Anal Bioanal Chem
DOI 10.1007/s00216-014-8424-1

NOTE

# Direct genotyping of C3435T single nucleotide polymorphism in unamplified human MDR1 gene using a surface plasmon resonance imaging DNA sensor

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Received: 20 November 2014 / Accepted: 16 December 2014


**Abstract** Optical genotyping of C3435T single nucleotide polymorphisms (SNPs) in unamplified human multidrug resistance (MDR1) gene was here performed by a surface plasmon resonance imaging (SPRi) dual-targeting DNA assay, allowing its selective detection down to 0.18 fM of the whole genomic DNA. The result was achieved by the combination of the rational selection of the DNA probe and an optimized sample pretreatment (i.e., ultrasound fragmentation and thermal denaturation). Some assay developments and tunings were reported in a previously published research, but here, for the first time, the biosensor reliability and its analytical performance were directly tested on the unamplified human DNA extracted from lymphocytes. The assay resulted to be able to differentiate among all the possible genotypes of C3435T (homozygote and heterozygote) in the diluted genomic samples using a label-free approach and by bypassing the classical PCR amplification of the target sequences. Moreover, the reusability of the DNA-based chip allowed up to 40 subsequent measuring cycles, opening new horizons in multi-SNP genotyping based on cheap and daily routine clinical monitoring by optical biosensing.

Published in the topical collection *Direct Optical Detection* with guest editors Guenter Gauglitz and Jiri Homola.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00216-014-8424-1) contains supplementary material, which is available to authorized users.

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Published online: 27 January 2015

**Keywords** Surface plasmon resonance imaging (SPRi) DNA sensor · Direct optical genotyping · C3435T single nucleotide polymorphisms (SNPs) · Human genomic DNA (hDNA) · MDR1 gene

## Introduction

Single nucleotide polymorphisms (SNPs) are the most common mutations occurring with a frequency of >1 % in the human DNA, and one of the biggest challenges in human genetics is to understand their role (in specific coding regions) in susceptibility to common diseases (e.g., cancer) [1].

Different approaches were developed to detect SNPs over the years especially based on molecular biology protocols coupled to PCR amplification [2] or on analytical instrumental approaches, i.e., mass spectrometry [3], hyphenated LC/MS [4], and electrophoresis [5].

Among the several transduction systems, optical-based biosensing represents a powerful tool for multi-analyte, sensitive, real-time, and label-free detection [6]. At the forefront of optical transducers is surface plasmon resonance imaging (SPRi) [7], implementation of conventional surface plasmon resonance (SPR) which exploits surface plasmon polaritons to investigate biointeractions in microarray formats [8]. The imaging advancement has opened new horizons to real applications of multi-analyte detection also reported and discussed in excellent and recent reviews dealing with SPR and SPRi-based sensing as ultrasensitive transducers for DNA sensing [9, 10] and, specifically, for SNP differentiation [11]. SNP mapping in the unamplified genomic DNA by SPRi was reported in the literature by associating SPR detection to gold nanoparticles to gain the SPR signal [12], making it possible

to bypass the amplification step traditionally performed by PCR [13, 14]. Alternatively, we investigated the sensitive and selective label-free detection of human genomic DNA sequences by fine-tuning of the multiple steps involved in the bioassay, from the selection criteria of DNA probes to the sample pretreatment [15]. We also demonstrated that the presence of SNP could be discriminated in matrices randomly amplified by whole genome amplification (WGA) [16] thanks to the tuning of the DNA probe features (length and functionalization). These recent researches represented the starting point for the ultimate and most challenging application of the method, i.e., the allele discrimination of the gene directly in the unamplified DNA samples extracted from human blood (lymphocytes), bypassing sample enrichment steps (e.g., PCR or WGA) which are time- and reagent-consuming [12].

We demonstrate here the direct genotyping of the human DNA extracted from lymphocytes, fragmented by ultrasounds, and thermally denatured, without the use of any amplification strategy for target enrichment or detection (by PCR or SPR signal enhancements by helping agents such as NPs or proteins) during the analytical step [11]. The reliability of the strategy was first tested on a set of genomically sequenced samples, carrying the three different genotypes associated to C3435T and used as controls. Afterward, blind analyses were performed on a second set of samples and the results obtained on the biosensor were finally confirmed by sequencing. C3435T polymorphism was selected as a case study for its relevance in theranostics and pharmacogenomics and for its correlation to susceptibility to many diseases such as breast and renal cancer [17].

## Experimental

### Detection and genotyping strategy

The assay design, a dual-targeting format (Fig. 1), involves a thiolated probe (hybridizing probe, HProbe, red sequence) immobilized on the chip to hybridize the fragmented DNA on a specific portion of the multidrug resistance (MDR1) gene and on three discriminating probes (single nucleotide polymorphisms C/T/A, SnpProbesC/T/A, dark gray sequence) to genotype the C3435T polymorphic site (yellow cross along the genomic DNA fragment, blue sequence) through their full matching or one-base mismatching discrimination of the specific base present in the tested allele genotype [16]. A thiolated negative control probe (NProbe, green sequence) was designed on the enhanced green fluorescence protein (EGFP) gene, not present in the human MDR1 gene, and was used to monitor the selectivity of the hybridization of HProbe toward the human genomic target.

### Reagents and buffers

Synthetic oligonucleotides involved in the assay (HProbe and SnpProbesC/T/A) were designed and selected as previously reported [16] by OligoWiz 2.0 with in silico evaluation [18] to maximize the selectivity of the affinity interactions. The NProbe was not selected by OligoWiz 2.0 but chosen as a control sequence from the EGFP gene. All sequences were purchased from Eurofins MWG Operon (Ebersberg, Germany) and reported together with their melting temperatures ($T_m$) at 250 nM in Electronic Supplementary Material Table A1. The 10-μM thiolated HProbe and NProbe were solubilized in 1 M $KH_2PO_4$ aqueous solution, pH 3.8, and then immobilized onto a gold chip surface as previously reported [15]. Phosphate buffered saline (PBS) buffer (300 mM NaCl, 20 mM $Na_2HPO_4$, 0.1 mM EDTA, pH 7.4) was used as a binding buffer in SPRi measurements and to dilute SnpProbesC/T/A and the human genomic DNA before testing them. Salts were purchased from Sigma-Aldrich (Milan, Italy) and dissolved in Milli-Q water (Millipore Corporation, MA, USA). Solutions were filtered using vacuum filter cups (Millipore Express Plus, 0.22 μm) and syringe filters (Puradisc cellulose acetate, 0.2 μm, from Whatman GmbH, Dassel, Germany) before use.

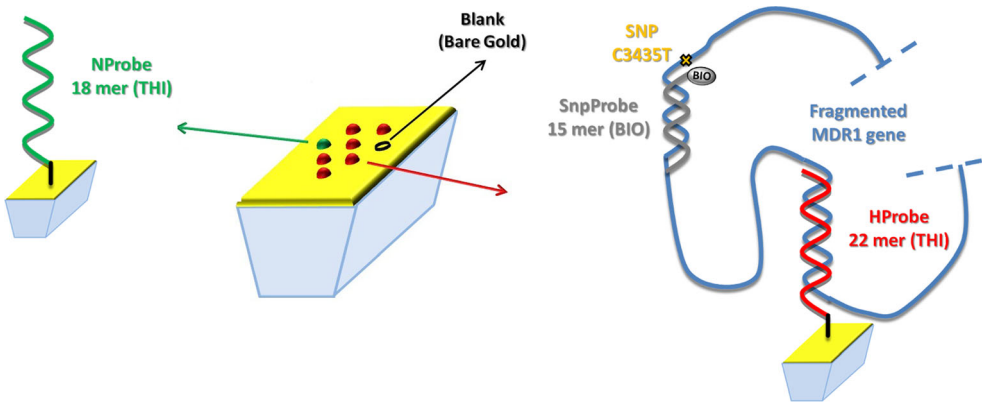


**Fig. 1** Probe deposition scheme and detection strategy. The fragmented MDR1 gene fragment (*blue*) first hybridized the HProbe (*red*), and then, it is genotyped using SnpProbesC/T/A (*dark gray*) in the polymorphic site C3435T. The NProbe (*green*) is exploited as a control probe to assess the selectivity of hybridizations

### Human genomic extraction and amplification for sequencing using molecular methods

Genomic DNA samples were extracted from 20 μL of the whole blood human lymphocytes using DNeasy 96 Blood & Tissue Kit from Qiagen, amplified by PCR (Mastercycler 5333, Eppendorf) on a 511-mer sequence (position 87138390–87138900 of chromosome 7, position 3673–4183 of coding sequence of the gene) flanking the C3435T SNP (position 87138645 of chromosome 7, position 3928 of coding sequence of gene) using an ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems by Life Technologies, Thermo Fisher Scientific) for the SNP identification. After SNP genotyping, three unamplified samples displaying three different polymorphisms were selected for SPRi assays: two homozygote samples (CC and TT) and one heterozygote sample (CT) in the polymorphic site (DNA sequencing interferograms are reported in Electronic Supplementary Material (ESM) Fig. S1). For the sake of clarity, we specify that the HProbe and SnpProbes were designed to specifically hybridize the complementary allele strands (with respect to those genotyped), therefore carrying the following genotypes: GG and AA for the homozygote samples and GA for the heterozygote samples. Samples (20 μL each, ~0.14 pM) were quantified using a GeneQuant RNA/DNA Calculator (Amersham Pharmacia Biotech Inc., UK). In order to have enough volume/sample to perform at least six replicates (100 μL for each injection/sample) and on the base of the sensitivity of the system [15], all the samples were diluted to 2.8 fM (~50-fold, 1000 μL final volume) before pretreatment and analysis.

### Pretreatment of genomic DNA samples

Before SPRi analysis, the whole human DNA was fragmented by a fast ultrasonic treatment (3 min with VC100, 100-W model; Sonics, Newtown, CT, USA) under optimized conditions (i.e., ultrasonic bath for 3 min with an amplitude of 60 % and pulses of 90 % with a frequency of 0.33 Hz) and was then denatured by a thermal treatment (95 °C for 10 min in C1000 Thermal Cycler; Bio-Rad, Italy) [15].

### Surface plasmon resonance imaging measurements

SPRi measurements were performed on SPRi-Lab$^{+}$ and SPRi-Biochips™ glass prisms coated with gold from Horiba Scientific (France). The detailed working conditions were reported in Electronic Supplementary Material ("Experimental" details).

## Results and discussions

### Sensitivity and selectivity of the biosensor

The DNA sensor was preliminary tested with the synthetic HTarget to confirm the homogeneity of the primary receptor immobilized on the surface and the reproducibility of measurements (ESM Fig. S3). Once the successful fabrication of the biochip is assessed, the biosensor was tested for its sensitivity and selectivity toward the GG homozygote genomic DNA since it represents the *wild-type* allele. Aliquots (100 μL, 2.8 fM; see "Experimental") of the whole DNA extracted from lymphocytes and diluted in a PBS buffer were pretreated by ultrasonication/denaturation and tested on the biosensor between 2.8 and 0 fM (ESM Fig. S4a). A marked dose–response effect was achieved with a linear correlation $R^2$=0.992 and an experimental sub-femtomolar detection limit, i.e., 0.18 fM (0.015±0.004 %RV, percent of reflectivity variation) (ESM Fig. S4b). The calculated detection limit defined from the ratio between $S$=0.015 %RV ($S$=3 N, where $N$=0.005 %RV) and slope of calibration curve (0.065 %RV/fM) was 0.23 fM. The percent of relative standard deviation % (%RSD) averaged on all calibration points of the genomic DNA samples was 14 % on the HProbe. The result pointed out the good repeatability of the measurements recorded on the HProbe (%RSD<15 %), despite the high complexity and the intrinsic variability (due to the random fragmentation) of the tested samples. On the NProbe, the nonhuman DNA sequence belonging to the EGFP gene, an unspecific hybridization of the genomic DNA, was recorded with %RSD=82 %. The high variability of the binding, compared to the lower one recorded on the HProbe, suggested the random nature of the binding. This finding was rigorously verified by injecting the secondary probe SnpProbeC, fully complementary only to the MDR1 gene (carrying the GG polymorphic site on the captured DNA fragments), on the preformed hybrid HProbe/genomic DNA at 2.8 fM. As expected, the SnpProbeC gave a SPR signal only on the HProbe spots and not on the NProbe (ESM Fig. S4d), confirming that only the HProbe spots were able to specifically hybridize genomic fragments of the MDR1 gene, while the NProbe bound sequences of the human genome in a randomly manner. No unspecific interactions were recorded on the bare gold surface (blank spot, B in ESM Fig. S4c).

As previously reported [15], the ultrasonic fragmentation resulted to be undoubtedly strategic also for the detection of the unamplified human genomic DNA at a femtomolar level. The generation of long fragments (~10,000/20,000 mers) of the genomic DNA carrying the sequence of interest provided a detectable reflectivity variation (%) also at very low molar concentrations (2.8 fM), much lower than that obtained for the 22-mer synthetic target; this is because although the hybridization of long genomic fragments to HProbe was probably

characterized by a low efficiency (due to steric hindrance at the surface and, in turn, a lower number of molecules caught by the receptor), the overall %RV resulted to be greater thanks to the huge molecular mass bound to the receptor.

## Discrimination of single nucleotide polymorphisms

After assessing the selectivity of the molecular recognition between the HProbe and MDR1 gene fragments, the direct polymorphism discrimination was tested on the three unamplified genomes previously genotyped for the polymorphic site of interest: GG sample (homozygote), AA sample (homozygote), and GA sample (heterozygote) (ESM Fig. S1). The samples were diluted in a PBS buffer to obtain a final concentration of 2.8 fM (~50-fold with respect to the starting concentration obtained after extraction from lymphocytes), pretreated as described above, and allowed to interact with the HProbe. Subsequently, the allele discrimination of each genomic sample was achieved by subsequent injections of 250 nM of the discriminating probes (SnpProbeC, SnpProbeT, and SnpProbeA), being full matching to the MDR1 gene fragment for the first 14 bases except for the 15th position, i.e., the SNP locus, which carried C, T, or A, respectively (ESM Table S1), to achieve the genotyping. In particular, the 15th base showed to be matching or mismatching (depending on the assayed genotype) to the polymorphic site (G/A) of the MDR1 fragment hybridized to the sensor through the HProbe. Illustrative sensorgrams of binding profiles of positive (binding of SnpProbe, match) or negative response (no binding of SnpProbe, mismatch) are reported in ESM Fig. S5. Therefore, for example, the SnpProbeC carrying a cytosine residue in the 15th position would be fully matched both to GG (both alleles) and GA (only G allele). The same for the SnpProbeT, this hybridized to AA (both alleles) and GA (only A allele). The SnpProbeA was tested as a negative reference since none of the tested samples, once bounded to the HProbe, carries a thymine residue in correspondence to the 15th base of SnpProbeA. As reported in Fig. 2, the three different polymorphisms GG/AA/GA were successfully genotyped by sequential injections of the secondary probes on the primary hybrids preformed on the biochip. The SPRi signals reported are relative only to the secondary recognitions, i.e., SnpProbeC/T/A sequences on the GG/AA/GA samples previously bound at 2.8 fM on the HProbe (the %RVs relative to the binding of the genomic DNA to the HProbe were previously subtracted). Green bars are relative to spots carrying the NProbe. The results shown in Fig. 2 are also summarized and schematized in ESM Table S2.

The %RV signals recorded after the hybridization with SnpProbes (C and T), together with the nonbinding SnpProbeA, used as a negative reference, proved that the three polymorphisms could be reliably differentiated by simple crossed injections of SnpProbes on the pre-captured genomic

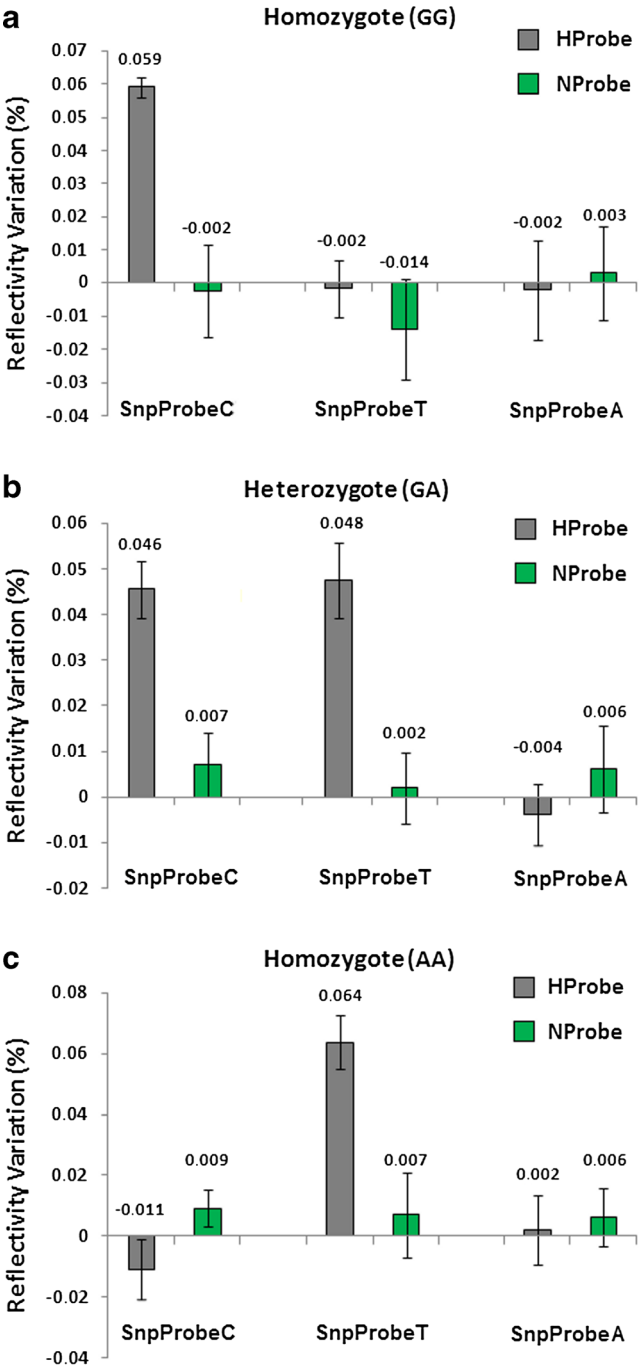


**Fig. 2** SNP discrimination. SPRi signals relative to (**a**) homozygote GG, (**b**) heterozygote GA, and (**c**) homozygote AA binding different SnpProbes (C, T, A) (single nucleotide polymorphism probes C, T, and A) on the hybridizing probe (*HProbe*) and the negative control probe (*NProbe*)

DNA carrying the MDR1 gene. This was a significant achievement because it was obtained, for the first time, on the unamplified whole genomic DNA. Moreover, this result was achieved without the need of signal amplifiers (proteins and/or NPs). %RSD values of the secondary hybridizations, calculated on six replicates for each sample, were all below

17 %, and the hybridization between the HProbe and MDR1 gene fragments occurred in a specific way, since no significant signals of molecular recognition with SnpProbes were recorded on gene fragments randomly hybridized on the (NProbe), as clearly shown in Fig. 2 (green bars). To our knowledge, this was the first example of label-free assay for the femtomolar-sensitive and direct SNP discrimination in the unamplified genomic DNA. In addition, the biosensor could be simply and efficiently regenerated by 100 mM HCl (an ordinary chaotropic agent) thanks to the basic and reversible DNA/DNA affinity interaction setup (ESM Fig. S5). Efficacy and effects of the regeneration were evaluated after 40 measurement/regeneration cycles on the DNA samples. To this aim, the HTarget was used as a calibrator to estimate both the surface regeneration efficiency and its possible degradation as follows: the averaged SPRi signal (%RV) obtained on the HProbe by injecting 250 mM HTarget at the beginning of the experiment was taken as 100 % of the recordable signal and finally compared with the same one obtained after 40 measurement/regeneration cycles of SNP detection. The hybridization signal recorded at the 40th measuring cycle was 1.872±0.144 %RV, a result that is not significantly different from the same one recorded at the first measurement cycle of the experiment, i.e., 2.010± 0.116 %RV (Student's $t$ test, six replicates, significance level of $p$=5 %) (see also ESM Fig. S6). This strongly proved that the regeneration was not only efficient, allowing tens of sequential measurements on the same biochip, but also not destructive for the sensing surface, since regenerations successfully occurred without a significant loss of signal, with dramatic lowering of time and cost analysis.

### Blind allele recognition of samples

Once the accuracy of the SNP detection on the samples previously genotyped is demonstrated, we further tested the blind samples, as a proof of concept of the real applicability of the biosensor for clinical purposes. The allele recognition was therefore accomplished on the blind genomically fragmented samples that were assayed using the developed method. The results obtained on the samples clearly revealed two different genotypes: the GG homozygote (wild type) and the GA heterozygote, as reported in ESM Fig. S7.

The results obtained on the biosensor were finally verified and confirmed using traditional molecular methods, i.e., PCR amplification of the MDR1 region carrying the SNP and subsequent sequencing of amplicons (DNA sequencing interferograms, ESM Fig. S8).

SPRi data inferred from the blind analysis were finally compared to those obtained on the genotyped samples used for the assay optimization, displaying a good inter-assay reproducibility not only in terms of the polymorphism discrimination but also in terms of the reproducibility of the SPRi signals (ESM Fig. S9).

## Conclusions

A DNA optical biosensor, based on SPR imaging transduction, is here presented for the direct discrimination of three genetic variants, GG (wild type)/AA/GA, of the C3435T SNP directly in the unamplified human genomic DNA extracted from the 20-μL whole blood by mini-invasive sampling and is here assayed at 2.8 fM after a dilution of ~50-fold.

The allelic discrimination was based on a dual-targeting assay based on the DNA probes tuned in previous researches and here tested in the unamplified genomic samples on the C3435T SNP. The assay is basic in the geometry but optimized by the rational choice of highly selective hybridizing and discriminating probes. The pre-analytical step was just a mild and tuned ultrasonic fragmentation followed by thermal denaturation. The combination of these steps is the key feature of the successful and selective detection of multidrug resistance (MDR1) gene fragments at a femtomolar level. The reported approach and results obtained on the C3435T SNP pointed out a significant improvement to the state-of-the-art SNP detection in the unamplified samples by SPRi, since here the direct sensitive detection is achieved without the employment of signal enhancers, with positive impact on the biosensor regeneration, speed of responses, and reduction of cost analysis, all required features for daily routine clinical monitoring. Indeed, the strategy lowered and amortized the cost analysis down to 10$/sample (materials, extraction kit, and gold chips excluding operators' costs), allowing to complete the entire analytical process, from sample extraction to instrumental outputs, in less than 2 h.

The assay could be easily transferred to the study of any other SNPs of clinical interest, opening new horizons to multi-SNP genotyping in parallel analysis through the employment of the multi-array format and emphasizing the suitability of SPRi for clinical and medical applications [19].

**Acknowledgments** The authors acknowledge Fondazione ARPA, Progetto Dolore, for the financial support.

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