

## Detection of *TP53* mutation using a portable surface plasmon resonance DNA-based biosensor

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Received 17 June 2004; received in revised form 25 August 2004; accepted 26 August 2004

Available online 3 October 2004

### 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™ SPR-EVM-BT, from Texas Instruments. A direct immobilisation procedure, based on the coupling of thiol-derivatised oligonucleotide probes (Probe-C6-SH) to bare gold sensor surfaces, was optimized using synthetic oligonucleotides. Hybridisation reactions between the immobilised 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 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. © 2004 Elsevier B.V. All rights reserved.

**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 and Wagener, 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 muta-

tions 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 and Taylor, 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

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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 hybridisation 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 hybridisation reaction between a probe immobilised on the transducer surface, which may be either an electrode, in particular a carbon paste (Palacěk et al., 1998) or a gold electrode (Wang et al., 1997a; Miyahara et al., 2002), a quartz crystal coated with gold (Wang et al., 1997b; Wittung-Shafshede et al., 2000) or a sensor chip (Nilsson et al., 1997, 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 hybridise with the immobilised 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, 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 to amplification by PCR.

## 2. Experimental

### 2.1. Apparatus and reagents

For all the experiments the SPR device Spreeta™ (Texas Instruments Inc., USA) and a bare gold Spreeta™ sensor

were used. All experiments were conducted at a flow rate of 5 µl/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:  $\text{KH}_2\text{PO}_4$  1 M, pH 3.8;  
hybridisation buffer: NaCl 150 mM,  $\text{Na}_2\text{HPO}_4$  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<sub>2</sub>)<sub>6</sub>-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 Fluorometer, Turner Biosystems from Analytical Control, Milan, Italy).

A 236 bp DNA fragment containing the target sequence *TP53* was amplified by using the functionalised sense (5'-CTTGCCACAGGTCTCCCCAA-3') and antisense (5'-AGGGGTCTAGCGGCAAGCAGA-3') primers (MWG-BIOTECH, Florence, Italy). The PCR conditions were as follows: 94 °C for 4 min, 56 °C for 1 min 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 Fluorometer, Turner Biosystems from Analytical Control, Milan, Italy). Screening of the PCR products was performed by gel electrophoresis and visualized through a UV 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 hybridisation with the immobilised probe. We first referred to the thermal denaturation method, which is well documented in many previous studies (Miyahara et al., 2002; Wang et al.,

1997; Wittung-Shafshede et al., 2000; Nilsson et al., 1997, 1999). It is based on a 5 min incubation step at 95 °C followed by 1 min 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 hybridisation 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 denaturing mixture. These oligonucleotides are complementary to some sequences on the strand which hybridise the immobilised 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 hybridisation can occur (Fig. 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 min incubation at 95 °C and then 1 min 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 in the denaturing step also.

#### 2.4. Immobilisation method

A direct immobilisation 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%  $\text{NH}_3$ /30%  $\text{H}_2\text{O}_2$ /MilliQ  $\text{H}_2\text{O}$  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 (1  $\mu\text{M}$ , in  $\text{KH}_2\text{PO}_4$  pH 3.8) solution was then allowed to flow in the system for 2 h, 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 h (in the dark). (3) Once the immobilisation procedure ended, the surface was finally washed by running the hybridisation buffer for 4 h.

#### 2.5. Hybridisation reaction

The DNA hybridisation measurement cycle comprised injection of the target sequence, which was left in contact for 9 min to allow hybridisation; washing of the surface for 3 min with hybridisation buffer to remove the unbound DNA material; and regeneration with 2.5 mM HCl for at least for 2 min to return to the baseline (flow rate is 5  $\mu\text{l}/\text{min}$ , 25 °C). The analytical signal, reported as refractive index (RI), is repre-

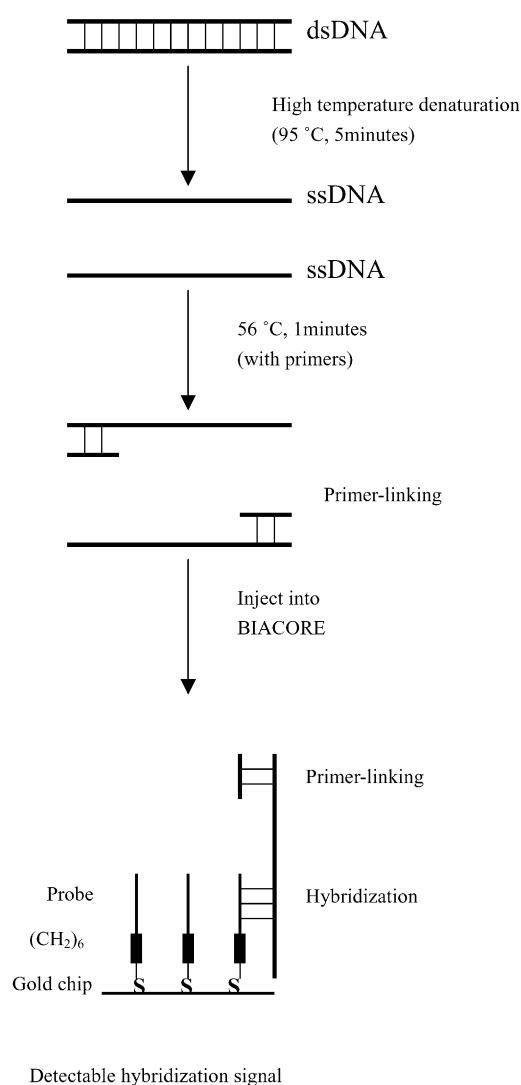


Fig. 1. Scheme of the denaturing procedure applied to PCR-amplified samples. To prevent the re-association of the complementary sequences in solution, small (20 bases) oligonucleotides are added to the denaturing mixture containing the PCR-amplified material. These oligonucleotides partially hybridise the complementary strands but do not overlap with the target sequence, which remains free to bind the immobilised probe.

sented 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 generate 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 optimized.

### 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., 2004a, 2004b). The total immobilisation time, starting from a bare gold sensor surfaces was of 3 h, 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  $\mu\text{M}$  26-mer solution of non-complementary sequence used as negative control. No detectable hybridisation signal was obtained [signal < 0.1 RI ( $\text{E}-5$ )], showing that the sensor was specific.

The reproducibility of the measurement was estimated using the same concentration (1  $\mu\text{M}$ ) of *TP53* target for nine different cycles of hybridisation/regeneration on the same sensor in 1 day, the average value obtained was 12.1 $\text{E}-5$  RI with S.D. = 0.8 $\text{E}-5$  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 optimized concentration of 2.5 mM. The single stranded probe was successfully regenerated by a 2 min treatment with 2.5 mM 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 hybridisation efficiency of the immobilised probe. We checked the hybridisation signal with the same concentration (1  $\mu\text{M}$ ) of *TP53* complementary target oligonucleotide after every 10 hybridisation/regeneration cycles and the results are listed in Table 1. A decrease in the hybridisation signal can be observed at around 60 measurements cycles.

Table 1  
Lifetime of sensor system

Measurement cycles	Hybridisation signals RI ( $\text{E}-5$ )
1	11.0
10	12.4
20	11.1
30	11.7
40	12.6
50	12.7
60	10.2
70	8.7

Hybridisation signals when testing 1  $\mu\text{M}$  of complementary oligonucleotide.

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 value 3.2 $\text{E}-5$  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  $\mu\text{M}$ ) of the complementary target oligonucleotide with the different surfaces on which the same probe had been immobilised. At least three replicates of this measurement for each surface ( $n \geq 3$ ) were performed. The results are shown in Fig. 2. The average hybridisation value obtained with 1  $\mu\text{M}$  complementary target oligonucleotide, calculated over five sensors, was 12.4 $\text{E}-5$  RI, with S.D. = 1 $\text{E}-5$  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 immobilisation method for SPR-based sensors. Reproducibility of the immobilisation step was also good: the average signal over five different immobilisation events was 15,294 $\text{E}-5$  with S.D. = 0.01 $\text{E}-3$  RI.

A calibration curve (0–2  $\mu\text{M}$ ) of *TP53* complementary target was produced and the results are shown in Fig. 3A. Each concentration tested represents at least three measurements ( $n \geq 3$ ). A linear region up to 0.5  $\mu\text{M}$  was found (Fig. 3B) and the experimental detection limit was 50 nM. However, a calculated detection limit of 10 nM was obtained (calculated as the concentration which would generate a signal which was three 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 hybridisation responses with fully complementary oligonucleotides, mismatch oligonucleotides and a mixture solution

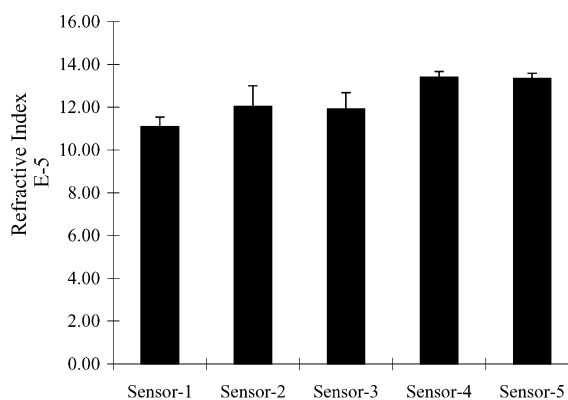


Fig. 2. Reproducibility among five different sensors. The average value obtained with 1  $\mu\text{M}$  complementary oligonucleotide target was 12.4 $\text{E}-5$  RI, with S.D. = 1 and CV% = 8.



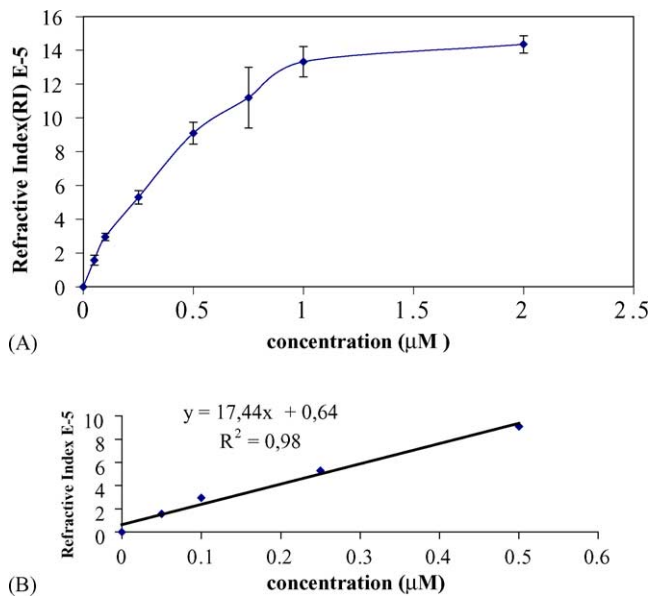


Fig. 3. Calibration curve for *TP53* (A) and zoom of the linear region (B). The probe (26 mer) complementary to the wild-type *TP53* is immobilised on the sensor surface. The fully complementary oligonucleotide (26 mer) is added in solution.

containing 50% of both the oligonucleotides. The 100% fully complementary, 50% mismatch and 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 min (instead of 9 min), to perform all the three different calibration curves on the same, freshly prepared sensor, on the same day. Results are shown in Fig. 4. The applied concentration range was 0–10  $\mu\text{M}$ , 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 Fig. 2 are due to the much shorter hybridisation times used here (2 min versus 9 min). A decreased signal in the presence of

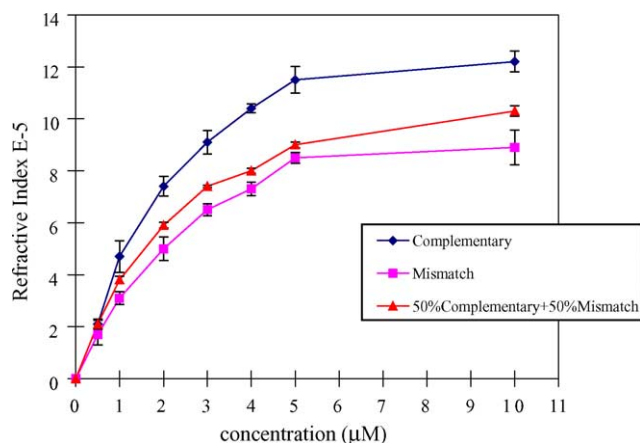


Fig. 4. Calibration curves with fully complementary oligonucleotides, mismatch oligonucleotides and a mixture solution containing 50% of both the oligonucleotides.

the mismatch was always observed (concentration  $> 0.5 \mu\text{M}$ ). The decrease percentage 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  $0.5 \mu\text{M}$ . An average decrease (calculated in the concentration range 2–10  $\mu\text{M}$ ) of  $21\text{E}-5$  RI with S.D. =  $3\text{E}-3$  for the 50% samples and of  $29\text{E}-5$  with S.D. =  $2\text{E}-5$  for the 100% mismatch sample was found, respectively. In particular the highest decrease for the 50% sample was observed at  $4 \mu\text{M}$  (23%) and for the 100% mismatch at  $2 \mu\text{M}$  (32%). The data obtained showed that the hybridisation response variations corresponding to the complementary oligonucleotides, the mismatch and the mixture solution were influenced by the concentrations used. The difference in the hybridisation 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 hybridisation with PCR-amplified samples

The system was finally applied to complementary and the mismatch sequences (C  $\rightarrow$  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 hybridisation with the probe immobilised on the sensor surface. The PCR-amplified samples were denatured 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 min incubation step at  $95^\circ\text{C}$  and then cooling on ice for 1 min. However, SPR testing showed no detectable hybridisation 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 Section 2.3) for the PCR samples before injection in the instrument. The denaturing 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 ( $95^\circ\text{C}$ ) followed by a 1 min 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  $56^\circ\text{C}$ , 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

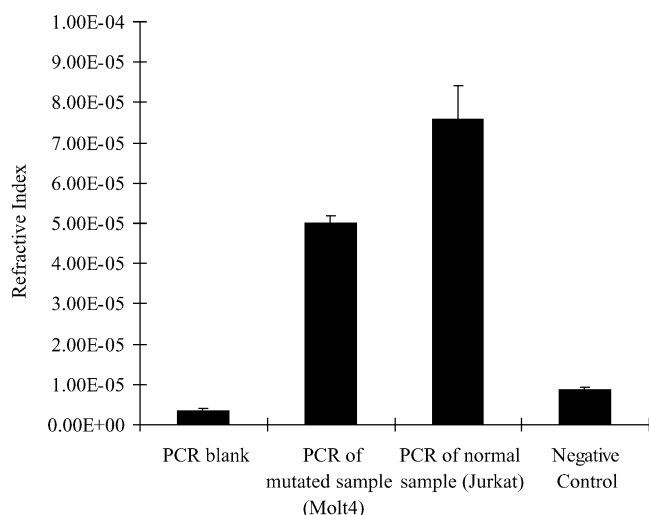


Fig. 5. Results with PCR-amplified samples. DNA extracted from cell cultures wild-type (Jurkat) and mutated for codon 248 (Molt 4).

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 hybridisation shifts obtained from the testing of PCR-amplified sample after applying this new denaturing treatment are shown in Fig. 5. The sample was diluted in hybridisation buffer up to a final concentration of 0.15  $\mu$ M. 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 ( $3.33\text{E}-6$  RIU) was obtained. The normal wild-type sample (Jurkat) resulted in a measurable shift of  $7.57\text{E}-5$  RIU with S.D. =  $8.39\text{E}-6$ , and CV = 11%. The sample carrying the mutation at codon 248 (Molt 4) showed an average signal of about  $5.00\text{E}-5$  RIU with S.D. =  $2.00\text{E}-6$  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  $\mu$ M) and the same denaturation procedure. The signal from the negative control was very low (average value was  $8.57\text{E}-6$  RIU with S.D. =  $7.02\text{E}-7$  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 min) 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).

#### 4. Conclusions

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

Probe immobilisation 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 hybridisation 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 demonstrated the potential of the method for routine analysis.

#### Acknowledgements

We thank Dr. Lucia Mosiello, Enea-Casaccia, Italy, for cultivating Jurkat and Molt 4 cell lines and for DNA extraction. One of the authors (Maria Minunni) would like to thank the Italian Ministry of Health (Ministero della Sanità) for financial support: Project “Inibitori della ciclossigenasi-2 (COX2) nel trattamento dei tumori colorettali” for financial support.

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