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Detection of *TP53* mutation using a portable Surface Plasmon Resonance DNA-based Biosensor

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Abstract

A DNA-based Surface Plasmon Resonance (SPR) biosensor has been developed for the detection of *TP53* mutation using the inexpensive and commercially available instrument, SPREETA^{TM} SPR-EVM-BT, from Texas Instruments. A direct immobilization procedure, based on the coupling of thiol-derivatised oligonucleotide probes (Probe-C6-SH) to bare gold sensor surfaces, was optimized using synthetic oligonucleotides. Hybridization reactions between the immobilized probe and a short sequence (26 mer) complementary, non-complementary and one point mutation DNA were then investigated. The main analytical parameters of the sensor system were studied in detail including selectivity, sensitivity, reproducibility and analysis time. Finally, the sensor system was successfully applied to polymerase chain reaction (PCR) amplified real samples, DNA extracted from both normal, wild type, (Jurkat) and mutated (Molt 4), carrying the mutation at codon 248 of the *TP53* cell lines. The results

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obtained demonstrate that the DNA-based SPR biosensor was able to distinguish sequences present in the various samples that differ only by one base and hence it appears to be a strong candidate technique for the detection of gene mutation.

Keywords: SPR, DNA biosensor, *TP53*, mutation, Spreeta™

1. Introduction

It has been estimated that 60% of all humans have been affected by gene mutation in their lifetime. This rather dramatically demonstrates the importance of increasing demand for new high-throughput methods for mutation detection.

A variety of methods are currently used to assess the mutation status of individual tumours (Nollau *et al*., 1997). TP53 gene is mutated in most types of human cancers and is one of the most studied genes in cancer research. Moreover, many studies have suggested that TP53 mutations have prognostic importance and sometimes are determinant in the response of tumours to therapy. The role of TP53 as an important early diagnostic marker and its mutation spectrum have emerged and traditional methods for detecting point mutations as well as new approaches based on biosensors and DNA chips for detecting and recognizing mutations of TP53 have been developed. This has been reviewed very recently by Jiang *et al*. (2004).

Among traditional methods for molecular diagnosis and also for TP53, distinction is made between point mutation scanning and screening technologies. Scanning technologies aim at finding unknown mutations in candidate or known disease genes, such as direct DNA sequencing. Screening techniques aim at finding known mutations, preferably with high throughput (Jiang *et al*., 2004). For example, denaturing high-performance liquid

chromatography (DHPLC) (Narayanaswami *et al*., 2002; Gross *et al*., 2001), Single-strand conformation polymorphism (SSCP) (Miyajima *et al*., 2001; Ru *et al*., 2000), and denaturing gradient gel electrophoresis (DGGE) (Sheffield *et al*., 1989; Van Orsouw *et al*., 1998). However, some of these approaches are time consuming and require highly skilled labor, while some are less sensitive or use expensive equipment.

In recent years, a new trend for the detection of TP53 mutations has aimed at label free, high sensitivity and specificity, real-time and rapid detection. Biosensors, in particular DNA-based sensors, and gene chips are of considerable recent interest due to their tremendous promise for obtaining sequence-specific information in a faster, simpler and cheaper manner compared to traditional hybridization assays. Different transduction principles have been employed for TP53 DNA detection including electrochemical, piezoelectric and optical (SPR) techniques. All these systems are based on the hybridization reaction between a probe immobilized on the transducer surface, which may be either an electrode, in particular a carbon paste (Palacek *et al*., 1998) or a gold electrode (Wang *et al*., 1997; Miyahara *et al*., 2002), a quartz crystal coated with gold (Wang, *et al*., 1997; Wittung-Shafshede *et al*., 2000) or a sensor chip [Nilsson *et al*., 1997; Nilsson *et al*., 1999) made of glass with evaporated gold on one side in the case of SPR sensing. Most of the papers relating to TP53 detection in the literature use only standard solutions containing synthetic oligonucleotides, respectively complementary and containing a mutation (mismatch: point mutation in one base). Only Miyahara *et al*. and Nilsson *et al*. deal with real samples consisting of PCR amplified DNA from blood or tissues, in particular, from microdissected tumor biopsies, containing the target sequence able to hybridize with the immobilized probe.

In the current work, we report a method for detecting *TP53* mutations using a new portable Surface Plasmon Resonance-based Biosensor. In particular, we employed the inexpensive, portable and commercially available

instrument, SPREETA™ SPR. The system is based on the hybridisation reaction between the immobilised probe and its complementary or mismatched sequence in solution. The probe immobilisation is based on the coupling of thiol-derivatised oligonucleotide probes (Probe-C6-SH) to bare gold sensor surfaces. This procedure has been successfully employed with SPR DNA-based sensing in previous work by our group using both the Spreeta™ and Biacore X™ instruments (Wang *et al*., 2004a; Wang *et al.,* 2004b). The system was optimized using synthetic oligonucleotides and the main analytical parameters of the sensor (selectivity, sensitivity, reproducibility, analysis time etc) were studied in detail. The system was applied to complementary and a mismatch sequences (C→A) corresponding to codon 248 of the *TP53* gene. DNA extracted from "normal" wild type cell line (Jurkat) containing the fully complementary sequence and DNA extracted from cell line (Molt 4) carrying that mutation was also tested prior amplification by PCR.

2. Experimental

2.1. Apparatus and reagents

For all the experiments the SPR device Spreeta™ (Texas Instruments, Inc., United States of America) and a bare gold Spreeta™ sensor were used. All experiments were conducted at a flow rate of 5µl/min and 25°C.

6-mercapto-1-hexanol (MCH) was purchased from Sigma Aldrich (Milan, Italy). Other reagents for the buffers were purchased from Merck (Darmstadt, Germany). The composition of the buffers used for the experiments is as follows:

Immobilisation solution: KH_2PO_4 1M, pH 3.8.

Hybridisation buffer: NaCl 150 mM, Na_2HPO_4 20 mM, EDTA 0.1 mM, Tween 20 0.005%, pH 7.4.

Oligonucleotides were purchased from Sigma-Genosys (Cambridge, UK). The base sequences of the 5'-functionalised probes (26-mer), and 26-mer synthetic target oligonucleotides, are described below:

Thiolated Probe (*TP53*): 5' HS-$(CH_2)_6$- TGG GCG GCA TGA ACC GGA GGC CCA TC 3'

Target (*TP53*): 5'-GAT GGG CCT CCG GTT CAT GCC GCC CA-3' (complementary to probe *TP53*);

The relative one base mismatch oligonucleotides (at the codon of interest (248), underlined in probe sequence) and a non-complementary strand (negative control) were also used.

2.2 Samples

DNA extraction from the mutated (Molt 4) and normal (Jurkat) cell lines was performed. Molt 4 is a cell line derived from a patient (human male 19 years old) with acute lymphoblastic leukemia in relapse, and carrying the mutation of interest at codon 248 of the *TP53*. The concentration of the extracted DNA material was determined with fluorescence assay by using Picogreen ® dye (TD-700 Fluorometor, Turner Biosystems from Analytical Control, Milan, Italy).

A 236bp DNA fragment containing the target sequence *TP53* was amplified by using the functionalised sense (5'-CTTGCCACAGGTCTCCCCAA-3') and antisense (5'-AGGGGTCAGCGGCAAGCAGA-3') primers (MWG-BIOTECH, Florence, Italy). The PCR conditions were as follows: 94°C for 4min, 56°C for 1min and 72°C for 2 min, 40 cycles. All PCR experiments were conducted using a Perkin Elmer Thermal cycler (model 9600) (Perkin Elmer, Shelton, USA).

The DNA concentration of the amplified products was also determined with fluorescence assay by using Picogreen ® dye (TD-700 Fluorometor, Turner Biosystems from Analytical Control, Milan, Italy). Screening of the PCR

products was performed by gel electrophoresis and visualized through a U.V. transilluminator. The control solution (blank) contained all the PCR reagents except the DNA template.

2.3 PCR samples treatment

Prior to SPR testing, the amplicons obtained from PCR need to be pre-treated to obtain single-stranded DNA for hybridization with the immobilized probe. We first referred to the thermal denaturation method, which is well documented in many previous studies (Miyahara *et al*., 200; Wang *et al*., 1997; Wittung-Shafshede *et al*., 2000; Nilsson *et al*., 1997; 1999). It is based on a 5 minute incubation step at 95°C followed by one minute on ice.

Moreover, a new denaturation approach (High temperature denaturation with primers method) was used. This method was optimized (Wang *et al*., 2004b) and found to be a simple and useful way to obtain single-stranded DNA for hybridization coupled with a symmetric PCR amplification system. The principle of this method relies on the use of small (20 bases) oligonucleotides (in this case corresponding to PCR reaction primers) added to the denaturating mixture. These oligonucleotides are complementary to some sequences on the strand which hybridise the immobilized probe, but are positioned laterally and do not overlap with the portion forming the complex with the probe. By the interaction between the thermally separated DNA strands and these oligonucleotides, re-association between dsDNA strands of PCR amplicons is prevented and surface hybridization can occur (Figure 1). The effect of the oligonucleotide binding is to prevent re-annealing of the denatured DNA strands before they come into contact with the sensor surface. The whole denaturation procedure was combined with sense and antisense primers. It comprised 5 minutes incubation at 95°C and then 1 minute incubation at a certain temperature determined as suitable for primer linking in the PCR procedure. These oligonucleotides were chosen on the basis of the particular sequence of the PCR fragment to be analyzed. In this case the same oligonucleotide used as

primers in the PCR step were employed also in the denaturating step.

2.4 Immobilization method

A direct immobilization method based on the formation of gold-thiolated DNA probes was used. 1). The gold sensor surface was first cleaned with ethanol, and then further cleaned with a solution of 30% NH_3/30%H_2O_2/MilliQ H_2O in ratio 1/1/5 for 10 min, then surface was rinsed with MilliQ water and allowed to dry. 2). The Spreeta sensor was inserted into the instrument socket. The thiolated probe (1μM, in KH_2PO_4 pH 3.8) solution was then allowed to flow in the system for 2 hours, by connecting the inlet of the cell to a peristaltic pump (Gilson), then the probe was replaced by MCH (6- mercapto-1-hexanol) solution which was left in contact with the surface for 1 hour (in the dark). 3). Once the immobilisation procedure was ended, the surface was finally washed by running the hybridization buffer for 4 hours.

2.5 Hybridization reaction

The DNA hybridisation measurement cycle comprised injection of the target sequence, which was left in contact for nine minutes to allow hybridisation, washing of the surface for three minutes with hybridization buffer to remove the unbound DNA material and regeneration with 2.5 mM HCl for at least for two minutes to return to the baseline (flow rate is 5μl/min, 25°C). The analytical signal, reported as Refractive Index (RI), is represented by the value observed before (baseline) and after the hybridisation reaction, once the surface had been washed by the buffer. All the measurements were performed in the same solution (hybridisation buffer).

3. Results and discussion

Preliminary experiments were conducted with the 25-mer oligonucleotides to verify the ability of the sensor to

generating a specific and reproducible signal when in contact with the fully complementary sequence solution. Initially, the probe was immobilised on the sensor surface and the operating conditions optimised.

3.1. Sensor optimization

The TP53 thiolated probe was successfully immobilised on the sensor surface and this was in agreement with our previous work conducted with different thiolated probes [Wang *et al*., 2004a; 2004b). This immobilisation procedure decreases significantly the immobilisation time without loss of sensor performance [Wang *et al*., 2004]. The total immobilisation time, starting from a bare gold sensor surfaces was of 3 hours, which is much less than the 5 days required to modify the chip with thiol/carboxylated dextran based on streptavidin binding to biotinylated probes [Mariotti *et al*., 2002; Minunni *et al*., 2001; Giakoumaki *et al*., 2003).

The sensor system was first optimized with synthetic oligonucleotides and the performance of the SPR-based DNA biosensor resulting from direct coupling of thiol-derivatised DNA probes onto gold chip was studied in terms of the main analytical parameters, i.e specificity, sensitivity, reproducibility, stability, analysis time, etc.

The specificity of the system was first evaluated by injecting into the system 1 μM 26-mer solution of non-complementary sequence used as negative control. No detectable hybridization signal was obtained [signal < 0.1RI(E-05)], showing that the sensor was specific.

The reproducibility of the measurement was estimated using the same concentration (1μM) of *TP53* target for nine

different cycles of hybridization/regeneration on the same sensor in one day, the average value obtained was 12.1E-05 RI with SD=0.8E-05 and CV=7%.

To optimize the regeneration procedure, different concentrations of HCl and NaOH were tested. Among them, HCl showed higher regeneration capacity at the optimised concentration of 2.5mM. The single stranded probe was successfully regenerated by a two minute treatment with 2.5mM HCl and was then ready for a new hybridisation cycle (data not shown). Such treatment could be performed up to 50 times without affecting the hybridization efficiency of the immobilized probe. We checked the hybridization signal with the same concentration (1μM) of *TP53* complementary target oligonucleotide after every 10 hybridization/regeneration cycles and the results are listed in Table 1. A decrease in the hybridisation signal can be observed at around 60 measurements cycles.

The stability of the sensor system was also studied, by monitoring the baseline value after each measurement cycle. This was estimated first, by comparing the baseline signal over different hybridisation cycles. In particular, the difference between the maximum and the minimum baseline was found to be only value3.2E-05 RI, indicating good stability of the system.

The reproducibility of the measurements between different sensors (N=5) was estimated, comparing the hybridisation signals obtained with the same concentration (1 μM) of the complementary target oligonucleotide with the different surfaces on which the same probe had been immobilised. At least 3 replicates of this measurement for each surface (n≥3) were performed. The results are shown in Figure 2. The average hybridisation value obtained with 1μM complementary target oligonucleotide, calculated over 5 sensors, was 12.4E-5 RI, with SD=1E-5 RI and CV%=8, indicating a very good. reproducibility of the measurements on different surfaces. This

result confirms that the direct coupling of probes by self-assembly of terminally thio-labeled oligonucleotides onto gold surfaces was a suitable immobilization method for SPR-based sensors. Reproducibility of the immobilisation step was also good: the average signal over 5 different immobilisation events was 15,294E-5 with SD=0,01E-3 RI.

A calibration curve (0-2μM) of *TP53* complementary target was produced and the results are shown in Figure 3A. Each concentration tested represents at least 3 measurements (n≥3). A linear region up to 0.5μM was found (Figure 3B) and the experimental detection limit was 50nM. However, a calculated detection limit of 10 nM was obtained (calculated as the concentration which would generate a signal which was 3 times the standard deviation of the baseline signal). The difference between the calculated and the experimental detection limit could be due to the instrument or the whole experimental procedure.

Further experiments were performed to compare the hybridization responses with fully complementary oligonucleotides, mismatch oligonucleotides and a mixture solution containing 50% of both the oligonucleotides. The 100% fully complementary, 50% mismatchand-50% fully complementary and 100% mismatch, reflect the possible genomes that can be found in patients. The hybridisation time, in this series of experiments, was decreased down to 2 minutes (instead of 9 min), to perform all the 3 different calibration curves on the same, freshly prepared sensor, on the same day. Results are shown in Figure 4. The applied concentration range was 0-10μM, and samples were tested in triplicate (n=3). The lower hybridisation signals observed here with the fully complementary sample, compared to the values observed in Figure 2 are due to the much shorter hybridisation times used here (2 minutes vs 9). A decreased signal in the presence of the mismatch was always observed (concentration > 0.5μM). The decrease % is calculated comparing the signal observed with the 100% fully

complementary sample and the ones obtained with the 100% mismatch or 50% samples. A significant decrease is observed at concentrations higher than 0.5μM. An average decrease (calculated in the concentration range 2-10μM) of 21E-5RI with SD= 3E-3 for the 50% samples and of 29E-5 with SD=2E-5 for the 100% mismatch sample was found, respectively. In particular the highest decrease for the 50% sample was observed at 4μM (23%) and for the 100% mismatch at 2μM (32%). The data obtained showed that the hybridization response variations corresponding to the complementary oligonucleotides, the mismatch and the mixture solution were influenced by the concentrations used. The difference in the hybridization responses increased at higher concentrations. This result was important for identifying the concentration of DNA suitable for use in the assays with PCR amplified DNA.

3.2. Detection of hybridization with PCR-amplified samples

The system was finally applied to complementary and the mismatch sequences (C→A) of codon 248 of the *TP53* gene of living cells. DNA extracted from normal cell line (Jurkat) containing the fully complementary sequence and from cell line (Molt 4) carrying that mutation was first amplified by PCR and then tested on the sensor.

The amplicons obtained by PCR have a double helix structure and the two strands should be separated (denatured) to allow the hybridization with the probe immobilized on the sensor surface. The PCR-amplified samples were denaturated first with the thermal denaturation method, since this method was well documented in many previous studies [Wang *et al*., 2004a; 2004b; Mariotti *et al*., 2002; Minunni *et al.,* 2001; Giakoumaki *et al*., 2003). It involved a 5 minute incubation step at 95°C and then cooling on ice for 1 minute. However, SPR testing showed no detectable hybridization shift (data not shown). This was attributed to the re-annealing of the denatured DNA strands before coming in contact with the sensor surface, as also demonstrated by Mariotti and co-workers.

To overcome this problem, it was necessary to use a new treatment (described in paragraph 2.3) for the PCR samples before injection in the instrument. The denaturating conditions have been previously optimized (Wang *et al.,* 2004b) using symmetrically amplified PCR. The method is very simple and it is based on high temperature denaturation (95°C) followed by a 1 minute incubation step with oligonucleotides at a suitable temperature. The oligonucleotides (20 bases) chosen in this work were exactly the primers used in the corresponding PCR reaction to amplify the *TP53* fragment. The one-minute incubation was performed at 56°C, which is the suitable temperature to use for these specific primers linking in the relative PCR reaction. The first high temperature treatment (5 min) dissociates the PCR fragments (dsDNA) into the ssDNA complementary strands and then these oligonucleotides (primers) bind them leaving the target sequence free to hybridise the immobilised probe. The effect of the oligonucleotides binding is to prevent the re-annealing denatured DNA strands before coming in contact with the sensor surface.

The hybridization shifts obtained from the testing of PCR-amplified sample after applying this new denaturing treatment are shown in Figure 5. The sample was diluted in hybridization buffer up to a final concentration of 0.15 μM. PCR blanks, containing all the reagents of the PCR mixture except the template DNA and also containing the oligonucleotides for denaturation procedure, were tested to check any non-specific effect and negligible response (3.33E-6 RIU) was obtained. The normal wild type sample (Jurkat) resulted in a measurable shift of 7.57E-05 RIU with S.D=8.39E-06, and CV=11%. The sample carrying the mutation at codon 248 (Molt4) showed an average signal of about 5.00E-05 RIU with S.D=2.00E-06 and CV=4%. Comparing the signal observed with PCR amplified sample obtained with the fully complementary wild type sequence with the one observed with the mutated sample, a decrease of about 34% could be observed in the case of the second one (100% mismatch).

Negative control (GM maize PCR sample) was also tested using the same concentration (0.15 μM) and the same denaturation procedure. The signal from the negative control was very low (average value was 8.57E-06 RIU with S.D=7.02E-07 and CV=8%). These results clearly demonstrate the ability of the sensor to detect *TP53* gene point mutations. A great improvement was obtained by coupling SPR-based devices with the new denaturation method applied to PCR amplicons (Wang *et al*., 2004a; 2004b). After the interaction of the surfaces with the PCR-amplified samples, the regeneration with HCl 2.5 mM, which had been used for the experiments with the synthetic oligonucleotides, was not sufficient (data not shown). Higher concentrations (5, 10 and 25 mM) of HCl and NaOH were tested to find the optimal regeneration conditions; NaOH 25 mM (treatment for 1 minute) proved to be the most efficient. With this regeneration procedure, the sensor could be used up to 50 times, using both oligonucleotides and PCR real samples (data not shown).

3. Conclusions

We have successfully applied a portable Surface Plasmon Resonance (SPR) biosensor to point mutation detection. The system is based on hybridization detection between a DNA probe immobilised on the sensor surface and the target sequence in solution. The commercially available instrument SPREETA^{TM} SPR-EVM-BT was used for the analysis of PCR-amplified samples to detect *TP53* mutation.

Probe immobilization was achieved by direct coupling of thiolated probes on gold sensor surfaces. The SPR-based sensor developed showed high specificity, high sensitivity, good reproducibility and good stability.

It has been demonstrated that the biosensor was suitable for DNA hybridization detection both with synthetic

oligonucleotides and PCR-amplified real samples. The sensor system was able to distinguish between sequences differing only in one base both using standard solutions of synthetic oligonucleotides (26 bases) or real samples (236 bp).

A new, fast and improved denaturing procedure was employed for the analysis of real samples consisting of PCR-amplified DNA , which is based on the use of small oligonucleotides coupled to a high temperature thermal PCR sample treatment. This new sample treatment combined with the developed DNA-based SPR biosensor allowed the analysis of gene mutation in real samples and demonstarted the potential of the method for routine analysis.

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Table 1: Lifetime of sensor system.

Measurements Cycles	1	10	20	30	40	50	60	70
Hybridization Signals RI (E-5)	11.0	12.4	11.1	11.7	12.6	12.7	10.2	8.7

Hybridization signals when testing 1μM of complementary oligonucleotide.

Caption to Figures

Figure 1: Scheme of the denaturating procedure applied to PCR amplified samples.

To prevent the re-association of the complementary sequences in solution, small (20 bases) oligonucleotides are added to the denaturating mixture containing the PCR amplified material. These oligonucleotides partially hybridize the complementary strands but do not overlap with the target sequence, which remains free to bind the immobilized probe.

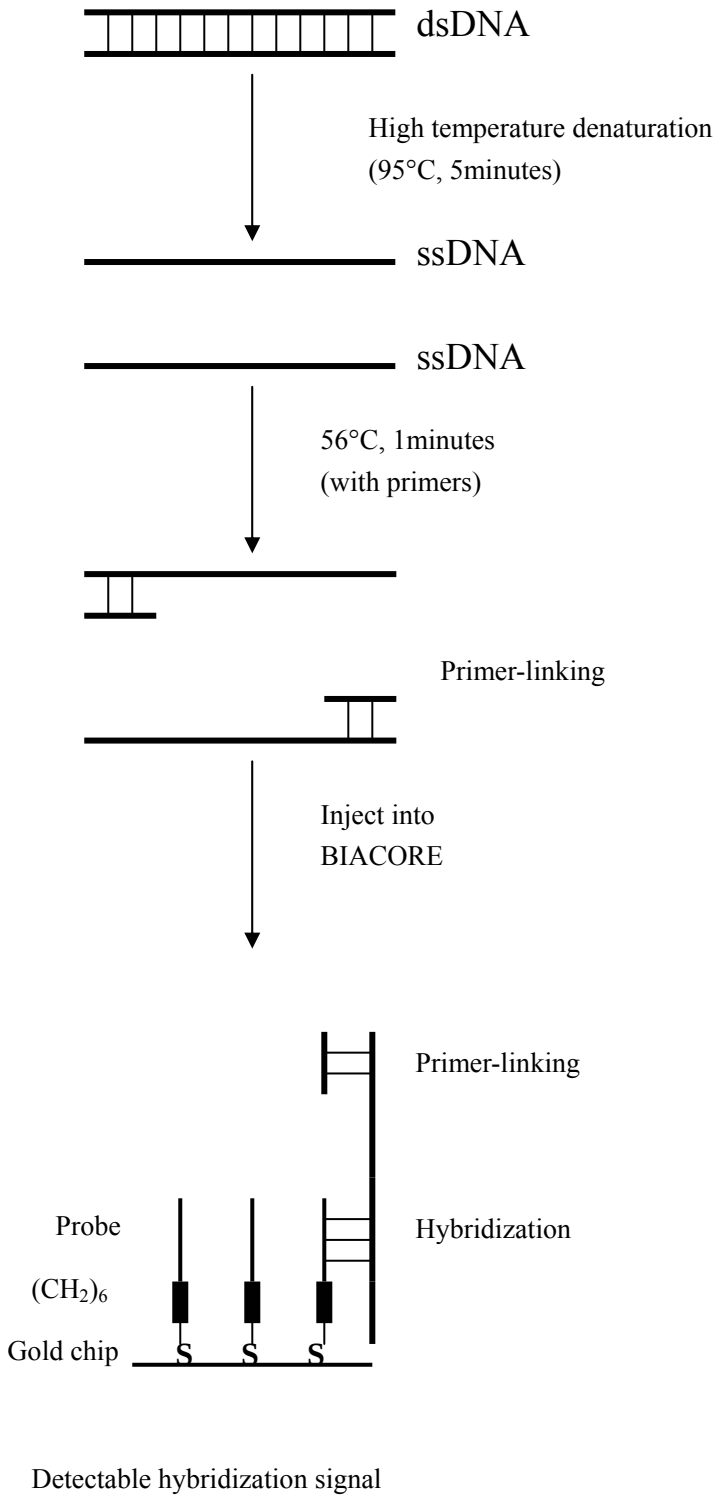
Figure 2: Reproducibility among 5 different sensors. The average value obtained with 1 μM complementary oligonucleotide target was 12.4E-5 RI, with SD=1 and CV%=8.

Figure 3: Calibration curve for *TP53* **(A)** and zoom of the linear region **(B)**. The probe (26 mer) complementary to the wild type TP53 is immobilised on the sensor surface. The fully complementary oligonucleotide (26 mer) is added in solution.

Figure 4: Calibration curves with fully complementary oligonucleotides, mismatch oligonucleotides and a mixture solution containing 50% of both the oligonucleotides.

Figure 5: Results with PCR-amplified samples. DNA extracted from cell cultures wild-type (Jurkat) and mutated for codon 248 (Molt 4)

Figure1



Detectable hybridization signal

Figure 2

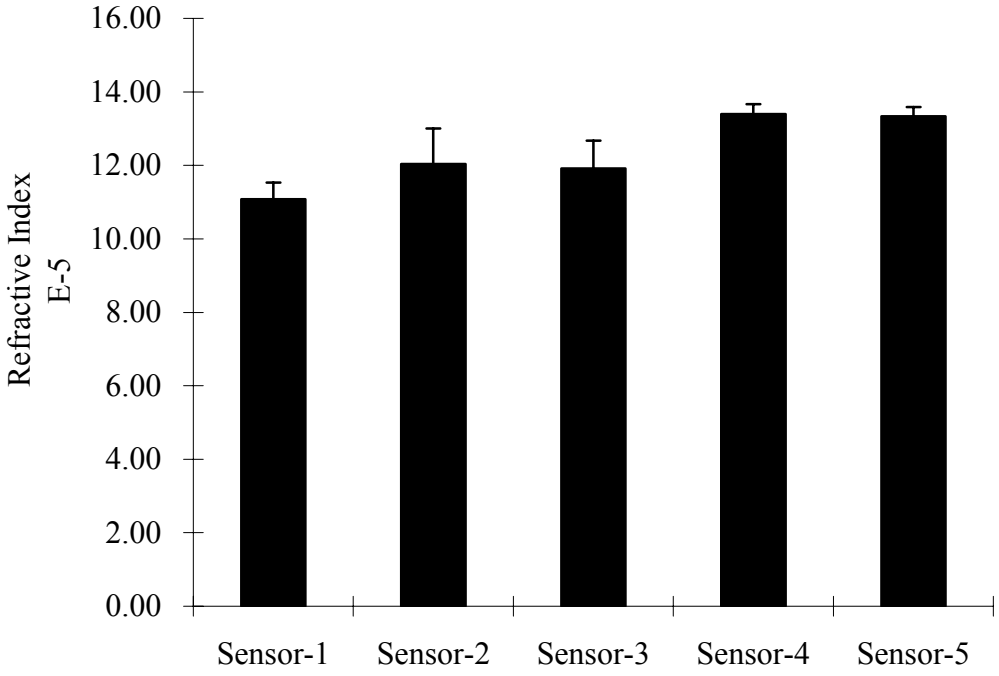
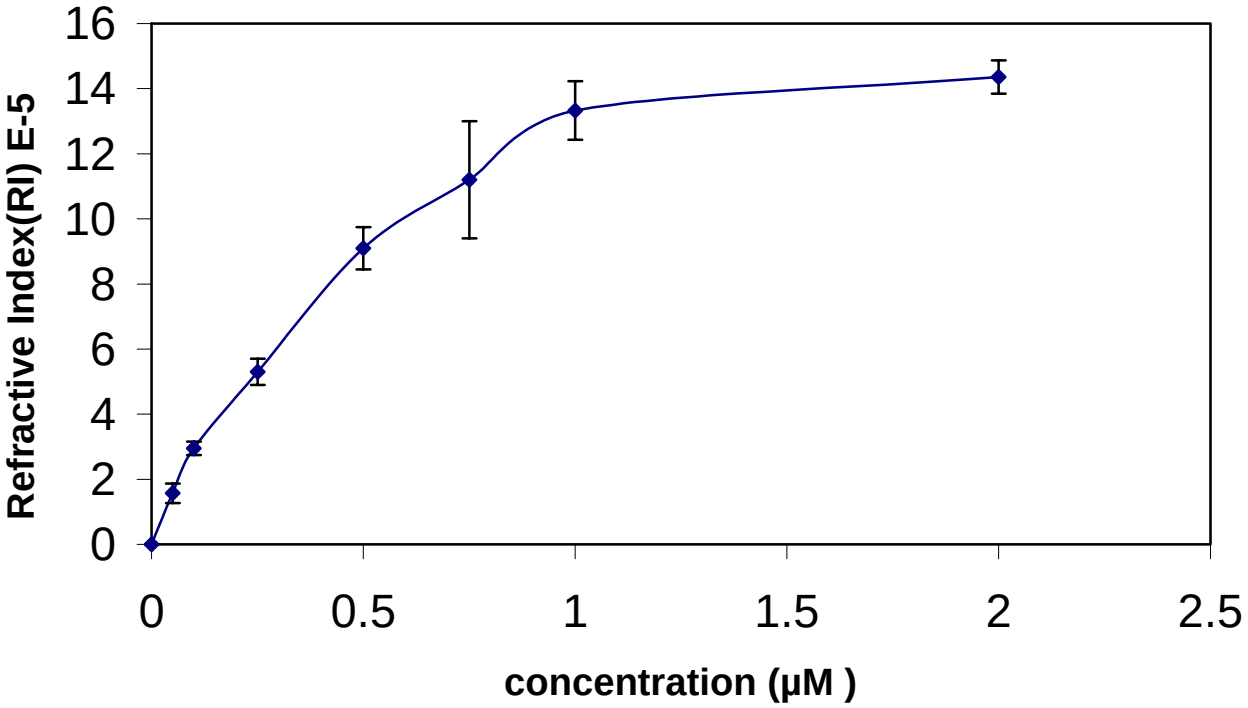

16.00
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Refractive Index
E-5
Sensor-1
Sensor-2
Sensor-3
Sensor-4
Sensor-5

Figure 3

A)


Refractive Index(RI) E-5
16
14
12
10
8
6
4
2
0
0
0.5
1
1.5
2
2.5
concentration (µM)

B)

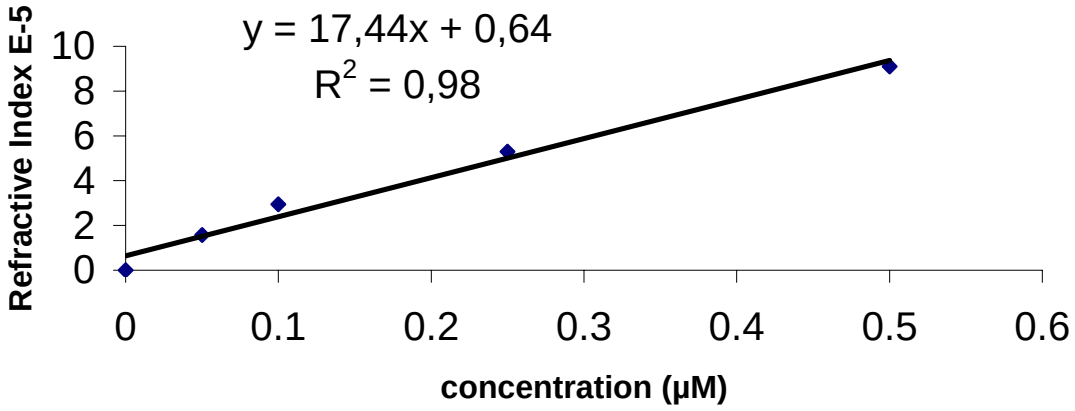

Refractive Index E-5
y = 17,44x + 0,64
R2 = 0,98
10
8
6
4
2
0
0
0.1
0.2
0.3
0.4
0.5
0.6
concentration (µM)

Figure 4

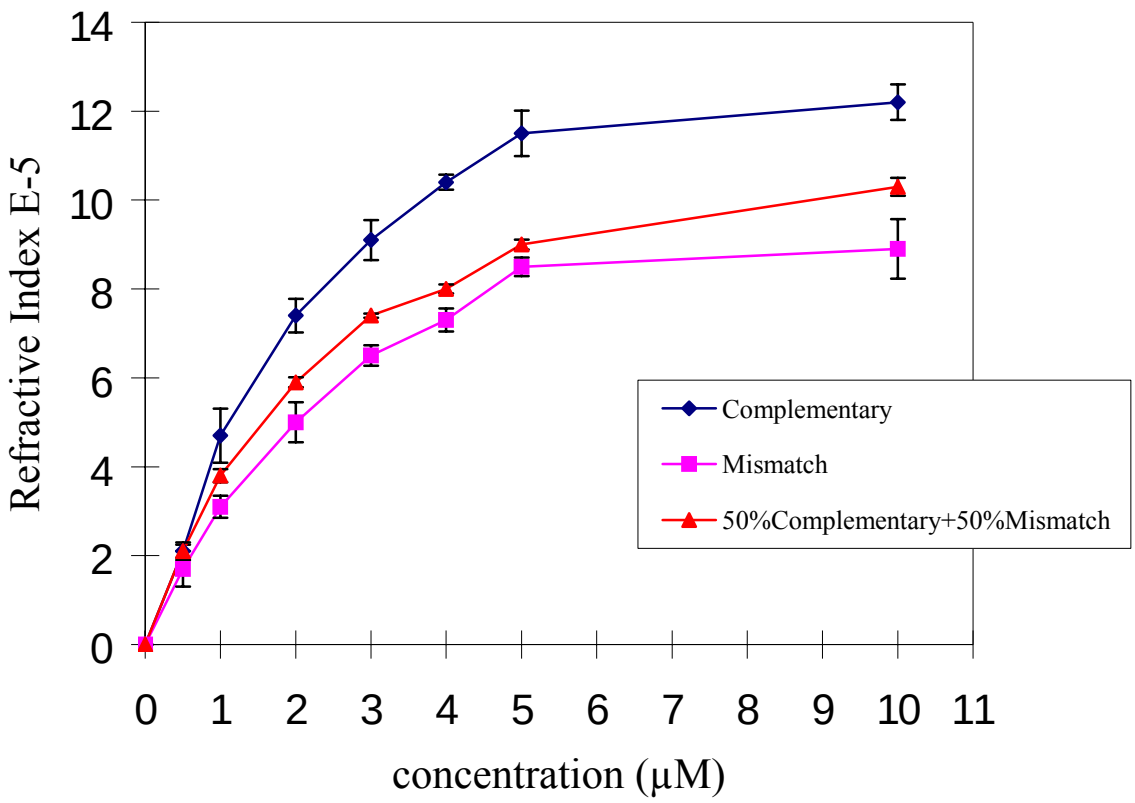
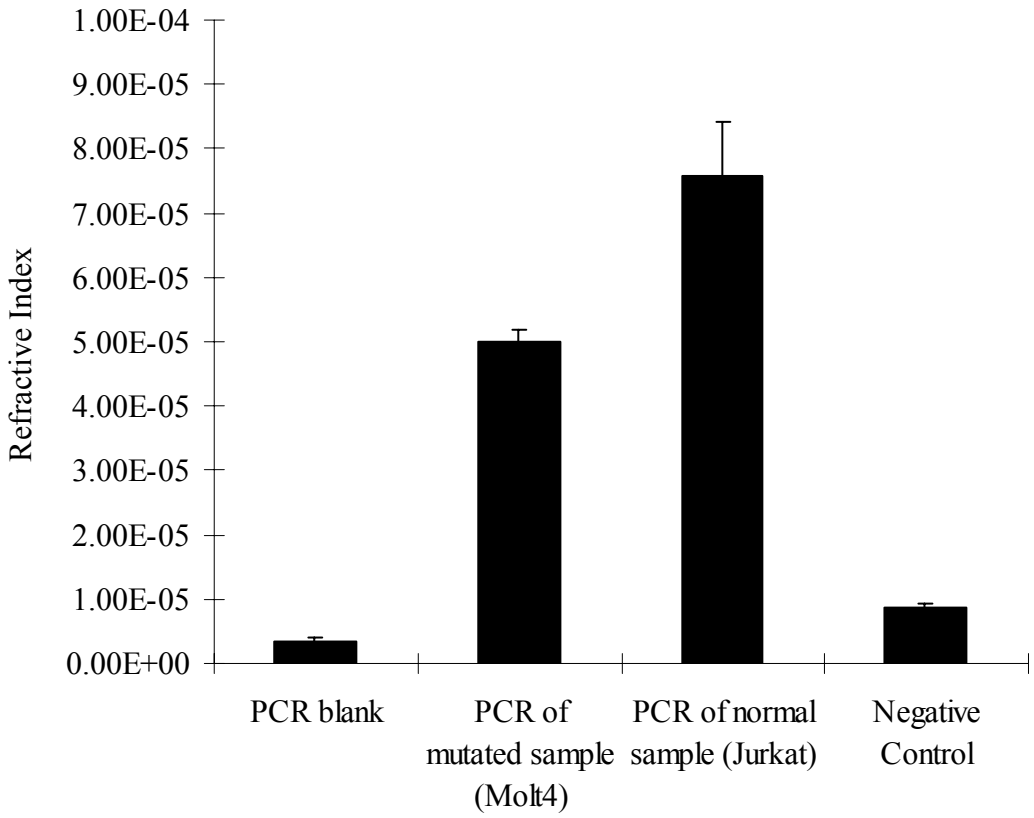

Refractive Index E-5
14
12
10
8
6
4
2
0
0 1 2 3 4 5 6 7 8 9 10 11
concentration (μM)
Complementary
Mismatch
50%Complementary+50%Mismatch

Figure 5


1.00E-04
9.00E-05
8.00E-05
7.00E-05
6.00E-05
5.00E-05
4.00E-05
3.00E-05
2.00E-05
1.00E-05
0.00E+00
Refractive Index
PCR blank
PCR of mutated sample (Molt4)
PCR of normal sample (Jurkat)
Negative Control