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Manuscript Draft

Manuscript Number:

Title: DNA microarray-based solid-phase PCR on copoly (DMA-NAS-MAPS) silicon coated slides: an example of relevant clinical application

Article Type: Full Length Article

Section/Category: The others

Keywords: DNA microarray; Solid-phase PCR; Polymer coated silicon slide; Genotyping

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***Highlights (for review)**

- The coating stability allows to perform effective solid-phase PCR on silicon chips.
- We optimized the SP-PCR protocol for the amplification of KRAS codon 12.
- The amplification efficiency on the surface is highly effective.
- The method proposed allows for the individuation of the genotype of all the samples.
- All theassayscould be integrated in an automatic microfluidic platform.

DNA microarray-based solid-phase PCR on copoly (DMA-NAS-MAPS) silicon coated slides: an example of relevant clinical application

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ABSTRACT

In a previous study we developed a highly sensitive DNA microarray for the detection of common KRAS oncogenic mutations, which has been proven to be highly specific in assigning the correct genotype without any enrichment strategy even in the presence of minority mutated alleles. However, in this approach, the need of a spotter for the deposition of the purified PCR products on the substrates and the purification step of the conventional PCR are serious drawbacks. To overcome these limitations we have introduced the solid-phase polymerase chain reaction (SP-PCR) to form the array of PCR products starting from the oligonucleotide primers. This work was possible thanks to the great thermal stability of the copoly (DMA-NAS-MAPS) coating which withstands PCR thermal cycling temperatures. As an example of the application of this platform we performed the analysis of six common mutations in the codon 12 of KRAS gene (G12A, G12C, G12D, G12R, G12S, and G12V). In conclusion solid-phase PCR, combined with dual-color hybridization, allows mutation analysis in a shorter time span and is more suitable for automation.

INTRODUCTION

DNA microarray technology represents an ideal platform for rapid, inexpensive and high-throughput clinical identification of relevant SNPs or mutations.

In a previous paper, we introduced a sensitive silicon substrate for microarrays, functionalized with a polymer, named copoly (DMA-NAS-MAPS). The method was based on the immobilization of amino modified PCR products onto crystalline silicon slides, coated by a thermally grown silicon dioxide (SiO_2) layer that promotes the intensification of the fluorescence signals on the surface as a result of optical constructive interference between the incident and reflected lights of the fluorescent radiation (Cretich et al., 2009; Volle et al., 2003). This kind of substrate is

functionalized by adsorption of a copolymer of dimethylacrylamide (DMA), N-acryloyloxysucinimide (NAS) and meta-acryloyl propyl trimethoxy silane (MAPS), copoly (DMA-NAS-MAPS), which allows for the covalent binding between the amino modified amplicons and the surface (Pirri et al., 2004). This coating procedure is simple and reproducible, when compared to organo-silanization, a process that requires highly controlled conditions and suffers from poor reproducibility. This functional polymer has been widely applied in the biosensor field for the bio-functionalization of polystyrene nanobeads (Marquette et al., 2007), silicon microcantilevers (Oliviero et al., 2008), polydimethylsiloxane (Cretich et al., 2008), and nitrocellulose substrates (Cretich et al., 2010).

One of the drawbacks of our previous mutation detection platform was that the PCR products required a purification step before spotting to enhance the concentration of the PCR products and to replace the amplification buffer with a suitable spotting buffer. Moreover, in the amplicon down approach, the purified PCR amplicons had to be spotted by an expensive commercial arrayer not available in most clinical chemistry laboratories. To overcome these disadvantages we tested the feasibility of introducing the solid-phase PCR (SP-PCR) in order to directly bind the PCR products onto the substrates. The PCR reaction takes place on the surface from immobilized primers (Adessi et al., 2000; Cheng et al. 2010; Kranaster et al., 2008). In perspective the chips with the immobilized primers could be sold to the user already printed as a part of a diagnostic kit, so as to make the printer quite unnecessary. In literature there are very few papers dealing with the analysis of relevant human mutations with platforms that perform SP-PCR in combination with microarrays. In this work we have applied this methodology to improve the KRAS mutation genotyping.

A key requirement for generating PCR products onto substrates is the grafting of the primers in a SP-PCR compatible manner. In this case the chemical bond between oligonucleotide primer and the array-substrate should withstand high temperatures and thermo cycling between 50 °C and 95 °C. Furthermore, the binding chemistry must ensure that the free 3'-OH end is accessible and extendable by a DNA polymerase; thus the substrate and immobilization chemistry to be used in SP-PCR have special requirements. To this aim, different immobilization protocols on glass (Ham et al., 2011; Khodakov et al., 2008; Sun et al., 2011; Von Nickisch-Rosenegk et al., 2008) and polymers (Carmon et al., 2002; Kinoshita et al., 2007) have been published. Most of the methods report on covalent immobilization of oligonucleotides through EDC chemistry, where 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) mediates the linkage of 5'-NH_2 modified DNA to hydroxylated substrates (Adessi et al., 2000). Another method is based on the UV-crosslink poly-dT modified DNA directly to glass (Sun et al., 2011) or to a 3-D hydrogel matrix on plastic substrates (Brandstetter et al., 2010). Besides that, acrydite-modified oligonucleotides in an acrylamide gel can be spotted on a substrate for oriented co-polymerization (Cheng et al., 2010). Indirect immobilization methods utilize homobifunctional linking molecules like glutaraldehyde (Khodakov et al., 2008) and 1,4-phenylene diisothiocyanate (PDITC) (Kranaster et al., 2008) for attaching oligonucleotides to activated or modified (mostly aminosilanized) surfaces. Another requirement for the PCR on a surface is a high surface density of spotted primers. To this aim a three-dimensional (3-D) hydrophilic-gel (*e.g.*, polyacrylamide) has been employed as a substrate to improve the surface area in SP-PCR (Strizhkov et al., 2000). The DNA attachment by this approach

requires a number of tedious steps. Therefore the whole process besides being complicated and time consuming and it has the drawback of producing arrays with a high background due to non-specific binding of unbound labeled probes which are difficult to remove from the 3-D matrix.
A need exists for a simple, robust, and versatile immobilization strategy compatible with SP-PCR. In this work for the first time we demonstrate the thermal stability of copoly (DMA-NAS-MAPS). Thanks to the stability of this polymer in thermal cycling conditions which entails the use of a temperature 95°C, we were able to successfully use SP-PCR to overcome the spotting of different PCR products. Only the common reverse primer is spotted on the surface. During thermal cycling the amplification reaction occurs and the PCR products are subsequently grafted to the substrate. All the reaction components (Taq polymerase, liquid primers, etc.) are removed by a simple washing step at the end of the amplification cycles. Due to the fact that all the mutations under study are in the same PCR fragment, the spotting of only one primer enables the identification of the genotype after hybridization with dual color fluorescent probes of all the mutations of interest. The first part of this article focused on experiments that demonstrate the thermal stability of the polymer coating whereas in the second part, after the optimization of the protocol for the solid-phase amplification reaction**,** we genotyped the six most common KRAS mutations in codon 12 and we evaluated the sensitivity of the system by means of a dilution curve.

EXPERIMENTAL SECTION

Materials and reagents

Tris, ethanolamine, ammonium sulfate, sodium dodecyl sulfate (SDS), Triton X100, bovine serum albumin (BSA), DMA, MAPS, and 20X standard saline citrate (SSC) were purchased from Sigma (St. Louis, MO, USA). NAS was obtained from Polysciences (Warrington, PA, USA). Oligonucleotides and primers were synthesized by MWG-Biotech AG (Ebersberg, Germany). DNA Polymerase was purchased from Roche Applied Science (Mannheim, Germany).
GeneFrames were purchased from Sigma (St. Louis, MO, USA).
Untreated silicon 1000Å Thermal Oxide (75 X 25 and 14 X 14 mm) slides were supplied by SVM, Silicon Valley Microelectronics Inc. (Santa Clara, CA USA).

Samples

Mutant-bearing plasmids were generated through the cloning of specific mutagenized PCR products harboring the six mutations tested in the assay and the corresponding wild-type fragment (Stenirri et al., 2004).

Procedure to evaluate the thermal stability of the polymer coating

See the online Supplementary information for a detailed description of the preparation of the silicon chip coating.
Synthetic 23-mer 5'-amine-modified oligonucleotides, OLIGO1 (5'-GCC CAC CTA TAA GGT AAA AGT GA-3'), and 3'-amino-modified oligonucleotides labeled with 5'-Cy3, OLIGO2 (5'-TCA CTT TTA CCT TAT AGG TGG GC-3') 100 μM stock solutions (MWG-Biotech AG Ebersberg, Germany), were dissolved in the printing buffer (sodium phosphate 150mM, pH 8.5, 0,01% Triton X100) to a concentration of 10 μM. The amino-modifications are necessary to bind the oligonucleotides

covalently to the substrate through a reaction between the amino groups and the active esters of the polymer coating. These solutions of oligonucleotides were printed on four copoly (DMA-NAS-MAPS) coated silicon chips (14 X 14 mm) to form a pattern of 2X2 subarrays (a row with 2 subarrays of OLIGO1 and the second row with 2 subarrays of OLIGO2) using a piezoelectric spotter, SciFLEXARRAYER S5 (Scienion Germany). Spotting was carried out at +20°C and 50% humidity. After the spotting the chips were placed in an uncovered storage box, laid in a sealed chamber, saturated with sodium chloride (40 g/100 mL H_2O), and incubated overnight. After incubation, all residual reactive groups of the coated silicon surfaces were blocked by dipping the slides in 50 mM ethanolamine/0.1 M Tris pH 9.0 a 50°C for 15 min. Then, the chips were washed with water and dipped for 15 min in 4 X SSC (3 M sodium chloride, 0.3 M sodium citrate, pH 7.0) /0.1% SDS buffer, pre-warmed at 50°C and rinsed with water and dried. After these steps the silicon chips were scanned with ProScanArray (Perkin Elmer, MA, USA) to determine the start fluorescence of the Cy3 labelled oligonucleotide (OLIGO2). Then, on the spotted chips a reaction chamber was created by GeneFrames. To evaluate the thermal stability of the polymer coating, 35 μL of the PCR reaction mixture was spread into each GeneFrame. After sealing the silicon chips with the provided plastic lid three chips (the fourth one is used as room temperature, RT, control) were placed in the slide thermocycler (Mastercycler Eppendorf) and incubated at 90°C for 1, 2 and 3 hours respectively. After the thermal incubation, GeneFrames were detached and chips were washed for 20 min at room temperature in 0.1X SSC, 0.1 % SDS, and for 10 min in 0.1X SSC, and finally nitrogen jet blown dry. Then the three chips and the RT control chip were scanned again to check the difference of the fluorescence signal before and after the thermal incubation. Subsequently a 23-mer oligonucleotide, complementary to OLIGO1, labeled at the 5’ terminus with Cy3, OLIGO1C (5'-TCACTTTTACCTTATAGGTGGGC-3') (MWG-Biotech AG Ebersberg, Germany), was dissolved in the hybridization buffer (2X SSC, 0.1% SDS, 0.2 mg/mL bovine serum albumin(BSA)) at a concentration of 1 μM and immediately spread onto microarray spotted area of the four silicon chips. All the chips were placed in the hybridization chamber, laid in a humidified incubator at 65°C for 2 h. Afterwards, the chips were shaken in 4X SSC at RT to remove the coverslip and then they were washed twice for 5 min with 2X SSC/0.1% SDS solution, pre-warmed at hybridization temperature (65°C). This operation was followed by another two washings with 0.2X SSC and 0.1X SSC, both carried out at RT for 1 min. Finally, the slides were dried using the nitrogen flux and then scanned for the last time. In all the scanning steps the fluorescence signals were measured with the laser power kept constant at 30% and the photomultiplier tube gain at 75%.

Solid phase PCR on silicon slides and genotyping

Exon 2 of the KRAS gene was amplified on the surface of the slides with the following primer set: 5’- GCC TGC TGA AAA TGA CTG AA -3’ (forward) and 5’- AGA ATG GTC CTG CAC CAG TAA-3’ (reverse) generating a 167 bp fragment. The reverse spotted primer is 5’-amino-modified and it was dissolved in the printing buffer (sodium phosphate 150mM, pH 8.5, 0,01% Triton X100) at a concentration of 20 μM and printed on a silicon slide (75X25 mm) using a piezoelectric spotter, SciFLEXARRAYER S5 (Scienion Germany). Spotting was carried out as previously reported. The printing was done in 4 different areas of the silicon slides distant enough to be separated by

Geneframes so as to be subjected to different amplification reactions with different DNA control template (wild-type, heterozygous, mutant homozygous) and blank control. After the spotting the amino-reverse primers were coupled to the arrays by incubating in an uncovered storage box, laid in a sealed chamber, saturated with sodium chloride (40 g/100 mL H_2O) and incubated at room temperature overnight.

After incubation, all residual reactive groups of the coating polymer were blocked by dipping the slides in 2% w/v BSA in PBS for 1 h, washed with water, and dried by nitrogen flux.

The PCR was performed in 35 µL reaction containing 100 ng of DNA, 200 uM of each deoxynucleotide, 2 U of FastStart Taq DNA Polymerase, 1X PCR Buffer without $MgCl_2$, 1.9 mmol/L $MgCl_2$, 20 pmoles of the forward primer and 5 pmoles of the reverse primer. To create a PCR reaction chamber on a DNA microarray, each silicon chip was sealed with a GeneFrame. The reaction mixture was spread into each GeneFrame using a filter pipette tip and sealed with the provided plastic lid, then the chips were placed in the slide cycler (Mastercycler Eppendorf). For enhancing thermal transfer and leak tightness of the GeneFrame, a laboratory paper towel was placed on top of each frame. Thereby, a constant pressure was applied to the GeneFrame upon closure of the lid. Cycling conditions for the solid-phase PCR entailed an initial denaturation at 95°C for 5 min followed by 40 cycles at 95 °C for 30 s, 50 °C for 30 s, 72°C for 30 s, and a final elongation at 72°C for 10 min. After SP-PCR the chips were treated as previously reported.

Detailed descriptions of the optimization of the solid-phase PCR protocol, microarray hybridization, image-scanning and data analysis steps are provided in Electronic Supplementary Information.

RESULTS AND DISCUSSION

Evaluation of the thermal stability of the polymer coating

A preliminary investigation was carried out to evaluate the stability of the polymer coating in PCR conditions. Solid-phase PCR requires that the oligonucleotide primer remains attached to the substrate during the 40 thermal cycles (25 min at 95°C, 30 min at 72°C and 20 min at 50°C in total). If some of the covalently bound primers detach or degrade, the surface density of amplicons may be lower. The binding stability was first analyzed by measuring the variation of fluorescence intensity of a spotted 3'-amino-modified oligonucleotide labeled with 5'-Cy3 (OLIGO2) before and after an incubation step at 90°C for 1, 2 or 3 hours in comparison to a control chip which was not submitted to the thermal treatment (RT control). Fig. 1A reports the percentage of spot fluorescence after thermal treatment of the silicon chip. The RT control was submitted only to the washing steps. As demonstrated by the high percentage (82.2%) of fluorescence intensity measured even after 3 hours of thermal treatment at 90°C, the bond between the oligonucleotides and the polymer is very stable compared to other substrates. There are data in literature that report fluorescence losses between 30-56% (Hoffmann et al., 2012) for oligonucleotides attached to polydimethylsiloxane (PDMS), polypropylene (PP), cyclic olefin polymer (COP), cyclic olefin copolymer (COC) or glass. A thermally induced loss of 60% of fluorescence was reported after 40 PCR cycles for oligonucleotides bound through BTA (benzene-1,3,5-triacetic acid) linker (Fedurco et al., 2006) and 40-60% after 50 PCR cycles for glass

functionalized by EDC (1- ethyl-3-(3-dimethylaminoprpyl)- carbodiimide hydrochloride), s-MBS (*m*-maleimidobenzoyl-*N*-hydroxysulfo-succinimide ester), s-SIAB (sulfosuccinimidyl(4-iodoacetayl)aminobenzoate), s-SMCC (sulfosuccinimidyl 4-(*N*-malimidomethyl)-cyclohexane-1 carboxylate), s-GMBS (*N*-(y-maleimidobutyryloxy)sulfo succinimide ester) and s-SMPB (sulfosuccinimidyl 4-(*p*-maleimidophenyl)-butyrate) (Adessi et al., 2000).

In an additional experiment, we evaluated the signal of a non-labeled oligonucleotide (OLIGO1) hybridized with a Cy3 labeled complementary oligonucleotide (OLIGO1C), obtained in the three chips incubated at 90°C in comparison with the RT control chip. The results of this experiment are summarized in Fig.1B. The fluorescence after hybridization in thermally treated chips is comparable, if not higher, than that on the control chip. These results confirm the stability of the oligonucleotide binding exposed to a temperature of 90°C for three hours.

A

	Starting fluorescence	Final fluorescence	% of remaining fluorescence
Control chip RT	24434.3 (± 5.4%)	22807 (± 14.3%)	93.3
Chip1	23365 (± 10.6%)	20898.9 (± 13.5%)	89.4
Chip2	25549.7 (± 8%)	20540.1 (± 13.5%)	80.4
Chip3	28879.8 (± 5.3%)	23742.9 (± 12.6%)	82.2

B

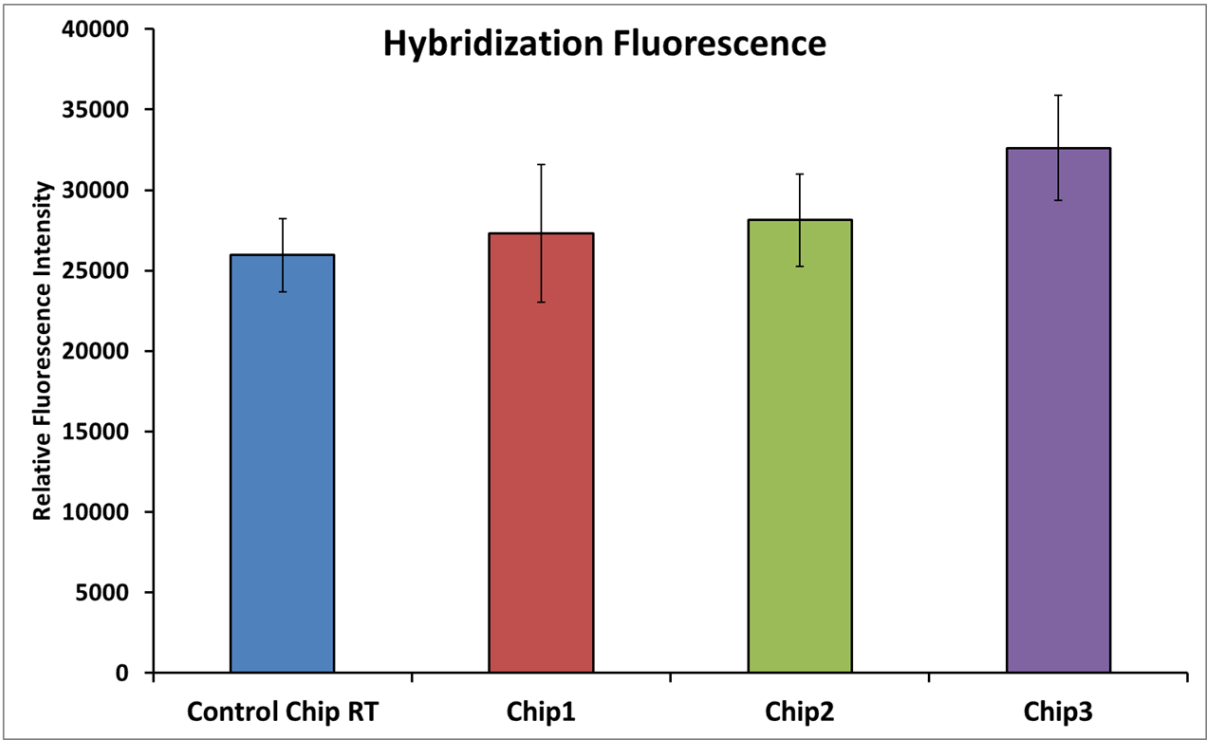

Fig. 1 Thermal stability of the polymer coating. *A) The thermal stability was calculated as a percentage of attached Cy3 oligonucleotide (OLIGO2) remaining after thermal incubation at 90°C for 1, 2 or 3 hours chip 1, chip 2 and chip 3 respectively. The relative fluorescence intensity acquired with ProScanArray (Perkin Elmer, MA, USA) at laser power 30% and photomultiplier gain 70% PMT before and after the thermal incubation was reported. B) The histogram shows the relative fluorescence intensities acquired at the same conditions after hybridization of oligonucleotide OLIGO1 spotted on the silicon surfaces, with its complementary oligonucleotide OLIGO1C labeled*

with Cy3. All the bars are the average of the intensity of 50 replicates of each subarray. The error bars are the standard deviations of the fluorescence intensity of each sample. Chip1= 1 hour incubation at 90°C; Chip2= 2 hour incubation at 90°C and Chip3= 3 hour incubation at 90°C.

Principles of the solid-phase PCR

The PCR occurs on solid-phase when at least one PCR primer is immobilized to a substrate or to a reaction compartment (Khan et al., 2008; Kohsaka and Carson, 1994; Palanisamy et al., 2010; Shapero et al., 2001). During the amplification reaction the generated amplicon remains bound to the surface via the immobilized solid-phase primer. To graft PCR products in a microarray format we used the approach followed by Hoffmann et al. where the PCR takes place in a reaction chamber formed by attaching a plastic frame to a microarray slide and filled with a buffer solution containing the primers in an asymmetric ratio together with the amplification additives. One of the two primers is immobilized by spotting on the slide surface (Fig.2A). The PCR reaction occurs in two phases. In the first step, the reaction proceeds preferably in the liquid phase, due to steric hindrance, until the reverse primer is depleted (Fig.2B). Then, the solid-phase PCR dominates and the amplicons that are formed bind preferentially to the surface (Fig.2C). The combination of the two steps allows for the simultaneous amplification and immobilization of the amplicons to the solid-phase (Fig. 2D).

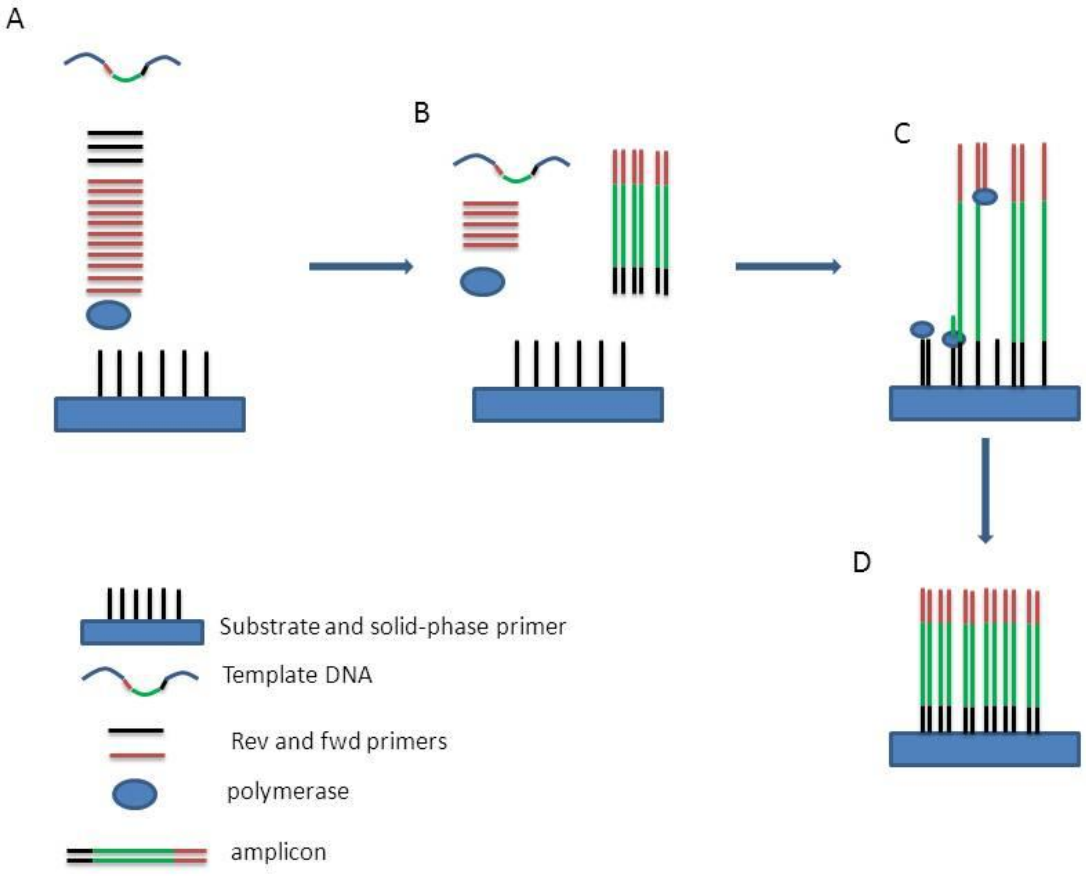

Fig. 2 Solid-phase PCR. *The reaction chamber contains reverse solid-phase primers attached covalently to the substrate as well as forward (fwd) and reverse (rev) primers in solution in asymmetric ratio. The forward primer is represented in red, the reverse primer in black.*

Several critical points in term of i) DNA template concentration, ii) number and duration of the cycles, iii) Mg concentration and annealing temperature affect the performance of the SP-PCR and need to be optimized to ensure efficient DNA amplification. Also, i) the concentration of the immobilized primer, ii) the presence of a poly(6)-dA linker at the 5' end of the spotted primer and, most importantly, iii) the asymmetric balance of liquid primers were optimized. The detailed optimization of the solid-phase PCR protocol is provided in Electronic Supplementary Information.

Genotyping of KRAS mutations

In our previous study (Galbiati et al., 2013) we have developed a microarray for genotyping of the most common KRAS gene mutations so we have all the know-how, the expertise and the optimized protocols to analyze these mutations. Furthermore, this is a good example to address. In the codon 12, the number of substitutions is high (six), making their detection more complex with allele specific techniques. We used the reporter and stabilizer probes and the hybridization protocol developed in the precedent work. After the spotting of the common poly-(A) reverse primer in concentration 20 μM on the coated silicon slides in a 6X6 pattern in 4 different areas distant enough to be separated by Geneframes, the chips underwent the optimized SP-PCR (40 cycles, 1.9mM $MgCl_2$, 1:4 ratio reverse:forward liquid primers, 100 ng DNA template, 50°C annealing temperature). On a single silicon slide (7.5 X 2.5 cm) 4 different SP-PCRs were performed, so for each variant only one chip is needed. Wild-type control sample, heterozygous and homozygous mutant DNA template and a control blank (without DNA template) were simultaneously amplified. Following denaturation of the resulting bound PCRs, the silicon slides were hybridized with the dual-colors protocol already described (Galbiati et al., 2013). An example of genotyping is illustrated in Fig.3.

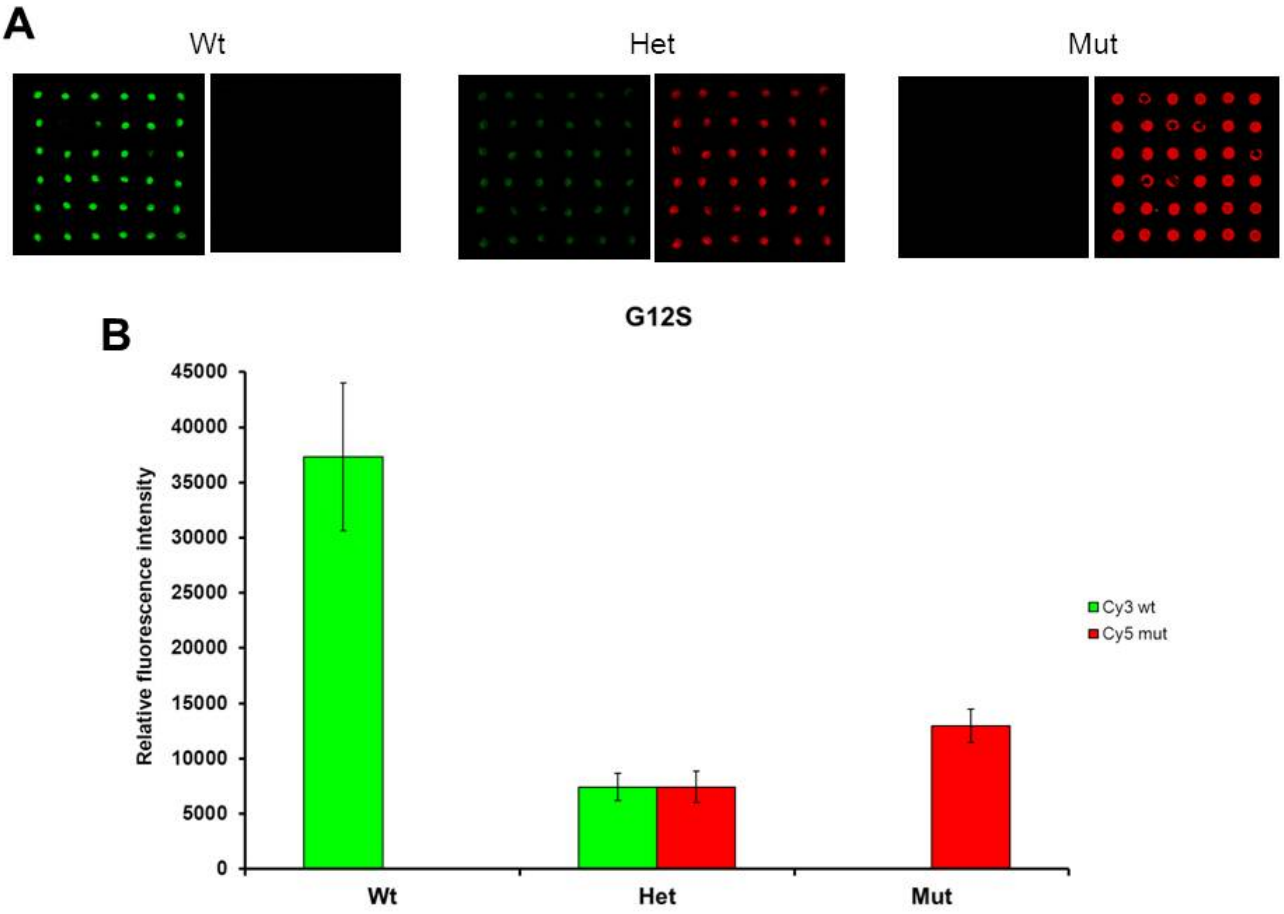

Fig.3 Microarray images for the genotyping of the G12S KRAS mutation. *(A) Microarray scanning of the Cy3 fluorescence (green) corresponding to the wild-type allele and Cy5 fluorescence (red) corresponding to the mutated allele. (B) Normalized relative fluorescence intensity after hybridization of known control samples with the reporters complementary to the G12S variation. Bars are the average of the intensity of the 36 replicates of each sample. The error bars are the standard deviations of the fluorescence intensity of each sample. Wt, wild-type control sample; Het, heterozygous control sample; Mut, homozygous mutated control sample.*

In the optimized system we succeeded to genotype all the six KRAS mutations with a high fluorescence signal, very low cross-hybridization and a good reproducibility from spot to spot. The results obtained for all the remaining five KRAS gene mutations (G12A, G12C, G12D, G12R and G12V) are shown in Fig.4.

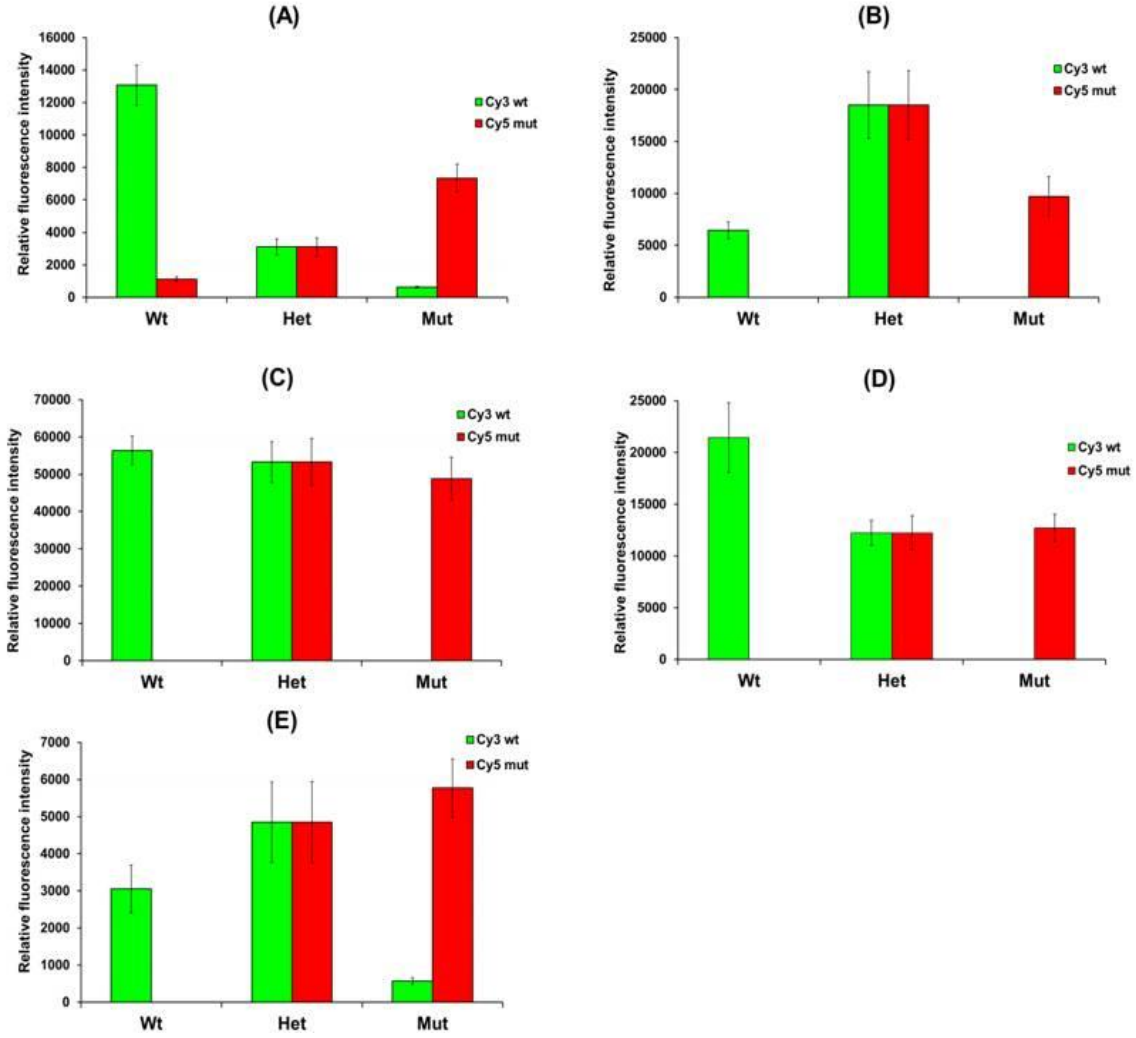

Fig.4 Plots for the genotyping of the codon 12 KRAS mutations. *Normalized relative fluorescence intensity after hybridization of control samples for the G12A (A), G12C (B), G12D (C), G12R (D) and G12V (E) KRAS mutations. Wt, wild-type control samples; Het, heterozygous control samples; Mut, homozygous mutant control sample. All the bars are the average of the intensity of the 36 spots of each sub-array. The error bars are the standard deviations of the fluorescence intensity of each sample.*

Detection limit

The sensitivity of the SP-PCR microarray assay in discriminating different proportions of mutated alleles was evaluated with serial dilutions (50%, 25%, 12.5%, 6.25%, 3.12%, 1.6%, 0.8%, 0.4%, 0.2%, 0.1% and 0.05%) of mutated DNA opportunely mixed with wild-type DNA.

In particular we used the CCRF-CEM cell line as a reference for KRAS mutation G12D (heterozygous) and the human breast cancer cell line MCF-7 as wild-type control. We analyzed by dual color hybridization twelve (11 dilutions and 1 wild-type control) silicon coated chips (14X14 mm) where a single SP-PCR was obtained starting form one of the dilutions under study. The microarray system proposed here was able to detect a minimum of about 0.8% of mutated allele in a background of wild-type DNA for the G12D mutation (Fig.5). A lowest detection limit (0.1%) was obtained, in our previous work, using the chip spotted with amplicons produced in solution;

although not too different, the amplicon density obtained with the SP-PCR is lower than the one could obtain by spotting a purified PCR product. We have quantified this difference by directly comparing the fluorescence signals obtained in the detection of G12D KRAS mutation in the two conditions. The Cy5 fluorescence signals on the solid-phase PCR-based array was found to be about 15% (data not shown) lower than the fluorescence signals on spotted PCR. This decreased density can be the cause of the lowest detection limit for the G12D mutation. This lower density is a phenomenon well known in literature (Adessi et al., 2000; Shapero et al., 2001) generally attributed to a low concentration of surface primers, the inability of the liquid primers to diffuse at the binding site, or to inefficient enzymatic extension. Overall, in our case, the amplification efficiency on the surface is highly effective, and the method proposed allows for the correct individuation of the genotype of all the samples with no or very little cross-hybridization and high fluorescence signal.

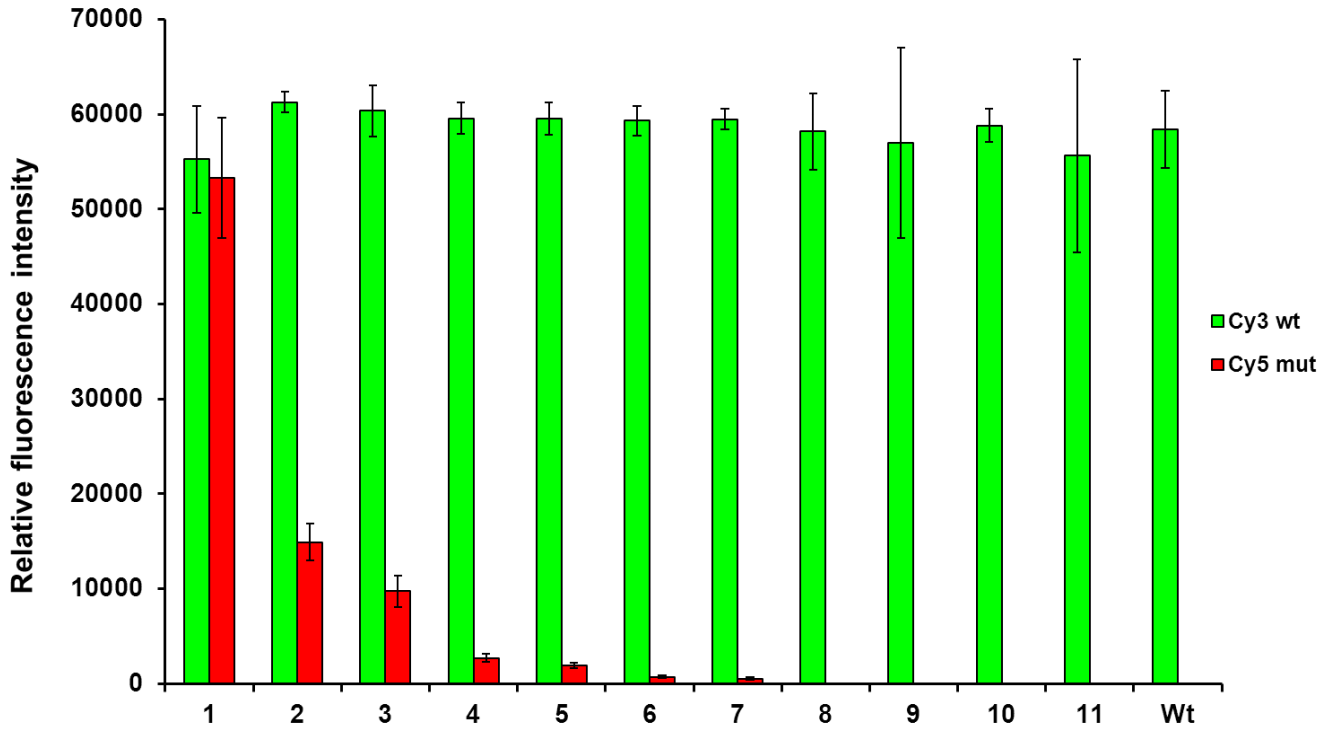

Fig.5 Detection limit of the G12D KRAS mutation. *Relative fluorescence intensity after hybridization. Numbers from 1 to 11: serial dilutions, 1 = 50%, 2 = 25%, 3 = 12.5%, 4 = 6.25%, 5 = 3.12%, 6 = 1.6%, 7 = 0.8%, 8 = 0.4%, 9 = 0.2%, 10 = 0.1%, 11 = 0.05%; Wt, wild-type control samples. All the bars are the average of the intensity of the 36 spots of each sub-array. The error bars are the standard deviations of the fluorescence intensity of each sample.*

CONCLUSIONS

In summary, we developed a DNA microarray-based solid-phase PCR method for the genotyping of the six most common mutations in the codon 12 of KRAS gene (G12A, G12C, G12D, G12R, G12S and G12V). The stability of this inexpensive, rapid and easy to use 3-D coating made by copoly (DMA-NAS-MAPS) is essential to achieve the goal of maintaining about 80% of the primers

immobilized on the surface after three hours of exposure of the chip at 90°C. The coating stability combined with its tridimentionality allows to perform highly effective solid-phase PCR on the silicon chip. Appling the dual-color fluorescent hybridization to the amplicon bond to the silicon chips, the genotyping was successfully achieved for all the codon 12 mutations in the KRAS gene tested. Overcoming PCR purification, concentration and spotting is of great importance in the perspective of using DNA microarrays as diagnostic tool with the only need to provide the operator with the silicon chip spotted with one primer necessary for the amplification reaction. Moreover, all the assays could be integrated in an automatic microfluidic platform, equipped with an appropriate temperature control unit, able, for example, to genotype all the KRAS mutations on a single chip. The genotyping of the KRAS mutations shown in this study is just one of the many potential applications of solid-phase PCR in the context of a microarray assay. The experiments clearly prove the potential of this substrate and opens new opportunities for the development of high-sensitive microarray assay.

AUTHOR INFORMATION

Notes

The authors declare no competing financial interest.

ACKNWOLEDGEMENTS

This work was partially supported by Regione Lombardia through Project LOCSENS ID30071637.

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