the normal allele (probe N), 5′-Ph-TGGACTCCGAGACTCCAGAC-D-3′, labeled with hapten digoxigenin (D) at the 3′ end; and a probe specific for the mutant allele (probe M), 5′-Ph-CGGACTCCGAGACTCCAGAC-F-3′, labeled with the hapten fluorescein (F) at the 3′ end. Both probes N and M carried a phosphate group (Ph) at their 5′ end to allow ligation. Finally, two oligonucleotides were used as controls for biosensor construction: B-CN-D, 5′-B-TGGTTGCTCTAGGAAAGCAGCATTCTGAAGA GGCTTCTAA-D-3′; and B-CM-F, 5′-B-TGGTTGCTCTAGGAAAGCAGTATT CTGAAGAGGCTTCTAA-F-3′.

The developing solution contained 60 mL/L glycerol, 10 mL/L Tween-20, 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).

Preparation of Antibiotin-Conjugated Gold Nanoparticles. A 1-mL aliquot of the Au NP solution (0.15 pmol/mL) was adjusted to pH 9 by adding 200 mM borax solution. In parallel, 4 μg of antibiotin antibody was diluted in 2 mM borax solution (final antibody concentration, 10 mg/L) and added to the previous gold solution, gradually by stirring. The mixture was incubated at ambient temperature for 45 min, and a solution of 100 g/L BSA in 20 mM borax was added. The solution was incubated at ambient temperature for 10 min, and the excess of reagents was removed by centrifugation at 4500*g* for 15 min. The supernatant was discarded, and the pellet was redispersed in 1 mL wash solution (10 g/L BSA in 2 mM borax), followed by centrifugation at 4500*g* for 5 min. The supernatant was discarded, and the red pellet was redispersed in 100 μL of an aqueous solution containing 1 g/L BSA, 1 g/L NaN_3, and 2 mM borax. A 5-μL aliquot of antibiotin antibody-conjugated gold nanoparticles was deposited on the conjugate pad of the biosensor.

Preparation of Dry Reagent Dipstick-Type Biosensor. The dry reagent strip biosensor (4×70 mm) consisted of an immersion pad, a glass-fiber conjugate pad, a nitrocellulose diagnostic membrane, and an absorbent pad. The parts were assembled on a plastic adhesive backing as follows: The membrane (25 mm in length) was first placed on the backing. The absorbent pad (15 mm) was then positioned above the membrane, overlapping by 2 mm. The conjugate pad (15 mm) was placed below the membrane, overlapping by 2 mm. The immersion pad (15 mm) was placed below the conjugate pad, overlapping by 2 mm. The TLC applicator, Linomat 5, was employed to construct two test zones and a control zone by loading antifluorescein antibody (anti-F), antidigoxigenin antibody (anti-D), and biotinylated BSA (B-BSA) on the membrane, respectively. The anti-F, anti-D, and B-BSA zones were constructed at distances of 10, 15, and 20 mm from the edge of the membrane, respectively. For the anti-F zone, a solution consisting of 250 mg/L antifluorescein antibody, 50 mL/L methanol, and 20 g/L sucrose in freshly prepared 100 mM $NaHCO_3$ buffer (pH 8.5) was loaded at a density of 75 ng per 4-mm membrane. For anti-D zone, a solution containing 500 mg/L antidigoxigenin antibody, 50 mL/L methanol, and 20 g/L sucrose in 100 mM $NaHCO_3$ buffer (pH 8.5) was loaded at a density of 500 ng per 4-mm membrane. Finally, for the control zone, a solution consisting of 4 g/L B-BSA, 50 mL/L methanol, and 20 g/L sucrose in PBS (pH 7.5) was loaded at a density of 1.6 μg per 4-mm membrane. The membrane was dried in an oven for 1 h at 80 °C, and the sensors were assembled as described above.

Extraction of Genomic DNA from Whole Blood. Whole-blood specimens were obtained from the University Hospital of Patras. Genomic DNA was isolated by using the Nucleospin Blood L kit and stored at −20 °C. The typical concentration range of genomic DNA extracted using the Nucleospin Blood L kit is 200−500 ng/μL.

Polymerase Chain Reaction. The PCR mixture (50 μL) contained 10 mM Tris−HCl (pH 8.8), 50 mM KCl, 0.1% Triton X-100, 2.5 mM $MgCl_2$, 200 μM of each dNTP, 0.5 μM each of the upstream and downstream primers, 2−5 μL of genomic DNA, and 1.25 U of DyNAzyme II DNA polymerase. The cycling conditions were 97 °C for 2 min, followed by 35 cycles at 97 °C, 15 s; 65 °C, 15 s; and 72 °C, 30 s. At the end of the cycling, the mixture was incubated at 72 °C for 10 min and cooled down to 4 °C. Negative controls (containing water instead of DNA) were included in each series of PCRs to confirm the absence of contamination. The PCR products were visualized by 2% agarose gel electrophoresis and ethidium bromide staining.

Oligonucleotide Ligation Reaction (OLR). The OLR mixture (12.5 μL) consisted of 20 mM Tris−HCl, 25 mM KCl, 10 mM $MgCl_2$, 5 mM NAD, 0.1% Triton X-100, (pH 8.3), 2.5 U of Ampligase, 500 fmol of each probe (probes C, N, and M), and 50 fmol of amplified PCR product. 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 65 °C for 2 min. The mixture was cooled down to 4 °C.

Simultaneous Visual Detection of Ligation Products by the DNA Biosensor. Prior to detection, the OLR products were denatured at 95 °C for 5 min and placed immediately on ice. A 12-μL aliquot of the solution was added to the conjugate pad, next to antibiotin−nanoparticle conjugate. The immersion pad was then dipped into a microcentrifuge tube containing 250 μL of developing solution. Visual detection of the two allele-specific OLR products was complete within 20 min.

RESULTS AND DISCUSSION

Assay Principle. The principle of the proposed genotyping method is illustrated in Figure 1. The genomic region that spans the locus of interest is first amplified by PCR. The size of the PCR product is 201 bp. Subsequently, a single oligonucleotide ligation reaction is performed in which the target DNA is thermally denatured and allowed to hybridize to two oligonucleotide probes: a biotinylated (B) common probe (C) that terminates one nucleotide upstream of the interrogated site and a 5′ phosphorylated allele-specific probe (normal-N or mutant-M). Probes N and M contain the allelic base at the 5′ end and are conjugated at the 3′ end with the haptens digoxigenin (D) and fluorescein (F), respectively. If probes N or M are perfectly complementary to

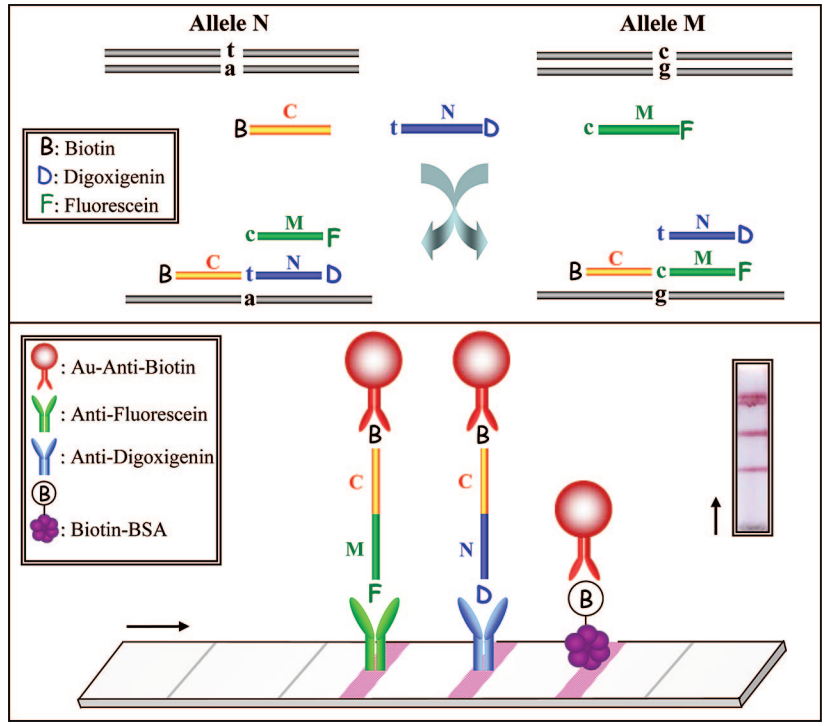

Figure 1. Principle of SNP genotyping by oligonucleotide ligation reaction (OLR) with simultaneous visual detection of both alleles by a DNA biosensor. Following PCR amplification, a single OLR is performed using a biotinylated common probe (C) and two allele-specific probes (N and M) labeled with digoxigenin (D) and fluorescein (F), respectively. Ligation products corresponding to the normal and mutant allele (B-CN-D and B-CM-F, respectively) are doubled-labeled with biotin and either digoxigenin or fluorescein, respectively. The products are captured by antidigoxigenin (anti-D) or antifluorescein (anti-F) antibodies that are immobilized at the test zones of the biosensor and detected by antibiotin-functionalized gold nanoparticles. The excess nanoparticles bind to biotinylated bovine serum albumin (BSA) that is immobilized at the control zone of the biosensor. The arrows show the direction of the flow.

the interrogated DNA, then ligase catalyzes the formation of a phosphodiester bond with the C probe. In contrast, a mismatch at the junction prevents ligation. Thus, two possible ligated fragments, B-CN-D or B-CM-F, are formed, depending on the alleles that are present in the sample. The ligated probes are double-labeled with biotin at the 5′-end and either digoxigenin or fluorescein at the 3′ end. For detection, the products are first heat-denatured to ensure separation of nonligated probes from the target and then are applied to the conjugate pad of the biosensor near the antibiotin-functionalized nanoparticles. The sensor is immersed in the appropriate buffer. As the buffer migrates along the strip, the OLR products bind to antibiotin-conjugated nanoparticles and are captured by immobilized anti-F, immobilized anti-D, or both. This leads to accumulation of nanoparticles on the test zones and the appearance of characteristic red lines. The excess nanoparticles bind to immobilized biotinylated BSA at the control zone of the strip, thus forming another red line that confirms the proper performance of the sensor. Free (nonligated) N or M probes are also captured at their respective test zones but not detected since they lack biotin and cannot bind to the nanoparticles. Free biotinylated common probe (C) is captured on the nanoparticles but not detected because it lacks the hapten. Consequently, the biosensor detects only ligated probes. The genotype is assigned visually by using a single biosensor strip: a red line at the anti-D zone indicates the presence of the normal allele (N). A red line at the anti-F zone signifies the presence of the mutant allele (M). Red lines at both test zones are obtained from heterozygotes. The size of the PCR product does not affect the development time required for detection, since the biosensor detects only the single-stranded ligation product (40 bases) and not the amplification product.

It should be noted that although the majority of OLR-based SNP genotyping methods require two separate OLRs per SNP (one with each probe N and M), the proposed method involves a single OLR with all probes C, N, and M in the same reaction mixture.

The biosensor described and evaluated in this work is fundamentally different from the one reported recently[20] for detection of OLR products. More specifically, (a) the sensor of ref 20 consists of a single test zone containing immobilized streptavidin, whereas the present sensor comprises two test zones containing antidigoxigenin and antifluorescein antibodies to enable the capture of both alleles in a single biosensor. (b) Detection in ref 20 is accomplished through oligonucleotide-functionalized gold nanoparticles, whereas the present biosensor employs antibiotin-coated gold nanoparticles as reporters for visualization of both alleles. As a result of this novel configuration, the genotyping for both alleles is achieved in a single ligation reaction, followed by detection with a single biosensor.

Optimization of the Biosensor. For the optimization of the biosensor, we prepared two synthetic control oligos (40-mers) with sequences of sizes identical to the two ligation products and double-labeled with biotin at the 5′ end and digoxigenin or fluorescein at the 3′ end (B-CN-D and B-CM-F, respectively). To investigate the specificity of the response of the biosensor to various products of the ligation reaction, we first constructed sensors comprising only one test zone, either anti-D or anti-F, at the specified distances from the edge of the membrane. Subse-

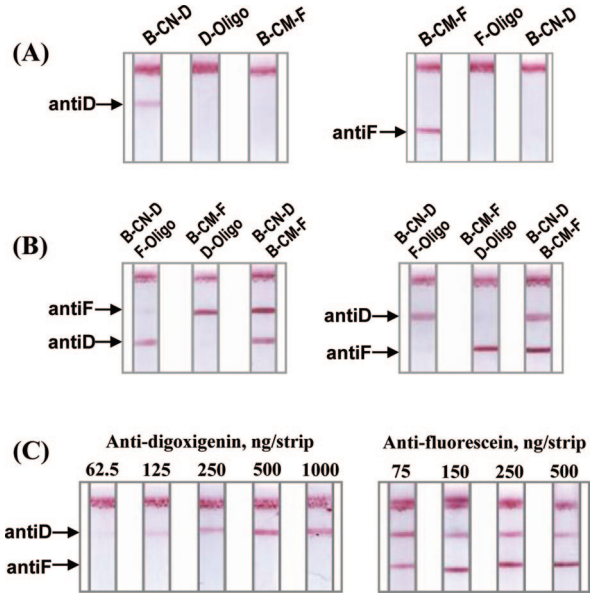

Figure 2. Response of the DNA biosensor to synthetic ligation products and nonligated probes. (A) Study of the specificity of the test zones of the sensor. Sensors containing either the antidigoxigenin (anti-D) or the antifluorescein (anti-F) zone were constructed. Two synthetic control oligos identical to the two ligation products and double-labeled with biotin (B) at the 5′ end and digoxigenin (D) or fluorescein (F) at the 3′ end were prepared (B-CN-D and B-CM-F, respectively). The synthetic ligation products, B-CN-D, B-CM-F, as well as nonbiotinylated oligos labeled with D and F at the 3′ end (D-oligo and F-oligo) were applied individually to the sensor (500 fmol of each). (B) Effect of the relative position of the two test zones on the membrane of the biosensor. Three mixtures of oligonucleotides, that is, F-oligo/B-CN-D, D-oligo/B-CM-F, and B-CN-D/B-CM-F were applied to the sensor. (C) Optimization of the amount of antibody deposited at the test zones of the strip. Control oligonucleotides B-CN-D and B-CM-F (500 fmol of each) were used for this study.

quently, the control oligos, B-CN-D and B-CM-F, as well as nonbiotinylated oligos labeled with D and F at the 3′ end (D-oligo and F-oligo) were applied individually to the sensor (500 fmol of each). The results are presented in Figure 2A. A red line at the anti-D zone is observed only when the control oligo B-CN-D is applied to the sensor. Neither the D-oligo that lacks a biotin moiety nor the control oligo B-CM-F gives a signal at the anti-D zone. Similarly, a red line at the anti-F zone is observed only when the control oligo B-CM-F is applied to the sensor, whereas no signal is obtained with the F-oligo and the control oligo B-CN-D. Consequently, each test zone of the biosensor responds only to the cognate double-labeled ligation product.

The effect of the relative position of the two test zones on the membrane was studied by constructing sensors in which the order of the zones (at the direction of the flow of the developing solution) was either anti-F, anti-D, or the reverse (anti-D, anti-F) and by applying three mixtures of oligonucleotides: (a) F-oligo/B-CN-D, (b) D-oligo/B-CM-F, and (c) B-CN-D/B-CM-F. The results (Figure 2B) show that the order by which the test zones anti-F and anti-D are positioned on the strip does not influence the color density of the sensor. F-Oligo and D-oligo are not detected because they lack biotin. Control oligos B-CN-D and B-CM-F are detected only at the corresponding test zones.

Finally, the amount of each antibody deposited on the membrane of the strip was optimized. The goal of the optimization was 2-fold; to determine the amount of antibody that gives a clear signal and also to adjust the relative amounts of the two antibodies in such a way that the color densities of anti-F and anti-D zones are similar when equimolar amounts of the corresponding analytes are applied to the biosensor. The effect of the amount of antidigoxigenin antibody was studied in the range of 62.5−1000 ng/strip using the B-CN-D control oligonucleotide (500 fmol/strip). The results are presented in Figure 2C. We observe that the color density increases with the amount of antibody up to 500 ng/strip. The zone becomes fainter at higher amounts of antibody. The decrease in the signal is attributed to the fact that the adsorbing capacity of the membrane is exceeded and the antibody remains free (not immobilized). As a consequence, the analyte (B-CN-D) is distributed between the immobilized and the free antibody. The fraction of the analyte that is bound to the free antibody migrates along the strip and remains undetectable.

For the optimization of the amount of antifluorescein, we used strips comprising a 500-ng anti-D zone and added the anti-F zone containing 75−500 ng of antifluorescein antibody per strip. The sensor was tested with an equimolar (500 fmol) mixture of B-CN-D and B-CM-F. In Figure 2C, it is observed that at 75 ng of antifluorescein antibody, the color densities of anti-F and anti-D zones are very similar. Thus, the final biosensors were constructed using 500 ng and 75 ng of antidigoxigenin and antifluorescein antibodies, respectively. The difference in the optimum quantities of anti-D and anti-F is attributed to the difference in the size; that is, Fab fragments of anti-D were used as opposed to the whole anti-F antibody. It turns out that the use of whole antibody leads to more efficient immobilization at the test zone and an orientation that facilitates the binding of the hapten.

Optimization of Oligonucleotide Ligation Reaction. The genotyping of ADRB3 polymorphism was used as a model for optimization of the OLR parameters. Amplified DNA from a normal individual (N/N) and a homozygote for the mutation (M/M) were used as target DNA fragments for optimization studies. In each series of ligation reactions, one parameter was studied and the others were kept constant, as described in Materials and Methods Section.

The annealing/ligation temperature was optimized in the range of 50−65 °C. The results for the N/N and the M/M samples are presented in Figure 3A. It is observed that the density of the cognate test zones for normal and mutant alleles is only slightly affected by the change in the temperature. The nonspecific signal, on the other hand, decreases dramatically in elevated temperatures. The nonspecific signal is defined as the presence of a red line at the anti-F zone when an N/N sample is tested or the appearance of a red line at the anti-D zone when an M/M sample is assayed. On the basis of the above findings, the optimum annealing/ligation temperature for OLR was set at 65 °C. Moreover, the density of each test zone was determined by scanning the strip with a simple desktop scanner. The allelic fraction of the normal allele was then calculated as the ratio of $D_{\text{anti-D}}/(D_{\text{anti-F}} + D_{\text{anti-D}})$, where $D_{\text{anti-F}}$ and $D_{\text{anti-}D}$ are the color densities at zones anti-F and anti-D, respectively. Therefore, the allelic fraction represents the fraction of the total color density that corresponds to the normal allele (zone anti-D). In Figure 3A, the allelic fraction is plotted as a function of the temperature. Ideally, the allelic fraction should be equal to 1 for a normal sample

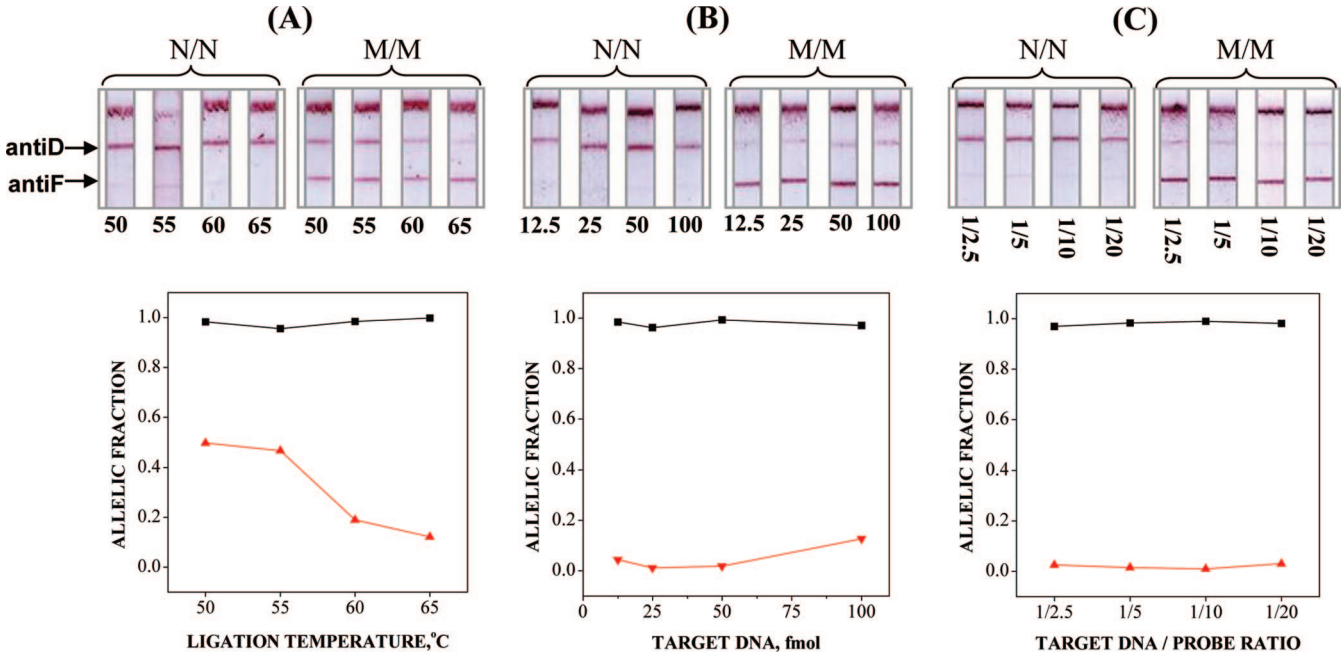

Figure 3. Optimization of OLR for N/N and M/M samples. For each study, the strips were scanned with a simple desktop scanner, and the allelic fraction was plotted against the optimization parameter. (A) Effect of annealing/ligation temperature. (B) Study of the amount of PCR-amplified DNA introduced into the OLR mixture. (C) Effect of the molar ratio of amplified DNA to oligonucleotide probe. The black line (squares) represents the N/N sample, whereas the red line (triangles) represents the M/M sample.

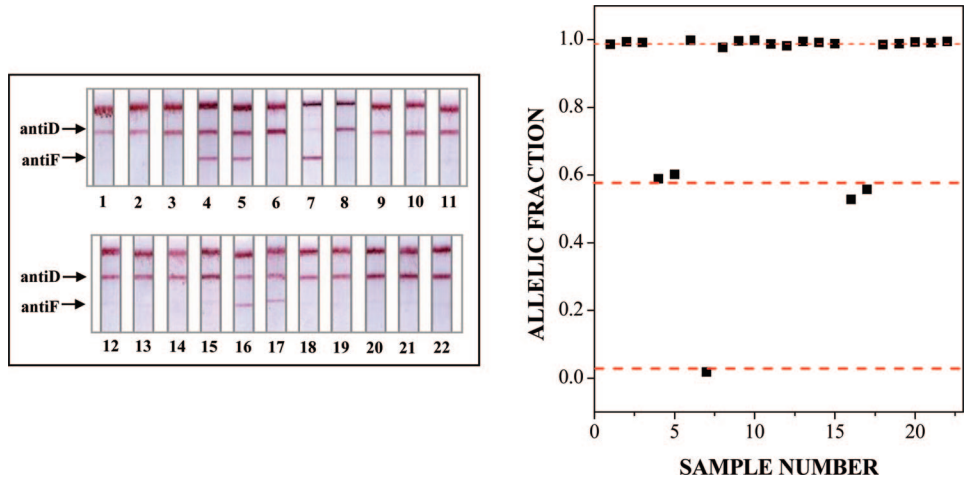

Figure 4. Genotyping of 22 blood samples for ADRB3 polymorphism by a single OLR followed by detection of both alleles by a single DNA biosensor. The strips were also scanned using a common desktop scanner, and the images were analyzed by densitometry. The allelic fraction of the normal allele was then calculated for each sample.

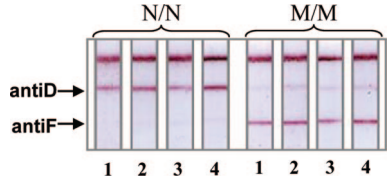

Figure 5. Study of the reproducibility of OLR-biosensor assay for a N/N and a M/M sample.

and equal to zero for a mutant sample. In practice, the larger the difference between the allelic fractions obtained from N/N and M/M samples, the higher is the discrimination ability between normal and mutant samples. The presence of nonspecific lines either at anti-F or anti-D zones results in a decrease in the difference between the allelic fractions and compromises the allele discrimination. This is because a nonspecific signal at the anti-F zone causes a decrease in the allelic fraction of an N/N sample, whereas a nonspecific signal at the anti-D zone leads to an increase in the allelic fraction of an M/M sample.

The amount of amplified DNA introduced into the OLR mixture was optimized in the range of 12.5−100 fmol. Representative sensor strips as well as the allelic fractions of the normal allele at various amounts of DNA template are shown in Figure 3B. We observe that the discrimination between the two alleles is excellent in the range of 12.5−50 fmol PCR product, but it deteriorates at levels higher than 50 fmol. This is because at high amounts of DNA template, there is competition between strand reannealing and probe hybridization, which leads to a decrease in the signal while the nonspecific binding increases. The nonspecific signal

at a high amount of target DNA may be attributed either to nonspecific ligation or to the accumulation of too much ligated product, which saturates the anti-F zone and therefore binds nonspecifically to the anti-D region.

The effect of the molar ratio of amplified DNA to oligonucleotide probe was studied in the range of 1/2.5 to 1/20 in a series of ligation reactions containing a constant amount of amplified DNA (50 fmol) and varying amounts of probes. It was found that the ability of the method to discriminate the two alleles is practically the same throughout the range of probe-to-target ratios (Figure 3C).

Genotyping of Clinical Specimens. The proposed assay was applied to the genotyping of 22 blood samples for ADRB3 polymorphism. Extraction of genomic DNA, PCR, and OLR and simultaneous detection of both alleles by the biosensor were all performed as described in the Materials and Methods section. Following PCR, the amplified DNA was quantified, and 50 fmol (0.5−2.5 μL PCR mixture) was introduced into the OLR mixture (12.5 μL). The genotype was assigned by a single strip, and the results are presented in Figure 4. The strips were also scanned using a common desktop scanner, and the images were analyzed by densitometry. The allelic fraction of the normal allele was then calculated for each sample. The allelic fractions obtained from the normal and mutant samples were higher than 0.95 and lower than 0.1, respectively. Heterozygous samples gave allelic fractions near 0.6. Therefore, in addition to visual detection, the calculated allelic fraction indicates clearly the genotype for each sample (Figure 4). All sample genotypes were confirmed by sequencing. The results of the proposed method were in total agreement with sequencing data.

Reproducibility. The reproducibility of OLR and the biosensor assay was assessed by analyzing ligation products from N/N and M/M genotypes of ADRB3. Four PCR amplifications per sample were carried out, followed by OLRs and detection by using the biosensor. The four biosensors were produced in a single batch using the same reagents. The results are presented in Figure 5. The color density of the test zones was estimated by scanning of the strips. For the N/N genotype, the CVs of the signals obtained from the anti-F and anti-D zones were 5.2% and 7.7%, respectively. For the mutant genotype, the CVs of the signals obtained from the anti-F and anti-D zones were 4.5% and 12%, respectively.

CONCLUSIONS

Although SNPs can be identified by direct hybridization with ASO probes, enzyme-based genotyping methods offer much higher specificity and robustness. Among the enzymatic methods (e.g., primer extension reaction), OLR offers the highest specificity for allele discrimination because two hybridization events are required for ligation. The proposed biosensor offers a number of significant advantages over currently available methods for detection of OLR products. First, it allows simultaneous visual discrimination of both alleles by a single biosensor. Only a single oligonucleotide ligation reaction is required for both alleles. In contrast to current methods, the detection of the OLR products does not require any specialized instruments and is complete within minutes. Unlike heterogeneous assays of OLR products (e.g., microtiter well-based methods), the proposed biosensor assay does not involve multiple incubation and washing steps. The assay is very simple, and the dry-reagent format minimizes the pipetting steps. The need for qualified personnel is much lower than current methods. The cost of genotyping is considerably lower than existing methods. Consequently, we believe that the proposed DNA biosensor will be particularly useful for point-of-care SNP genotyping.

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