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Blocked recombinase polymerase amplification for mutation analysis of *PIK3CA* gene

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ABSTRACT

A blocked recombinase polymerase amplification (blocked-RPA) approach has been developed for the enrichment of mutated templates in heterogeneous specimens as tumor tissues. This isothermal amplification technique opens alternative solutions for meeting the technological demand of physician office laboratories. Herein, the detection of mutations in *PIK3CA* gene, such as p.E545K, and p.H1047L, is presented. The main element was an oligonucleotide (dideoxycytidine functionalized at 3'-end) which matched with wild-type sequence in the target locus. The amplification was performed operating at 37 ºC during 40 min. The results demonstrated that the competition between the upstream primer and the blocker reduced the percentage of amplified wild-type allele, making the detection of the present mutation easier.

For mutation discrimination, a fast hybridization assay was performed in microarray format on plastic chip and colorimetric detection. This approach enabled the reliable discrimination of specific mutations against a background of up to 95% wild-type DNA. The applicability of the method, based on the combination of blocked-RPA and low-cost chip hybridization, was successfully proven for the genotyping of various cancer cell lines as well as tumor tissues. The assignations agreed with those provided by next-generation sequencing. Therefore, these investigations would support a personalized approach to patient care based on the molecular signature of human cancers.

Keywords: recombinase polymerase amplification; blocking agent; mutations in *PIK3CA* oncogene; gel electrophoresis; colorimetric array

INTRODUCTION

Neoplastic diseases are currently the leading cause of morbidity and mortality in developed countries, making cancer a public health problem of the first magnitude. The success of anticancer therapies depends on the correct assignation of disease subtype. Therefore, the detection of tumour biomarkers is important for the application of a personalized medicine [1,2]. Somatic mutations on oncogenes are excellent predictive biomarkers since the response to a particular line of treatment can be anticipated, reducing the adverse effects and improving efficiency [3]. They also can be prognostic biomarkers enabling the prediction of disease progression.

Several molecular techniques can be applied for knowing the mutational status of oncogene hotspots. In a DNA extract from patient sample (solid tissues and body fluids), the mutant variant is found in a low proportion compared to wild-type variant. This scenario presents an analytical challenge because wild-type variant can exhaust essential reagents and/or mask the mutant signal during detection assays [4]. Next-generation sequencing instruments holds great promise for point mutation detection, but currently, this technology is available in few health centers and clinics. The alternative solutions are based on introducing an enrichment method combined to more simple detection techniques. Particularly interesting are PCR-based methods proposed for a selective (or quasi-selective) amplification of minority alleles and mutations. These include the use of allele-specific primers to selectively initiate the amplification of the mutated genotype [5,6]; addition of oligonucleotide clamps to preferentially inhibit primer extension on wild type targets [7-9]; and control of temperature thermocycling to favor the preferential denaturation of mutant targets [10]. The differential behavior between wild-type and mutant variants are obtained using real-time or end-point amplification, fluorescence being the main detection principle. Most of described methods are able to detect the presence of mutation in the selected hotspot, but they do not provide information about their identification.

Herein, we developed an *in vitro* method based on a blocked isothermal amplification as enrichment technique for analyzing point mutations in *PIK3CA* gene (phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit alpha) as oncogene model. This gene codifies important cell membrane element and second messenger involved in cell signaling. The mutation has been located in human cancer as colorectal, breast, glioblastoma, gastric, ovary, lung, and skin [11,12]. There is an important basic and clinical research for understanding the impact of mutation on cancer cell growth,

survival, motility, and metabolism [13]. Additionally, novel inhibitor drugs are targeting this mutant protein [14].

Isothermal amplifications are revolutionizing the development of point-of-care testing due to their capability for the integration in portable, inexpensive instruments or devices [15,16]. Loop-mediated isothermal amplification (LAMP) is the most cited alternative to PCR. Their allele-specific option has displayed excellent results in the detection of a specific point mutation in *EGFR* gene [17], *BRAF* gene [18] and *KRAS* gene [19]. Rolling circle amplification (RCA) and recombinase polymerase amplification (RPA) have been used for the detection of point mutation [20, 21]. In our previous research, the genotyping of a single-point polymorphism was achieved using allele-specific primers for discriminating perfect-match and mismatch allele. In the current study, a new strategy is addressed for improving sensitivity, based on the addition of a blocking oligonucleotide to reduce the amplification of wild-type variant. Then, the presence of mutated variants can be established. Furthermore, an allele-selective hybridization is proposed in order to discriminate between different nucleotide changes. As proof of concept, the selected format involves a plastic chip as analytical platform and colorimetric imagining as detection approach. Microarrays represent an accurate tool for parallel identification of multiple markers, suitable for routine analysis in medical diagnostics [22].

EXPERIMENTAL

Material and reagents

Oligonucleotide sets were designed for the analysis of two important hotspots in *PIK3CA* gene (Table SI.1). In the helicase domain of exon 9, the most frequent mutations are in codon 542 (p.E542K) and codon 545 (p.E545K, p.E545A, and p.E545G). In the kinase domain of exon 20, they are in codon 1047 (p.H1047R and p.H1047L). Thermodynamic parameters were calculated for inducing the selective recognition of target regions [23]. The list of used oligonucleotides, supplied by Eurofins, is included as Supplementary Material (Table SI.2).

The reagents used for genomic DNA amplification were TwistAmp Basic RPA kit (TwistDx, UK). For microarray detection, the printing buffer composition was (2-(N-morpholino)ethanesulfonic acid at 0.1 M, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide at 20 mM, glycerol 10% (pH 5.5). The hybridization buffer was saline-sodium citrate (SSC) 2×: sodium chloride at 150 mM, sodium citrate at 15 mM, formamide 20% (pH 7.0). Hybridization washing buffer was a solution with NaCl 15 mmol

L^{-1}, trisodium citrate 1.5 mmol L^{-1}. Developing buffer was a phosphate buffered saline solution (PBS-T) containing 137 mM NaCl, 12 mM phosphate, 2.7 mM KCl, 0.05% Tween 20 (pH 7.4).

The hybridization chips were prepared by immobilizing the allele-specific probes on rectangular slides (25 mm × 75 mm). For covalent anchoring of probes, polycarbonate surface was activated by UV-ozone incubation (model FHR.Clean.150-Lab, FHR, Germany) using UV lamps (50 mW cm^{-1}) at 254 nm. After, the chips were immersed in a solution of NaOH 1M at 60 °C, washed with distilled water and dried. The dispensation of amine-DNA probes in printing buffer on the modified surface was performed by non-contact array printer equipment (AD1500, Biodot). The printing volume drop was 40 nL. Six arrays were printed per chip, including 4 replicates per target probe and controls. The chips were incubated for 1 hour, washed with PBS-T and water, dried and stored at 4 °C until use.

Samples and DNA extraction

Patients and several volunteers were recruited for the present study according to ethics and with informed consents. Genomic DNA was extracted from three 5-µm thick FFPE sections using Deparaffinization Solution and the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol. This isolation kit contains uracil-DNA glycosylase (UDG) that leads to the reduction of C>T sequence artefacts. Buccal cells were collected by rolling the swab (Catch-All sample collection swab, Epicenter) on the inside of the cheek. DNA extraction was performed using the PureLink Genomic DNA Mini Kit (Invitrogen). Briefly, the swab was incubated with Proteinase K, RNAse and lysate buffer. The purification was performed by using a spin column-based centrifugation procedure. Human cell lines SK-N-AS (ATCC CRL-2137) and HCT 116 (ATCC CCL-247) were used as native (wild type) and heterozygous (p.H1047R/c.3140A>G) controls, respectively. In these cases, DNA extraction was performed using PureLink kits (Invitrogen).

In addition, the assay included a negative control (DNA from *Salmonella Typhimurium*). The concentration of the DNA extracts (ng μL^{-1}) was obtained by spectrophotometry using NanoDrop 2000c, and by fluorimetry using Qubit dsDNA HS Assay Kit (ThermoFisher Scientific).

PCR amplification

PCR amplification in single format was performed for each target hotspot in *PIK3CA* gene (exon 9 or exon 20). Each reaction mixture contained 1 × DNA polymerase buffer, 3 mM of $MgCl_2$, 200 μM of each deoxynucleotide triphosphate, 200 nM of upstream primer and downstream digoxigenin-labeled primer, 4 ng of genomic DNA, and 1 unit of DNA polymerase (Biotools, Madrid, Spain). In certain experiments, a blocking oligonucleotide complementary to wild-type variant was added to each mixture (10 nM – 400 nM). The reaction was performed in thermocycler (United Nations, VWR) under the following conditions: initial denaturation cycle of 95 °C for 5 min, 35 cycles of denaturation at 95 °C for 30 s, annealing 57 °C for 30 s and elongation at 72 °C for 60 s, finally one cycle extension at the end for 5 min.

RPA amplification

RPA amplification in single format was performed for each target hotspot in *PIK3CA* gene (exon 9 or exon 20). The reaction mixtures (12.5 μL) were prepared with rehydrated buffer, 14 mM of magnesium acetate, 480 nM of upstream primer and downstream digoxigenin-labeled primer, 50 nM of blocking agent, 4 ng of genomic DNA, and the enzyme pellet. The reaction mixtures for conventional RPA did not contained the oligonucleotide complementary to wild-type variant. The heating system used was a thermocycler (United Nations, VWR) at 37 ºC for 40 min.

Gel electrophoretic detection

The RPA amplicons were cleaned for being visualized using agarose gel electrophoresis. Two methods were compared, the first being based on silica-gel membrane adsorption (PCR purification kit, Jena Bioscience, Germany). The RPA products, mixed with binding buffer and isopropanol, was transferred to the activated column and centrifuged at 10.000g for 30 s. After washing the column twice, the elution fraction was collected in distilled water. The second method was based on denaturation protocol. The RPA products were incubated at 65 °C in dry bath (model FB 15103, Fisher Scientific) for 10 min. After centrifugation at 10.000g for 20 s, the supernatant fraction was collected.

The electrophoretic separation was conducted in agarose gel 3 %. Amplification solutions were mixed with loading buffer and transferred to gel wells. After separation at 110 V, fluorescent dye (Realsafe Nucleic acid Staining Solution 2x, Real Lab.) was incubated for 30 min. Gel images were captured using a smartphone Iphone 7 and analyzed using ImageJ software.

Colorimetric chip detection

The RPA products were detected and identified by a hybridization assay using allele selective probes immobilized on polycarbonate chips. Amplified products (5 μL) were mixed with 45 μL of hybridization buffer, heated (95 °C, 5 min) and dispensed onto sensing arrays to perform the simultaneous analysis of 6 samples (four probe replicates). After incubation (37 °C, 60 min), the arrays were rinsed with progressive dilutions of hybridization washing buffer. To develop the probe-product duplex, an immunoreaction was used. PBST solution with 1:2500 monoclonal anti-Dig antibody (Abcam) and 1:400 monoclonal antisheep-HRP antibody in PBST, was dispensed (room temperature, 30 min). As HRP-substrate, 3,3',5,5'-tetramethylbenzidine solution (ep(HS)TMB-mA, SDT Reagents) was selected, generating a solid deposit.

Chips were directly scanned (Epson Perfection 1640SU office scanner), producing gray-scale images (Tagged Image File Format, color depth 16 bit, scale 0-65535). The optical intensity signals of each spot were quantified using in-home software. Image processing (feature gridding, addressing, segmentation and quality assurance) was automatically performed in less than 5 min.

Reference method

Several methods for detecting somatic mutations are currently available. NGS, considered to be an optimum method for mutation detection, was conducted employing the Ion Torrent PGM technology (ThermoFisher Scientific). The *Oncomine Solid Tumor DNA kit* was used for DNA analysis (ThermoFisher Scientific). This assay simultaneously screens hotspots mutations in 22 genes (included PIK3CA). For generating the DNA barcoded libraries, a multiplex PCR amplification of 10 ng of genomic DNA was performed. Sequencing was performed on the Ion Torrent PGM system on a 318v2 Ion Chip using Ion PGM Sequencing Hi-Q kit (Thermo Fisher Scientific). Data from sequencing runs were transferred to the Torrent Server, alignment to the hg19 human reference genome and variant calling was performed by the Ion Torrent Suite Software 5.4 (Thermo Fisher Scientific). In addition, all identified variants, such as PIK3CA mutations in exon 9 and 20 (p.E545K and p.H1047L), were visually checked using the Integrative Genomics Viewer (IGV) software.

RESULTS

Assay design

A flow diagram showing the main steps in our assay for the detection of point mutations is illustrated in Figure 1. From total genomic DNA extracted from human tissues, the target sequence is amplified following a clamp blocked RPA reaction. Compared to conventional RPA method, the reaction solution also contained an oligonucleotide complementary to wild-type DNA (blocker), the target nucleotide being in the central position of oligonucleotide. The upstream primer is in the vicinity of blocking oligonucleotide, and partially overlaps its 5'-end. The blocker oligonucleotide is designed for producing more stable complexes than upstream primer. The method operates by a competition for a common target site.

Figure 1. Schematic illustration of the RPA reaction for minority allele enrichment based on the addition of blocking agent. (Up) Wild-type blocked amplification. (Bottom) Mutant amplification.

The amplification mechanism of mutant variants is based on the action of recombinases (T4 uvsX and loading factor T4 uvsY), which form complexes with primers and bind them with their homologous sequences in duplex DNA [24]. A single-stranded DNA binding (SSB) protein binds to the displaced DNA strand and stabilizes the resulting D loop. Bsu polymerase (large fragment of *Bacillus subtilis* Pol1) produces the primer extension from the 3'-end, replicating mutant allele. The amplification of wild-type variants is impaired due to the presence of the blocking oligonucleotide. In this case, the blocking oligonucleotide dominates the binding to the template over the primer. Since the blocker also contains a chemical modification at the 3'-end, the oligonucleotide cannot be extended by Bsu polymerase. Then, the addition of this blocking agent during amplification of the target region induces the competition for the binding site in DNA template, leading to a preferential amplification of minority mutations. This clamping effect has been previously described for PCR-based methods [12,13], but this is the first time for RPA.

RPA amplification

The proposed assay was tested for the genotyping of the main hotspots in *PIK3CA* gene (codon 542/545 and codon 1047). An *in-silico* design was applied for the selection of the sequence-specific primers based on the requirements established for the product properties and method performances. Compared to the conventional RPA methods, short primers (< 30 nucleotides) were selected to facilitate the subsequent allele-specific assay. Even though they had little recombinase-mediated strand-invasion activity, short primers can still function via hybridization recognition [25]. An additional design restriction was taken into account. The length of products was limited to short amplification products (lower than 100 bp). As the conservation of biopsied tissues as formalin-fixed and paraffin-embedded can lead to an important degradation of nucleic acids, this selection criteria can minimize false-negative results.

Using the selected oligonucleotide sets (upstream and downstream primers), the first experiments were focused on the optimization of reaction conditions for the amplification of both hotpots in single format. Regarding kinetic behavior, the amplification process reached a stationary phase after 40 min. A similar product formation was obtained when the RPA reaction was produced within the 37-42 ºC range, showing high tolerance to temperature fluctuations. Therefore, the selected conditions were similar to previous studies for human cancer tissues [26].

RPA specificity was checked on the basis on the detection of target regions from human and non-human genomic DNA. Negative results were reported for the non-target genes or samples, demonstrating the absence of false-positive results. The matrix effect was also examined, analyzing different samples (cell culture, buccal swap, colon tissue) and storage conditions (fresh tissues and formaldehyde fixed-paraffin embedded tissues). Under the selected extraction and amplification conditions, excellent end-point amplification yields were obtained. A detectable signal was measured for $5 \cdot 10^2$ DNA copies. The results were comparable to those obtained with the PCR-based method, independently on sample source (paired t-test, p-value <0.05).

Selection of blocking oligonucleotides

A preferential RPA-based amplification of minority mutations was studied based on the addition of a blocking oligonucleotide to the reaction solution. Following the modalities described for blocked PCR amplification, two kinds of oligonucleotide sets were assayed. The first approach, both oligonucleotides (upstream primer and blocking agent) are

complementary to different template regions, preventing the amplification in the elongation step. In the second approach, also called clamp strategy, the blocker partially overlaps with the upstream primer, reducing the amplification in the primer annealing step.

As the number of possible oligonucleotides can achieve hundreds, the thermodynamic stability of DNA duplexes was examined. The parameters were the variation of free energy associated to the formation of DNA duplexes (ΔG) and melting temperature (T_m), or temperature at which half of the blocking oligonucleotides are single-stranded state (0.1 M NaCl, 25 ºC at pH=7). Firstly, the formation of upstream primer duplexes was studied in both hotspots of *PIK3CA* gene. The ΔG-values for the selected primers ranged between -19.8 kcal mol^{-1} and -20.5 kcal mol^{-1}, corresponding to T_m = 58.6-60.9 ºC. Secondly, the effect of design parameters on the blocking oligonucleotide/template hybrids was estimated. Blocking agents with length higher than 22 nucleotides produced DNA complexes more stable than the selected primers (T_m>62°C). The differential variation of free energy associated to the formation of DNA duplexes (wild-type vs. mutant variants) was calculated in function of clamp-region length and mutation position. The nucleotide number that overlapped with the primer produced a slight effect on the discrimination recognition for perfect match complex (wild-type template) respect to the mismatched complexes (mutant templates). However, the position of mutation on blocking agent sequence varied drastically the stability of the mismatched hybrids. In fact, the maximum difference between blockers and wild-type/mutant complexes was achieved when the mutations was located in a central position.

On the basis on these experiments, several design requirements of blocking oligonucleotide were defined for blocked RPA assay (Table 1). Applying *in silico* calculations, two primers and a blocking oligonucleotide compose the selected set per studied mutation. The estimated ΔG-values for wild-type complex were -23.6 kcal mol^{-1} for exon 9 and -21.9 kcal mol^{-1} for exon 20, corresponding to T_m = 64.5 ºC and T_m = 63.3 ºC, respectively. The selected blocking oligonucleotides should form a more stable complex with wild-type than with the mutated variant (about 4 kcal mol^{-1}). Regarding the clamp effect, the common nucleotides (3'-end of primer and 5'-end of blocking agent) were 2 in both targeted hotspots. Under these conditions, the expected reduction of upstream primer annealing was about ΔG = 1.9-2.0 kcal mol^{-1} for wild-type hybrid. Although RPA mechanism is based on the action of several enzymes, a differential behavior was expected. The blocker would preferentially hybridize onto the wild type template strand and the upstream primer would bind to the mutant template.

Table 1: (a) Design criteria of blocking oligonucleotide. (b) Optimized variables for blocked RPA.

Set-up of blocked amplification

The following step was the study of experimental conditions for blocked RPA, using DNA extracts from control/mutant cell lines and the selected olignucleotides. In a first set of RPA experiments, reaction mixtures contained the downstream primer and the unmodified blocker (without upstream primer). Positive responses were observed, indicating that the blocking oligonucleotide acted as upstream primer (Figure 2a). Later, the same RPA reactions were performed, including modifications at the 3'-end of the blocker (capped extension). The studied modifications were dideoxycytidine (ddC) and non-sense 3-mer tail (not complementary to template molecule), leading a signal reduction (t-test: p-value<0.002). Both chain terminator avoided the 3'-extension, yielding similar responses to negative controls, even for high concentrations of blockers (t-test: p-value>0.95). For further assays, ddC modified blocker was selected.

Human genomic DNA was added in PCR and RPA reactions containing three oligonucleotides (primers and blocker) for non-clamp and clamp approaches (Figure 2b). In case of PCR, the addition of the blocker reduced the amplification of wild-type variant, the effect being higher for clamp approach. These results agreed with those previously described for PCR-based methods, where the overlapping region between primer and blocker provided better assay performances [7,8]. In case of isothermal method, using a primer complementary to a different region than blocker, the primer elongation of wild-type DNA was produced (t-test: p-value = 0.89). The amplification of wild-type DNA only was reduced for clamp option (t-test: p-value = 0.01). This differential blocking effect compared to PCR agreed with the expected features of RPA mechanism. In the first approach, recombinase mediated in the formation of both complexes (primer/template and blocker/template). However, polymerase used in RPA (Bsu polymerase) has strand-displacing activity that means the ability to displace downstream DNA encountered during synthesis. Although the blocking oligonucleotide was initially bound, the primer elongation was possible, replicating wild-type templates. In case of second strategy, the observed behavior fitted with a physico-chemical competition for the same template region. The blocker operates a competitor of the upstream primer for the common target site. As the stability of blocker was higher, the primer annealing in RPA process was interfered. As the non-clamp option was incompatible with RPA biochemistry, further experiments were performed using the oligonucleotide set based on clamp effect.

*Figure 2: (a) Modification of blocker oligonucleotide for the prevention of Bsu extension in a RPA mixture without upstream primer. Blocker concentration: 300, 600 and 800 nM. (b) Amplifications (PCR and RPA) performed in conventional and blocked formats (blocker concentration 200 nM). T-test: * p-value<0.05, ** p-value<0.01, *** p-value<0.001. Wild-type genomic DNA: 1300 copies.*

Agarose gel electrophoresis was used for confirming the blocking effect on PCR and RPA reaction for PIK3CA (Figure SI.1). In case of PCR, the expected bands were detected (83 pb for exon 9 and 79 pb for exon 20). However, RPA products yielded smear bands due to mixture components (i.e. DNA binding proteins, detergents). Therefore, two post-amplification treatments were assayed (column purification and protein denaturation). The first method involved that silica-membrane columns retained short DNA molecules under high-salt conditions and eluted them using a low-salt buffer. The second method consisted in the denaturation of proteins at high temperature. Although both treatments provided a single band located at the estimated position, the separation obtained after heating strategy led to band intensities comparable to the PCR results. Further experiments showed that the band intensity decreased in the presence of blocking nucleotide in RPA reactions, being undetectable for high concentrations. Finally, the kinetic profile in the presence of blocker was compared to the conventional profile. Although amplification yield was lower than conventional value (about 50 %), the maximum signal was achieved after 40 min of reaction in both cases. Therefore, gel electrophoresis results proved the variation of replication activity as the consequence of blocker binding to the template.

For the selection of blocker concentration, genomic DNA extracted from human cell cultures (wild-type and mutant) were amplified, varying up to 400 nM (stoichiometric ratio of 1.33 compared to the upstream primer). End-point responses decreased as the concentration of blocking oligonucleotide increased for both kind of templates (Figure 3). As the amplification variation was also observed in mutated variants, an unspecific interaction of blocker was produced in the mismatched templates. Nevertheless, certain reaction mixtures produced a nearly null signal for native variant and perfectly detectable signal for mutant variant. Experimental data fitted to a typical four-parameter logistic curve with a suitable model goodness (R^2=0.94 and 0.98, exon 9 and 20, respectively). The equation of this nonlinear regression was $Signal = d + (a-d)/(1+([bloq]/c)^b)$, where [*bloq*] is the concentration of blocking agent, *d* is the background signal, *a* is the signal

for the absence of blocking oligonucleotide, *c* is related to the concentration at the inflection point and *d* is related to the curve steepness. Table 1 displays the estimated values for both hotspots of *PIK3CA* gene. Significative differences were found in the value of *c*-parameter that is related to half maximal effective concentration (EC50) or blocking concentration that reduced the signal to half. The EC50 values for wild-type templates were about 2 times lower than the values calculated for mutant templates for both target regions. The observed differential behavior confirmed that the addition of blocking agent produced a higher reduction of RPA reaction on perfect-match template (native) than a mismatched template (mutant). A concentration of 50 nM was selected for further experiments.

Figure 3: Effect of blocking concentration on the end-point response of RPA-based methods: Reactions for exon 9 (a) and exon 20 (b). Discontinue lines correspond to the regression curve applying four-parameter logistic model.

Table 2: Regression parameters obtained applying four-parameter logistic model to experimental data: blocking concentration vs. end-point response of RPA-based methods.

Identification of mutations

The presence of a mutation was established from the results (electrophoresis or fluorescence) for blocked RPA reactions (single format). Then, the detection of a band indicated that a mutated variant was amplified, achieving a selectivity of 100 %.

The determination of the specific genetic variant needed an additional end-point method (i.e. chip hybridization, bead hybridization, among others). In the present study, a simple colorimetric microarray assay based on using allele specific probes was developed for discriminating each specific mutation for a particular hotspot (Figure SI.2). The resulting RPA amplicons were hybridized to the chip which carried probes complementary to the targeted sequence. The use of microarray detection of RPA products has been previously described in previous studies [21,27]. Nevertheless, the method was further improved to serve the purpose of integrated mutational analysis.

Pursing the goal of a point-of-care test, a low-cost DNA chip combined with consumer electronic device for the readout of results, was developed. Firstly, specific probes were designed to maximize the selective recognition process, considering thermodynamic

calculations (Table SI.3). The estimated free energy variation for the complexes between the specific probes to its fully complementary target sequence varied from -21.4 to -22.4 kcal mol^{-1} for exon 9 and from -21.7 to -22.4 kcal mol^{-1} for exon 20. The values calculated for mismatched complexes ranged between -14.0 to -20.0 kcal mol^{-1}. Then, the estimated differences were large enough for a discriminatory assay. Secondly, the probe immobilization on polycarbonate chips was examined via photochemical surface activation [28,29]. Under selected conditions, the conjugation of amine-functionalized probes to carboxylate groups on chip surface produced an amide bond. The calculated immobilization probe density was 3.5 pmol cm^{-2}. A stable and strong bound was not affected by pH, temperature or microfluidic flows (losses < 5%).

Latterly, the hybridization experiments were performed by incubating the RPA products on chips with allele specific probes immobilized in microarray format. So, the discriminating elements were spatially separated, but integrated in a single assay. The optimization criteria were a high response signal for perfect-match probe and minimal response for the mismatched probes. Therefore, the mutation can be identified due to the formation of a perfect-match hybrid between the RPA product and one specific probe. The composition of hybridization solution (formamide 20 %, low ionic strength) was critical for the restrictive recognition. Another important factor was the volume of RPA product dispensed on chip. A beneficial effect of assay performed in a microarray format is the reduced distance that molecules need to travel from the bulk solution to the solid-liquid interface. Selected volume (45 μL) minimized the time required to obtain high spots signal, associated to shortened diffusion times. Compared to PCR chip, higher background signals were registered for RPA products. However, the signal-to-noise ratio (S/N), calculated as the ratio of the signal and the standard deviation of the background noise, was between 12 and 22.

Under the selected conditions, unblocked RPA products from human cell cultures were hybridized on the chip. Given the presence of the biorecognition product, a variation of the reflection properties of chip surface was measured (Figure 4). If there was no reaction product, the maximum intensity of the reflected beam was collected (background signal). If target gene-probe recognition and subsequent solid deposit formation occurred, the light would strike the product, modifying the optical response. Wild-type products yielded a high response for their specific probes (S/N>15), and were low or null for the remaining ones (S/N<6). Mutant products of exon 9 (p.E545K) were specifically recognized for the corresponding probe (S/N = 12), since the spot signals were significantly higher than controls (t-test: p-value = 0,005). Mutant products of exon 20 (p.H1074R) hybridized to

two probes (wild-type and p.H1074R probe), giving detectable signals (S/N>15) with similar intensities (t-test: p-value = 0,07). These values agreed with a genomic DNA coming from a human cell culture that is heterozygote for this locus. Therefore, a sensitive and selective hybridization assay was achieved. The results obtained using a planar polycarbonate chip and a simple optical detection also demonstrated that the assay is compatible with a typical mass produced material and a sensing technology of DNA devices [16,22,30].

Figure 4: Probe Selectivity: spot intensity of array probes obtained from different unblocked RPA reactions and samples. Dashed line indicates control negative response.

Patient analysis

There were important analytical challenges associated to the mutation detection and identification related to solid cancer screening. DNA from FFPET used to determine mutation status was highly degraded due to fixation. In fact, the nature of clinical sample and the conservation mode led to low amount/poor-quality of DNA in same samples. Intact double strand DNA ranged from 10 to 270 ng μL^{-1}, with a purity 260/280nm ranged from 1.7 to 2.1. In addition, the small proportion of mutated DNA can limit the success of the assay. So, assay sensitivity was determined by preparing heterozygous mixtures with increasing percentages of mutant DNA compared to the wild-type type (Figure SI.3). These experiments emulated the wide range of clinical scenarios regarding the variable proportion of tumor cells (mutant DNA) respect to non-tumor cells (native DNA) in a biopsied sample. Experimental data was adjusted to a linear regression with an excellent model goodness (R^2=0.994), indicating that blocked RPA produced a proportional amount of mutated sequences to the initial concentration of mutant genomic DNA. Mismatched DNA was detected up to 5 %, which indicated that the system was capable of detecting the mutant variant, even in low concentrations. In absence of blocker, the mutant variants were only detected when the percentage was 2-8 times higher. These results confirmed the formation of a stable duplex between blocker and wild-type template, limiting the primer hybridization and consequently producing the mutant enrichment.

Intra and inter-day reproducibility, expressed as the relative standard deviation of spot intensities for the replicated assays (five replicates), were 13 % and 17 %, respectively. The ANOVA test showed that the end-point responses were comparable for the four studied genes (p-value > 0.05).

The next experiments were focused on the analysis of blind samples collected from oncological patients (formalin fixed and paraffin embedded tissues). For each sample, a blocked RPA reaction per exon was performed. The arrangement of the microarray matrixes on the chip was designed in such a way that 6 samples (4 spot replicates) would be analyzed in parallel. The incorporation of quality controls (positive and negative) helped to ensure reliable results considering the possible variation of analytical process. Examples of the obtained microarray images are shown in Figure 5. Despite of the blocked amplification, positive responses for both wild-type probes were observed in all chips (S/N 6±2). Nevertheless, a clear assignation was achieved based on the spot signals for mutant probes. Most of samples were assigned as wild-type for both studied locus (exon 9 and exon 20) because the mutant probe intensities were comparable to negative controls. The exceptions were two chips that showed a detectable response for one of the mutant probes. The S/N were 10±2 for p.E545K mutant and 17±2 for p.H1047R mutant. Therefore, patients were classified as mutant in exon 9 and mutant in exon 20, respectively. The assignations agreed with those obtained using next-generation sequencing technology in all cases. However, sequencing analysis required higher amount of DNA, labor-intensive sample preparation and took longer to generate data compared with our method. The results highlighted that blocked RPA was an adequate approach since the copy number of mutated regions was high enough to be detected and discriminated in a hybridization assay. Furthermore, the proposed method fulfilled the requirements for a mutational analysis in a simple health system framework (i.e. short analysis time, low cost, and simple).

Figure 5: On-chip hybridization results for oncological patient samples: (a) Microarray layout, (b) Blocked RPA for exon 9, sample: wild-type, (c) Blocked RPA for exon 9, sample: mutant p.E545K, (d) Blocked RPA for exon 20, sample: wild-type, and (e) Blocked RPA for exon 20, sample: mutant p.H1074R.

CONCLUSIONS

Increasing knowledge on genetic variants and availability of specific therapeutic agents is enabling the development of a more personalized oncogenic medicine (e.g. specific monoclonal antibodies). However, a parallel technological development is required for translating them to clinic routine. Real-time PCR, droplet digital PCR and DNA sequencing are the most widely used method for mutational analysis. Although this objective has been effectively addressed, they involve a labor-intensive expensive

solution or a limited capability in multiplex analysis. This study is aligned with the research line of developing alternative solutions.

RPA-based methods are a successful approach for supporting portable diagnostic DNA assays due to their performances. However, this study is the first in demonstrating that blocked RPA is an adequate mutant enrichment technique. The results have shown that this blocking behavior is a less effective process compared to other approaches, but the achieved sensitivity (about 5 %) and reproducibility (about 15 %) are enough for their application to biopsied samples in solid tumors.

The other relevant issue approached in this study is the discrimination of point mutations. In large hospitals, several methods are applied for determining the presence of a mutation in certain locus, without identifying the specific variant. Others perform a reaction per each mutation. The consequences are a lack of information limiting the possibilities of modern oncology or an increase of laboratory efforts, reducing the sustainability of the health system. Chip-based detection approaches, such as we have introduced, show the advantage of multiplexing. All investigated variants can be implemented on the same microarray. In addition, the inclusion of controls guarantees the assay reliability. Indeed, the optimized protocol is faster (performed in less than 2.5 h) and easier to handle than sequencing.

According to the amplitude of described applications based on blocked PCR, the expectations of the blocked RPA are high. Furthermore, the method can potentially support the detection/discrimination of mutations in more health scenarios (e.g. small hospital or clinics). Demonstrated for *PIK3CA* mutational analysis, the next experimental activities are addressed to expand to other mutations. Then, a better stratification of patients, or division of patients into subgroups based on the molecular characteristics, can be achieved and a subsequent individualized treatment can be assigned.

Conflict of Interest Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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667 complexes between probes and templates. The bold numbers correspond to perfect-
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669

670 Figure SI.1: Images of agarose gel electrophoresis after amplifying *PIK3CA* gene exon
671 20: (a) PCR products and (b) RPA products after centrifugation-based purification. 1:
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673 Wild-type template (human cell line).

674 Figure SI.3: Identification of mutation based on chip hybridization assay: (a) Surface
675 activation (photochemical treatment), (b) immobilization of probes (amide bond
676 formation), (c) incubation with RPA products, (d) image developing.

677 Figure SI.4: Spot intensity of array probe for mutation p.H1047R obtained from different
678 concentrations of mutant DNA. Target region: exon 20. Sample: cell culture HCT 116.
679 Linear model: $y = (94\pm3)\,x + (10\pm160)$, $R^2 = 0.994$. Limit of detection: 5.1 %.

680

681 **TABLE 1**

682 (a)

Criteria	Restriction
Sequence[1]	Complementary to the wild-type variant
Mutation position	Central position of oligonucleotide
Melting temperature[2]	Higher to both primers (upstream and downstream primers)
Secondary structures	Low stability
Self-complementarity	None
Modification	3'-end to inhibit the extension
Upstream primer position	Overlap in order to induce the competition for the binding site in DNA template

683 [1] Oligonucleotide specificity must be checked, i.e. alignment against genes of NBCI databank.

684 [2] Temperature at which half of the blocking oligonucleotides are single-stranded (ssDNA) state.

685 (b)

Variable	Studied range	Selected
Magnesium acetate concentration (nM)	14-30	14
Primer concentration (nM)	42-420	420
Blocker concentration (nM)	0-600	50
DNA (ng)	0-40	4
Temperature(°C)	25-45	37
Time (min)	0-60	40

686

687

688 **TABLE 2**

		a	b	c	d	R^2
exon 9	wild-type	10000±300	1.5±0.3	62±8	700±300	0.990
	mutant p.E545K	10000±200	1.5±0.2	140±14	600±400	0.995
exon 20	wild-type	10500±400	1.5±0.2	22±2	800±400	0.980
	mutant p.H1047R	10000±300	1.5±0.2	44±5	800±300	0.982

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