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Nucleotide-selective amplification and array-based detection for identifying multiple somatic mutations

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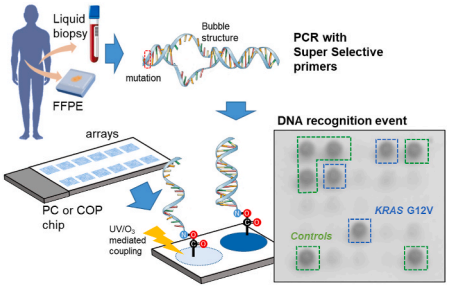
HIGHLIGHTS

- High-performance optical biosensing is achieved for mutation analysis.
- The nucleotide discrimination arises from selective amplification and array detection.
- The specific oligo design provides the genotyping of multiple variants in a single experiment.
- The registered signal is excellent as regards homogeneity, reproducibility, and intensity.
- Patients were correctly discriminated according to identified KRAS genotypes.

GRAPHICAL ABSTRACT



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ABSTRACT

In the context of personalized and cost-effective treatment, knowledge of the mutational status of specific genes is advantageous to predict which patients are responsive to therapies. As an alternative to one-by-one detection or massive sequencing, the presented genotyping tool determines multiple polymorphic sequences that vary a single nucleotide. The biosensing method includes an effective enrichment of mutant variants and selective recognition by colorimetric DNA arrays. The proposed approach is the hybridization between sequence-tailored probes and products from PCR with SuperSelective primers to discriminate specific variants in a single locus. A fluorescence scanner, a documental scanner, or a smartphone captured the chip images to obtain spot intensities. Hence, specific recognition patterns identified any single-nucleotide change in the wild-type sequence overcoming qPCR methods and other array-based approaches. Studied mutational analyses applied to human cell lines provided high discrimination factors, the precision was 95%, and the sensitivity was 1% mutant of total DNA. Also, the methods showed a selective genotyping of the KRAS gene from tumorous samples (tissue and liquid biopsy), corroborating results by NGS. The developed technology supported on low-cost robust chips and optical reading provides an attractive pathway toward implementing fast, cheap, reproducible discrimination of oncological patients.

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1. Introduction

The accurate identification of single point mutations is important for diagnosing cancer and determining prognosis, definitively, the selection of personalized therapy based on a genotype-driven approach [1]. Also, continuous monitoring of mutation occurrence is critical because new genetic alterations arise during the treatment (i.e., drug resistance), leading to modifying the best therapy [2]. Currently, different technologies perform mutational analysis, even identifying the single base replaced [3]. However, their high cost, time-consuming, labor-intensive, and low portability limit the implementation of frequent testing in the health system.

As an alternative to sequencing, novel DNA amplification methods incorporate an enrichment of mutant variants to enable their selective exponential synthesis [4,5]. A reported solution is to suppress the wild-type primer extension, adding a blocking oligonucleotide designed to bind complementarily to this sequence [6,7]. Nevertheless, most described methods report false positives due to unspecific amplification and cannot provide information about the specific mutant variant. For improving assay selectivity, specific-sequence primers, called Super-Selective primers, have been developed to destabilize mismatched interactions [8]. In these approaches, the unique design aids in correctly identifying a mutation in the selected hotspot based on real-time fluorescence signals in a qPCR instrument.

Regarding the simultaneous detection of several hotspots, array technology remains the best-suited approach in the diagnosis field [9,10]. Overcoming traditional glass chips and fluorescence scanners, relevant scientific advances come from thermoplastic polymer chips that enable low-cost optical sensing in platforms with high-volume fabrication, e.g., micro-injection moulding and hot-embossing [11]. Other advantages are high sensitivity, versatility in sample handling, fluidic manipulation, and biosensing detection.

In the last decade, new integrated systems and sensors fulfilled the requirements for point-of-care devices aimed at genotyping multiple loci and the massive monitoring of cancer patients [12,13]. Although ingenious approaches have been published based on novel techniques (i.e., isothermal reactions, CRISPR technology), few have been applied to complex clinical samples, i.e., solid tissues or liquid biopsies. The relevant challenge is reaching the required performances for low-abundant mutant sequences in a multiplexed mode. A reliable strategy can be to integrate a selective DNA amplification method and an effective process able to differentiate specific single-nucleotide changes.

The present study aims to develop a selective amplification process (blocked PCR and SuperSelective primer-PCR), multiplexed hybridization assays, and optical sensing addressed to mutational analysis and supported in a tailored oligonucleotide design. The research focused on the recognition events (template/primer/probe) that allow the detection of somatic mutations in the presence of abundant wild-type sequences. In fact, it is the first time that products of PCR with SuperSelective primers are discriminated against and detected using a DNA array. The selected materials are common substrates for manufacturing IVD devices, such as polycarbonate (PC) and cyclic olefin polymer (COP). The studied immobilization method is a smart soft photochemical activation to attach amine-functionalized oligonucleotides on demand with precise spatial control and low background signal. For low-cost array imagining, the proposed solution is a documental scanner or a smartphone [14].

Concerning the clinical impact, the research objective was to confirm that the array-based method can allow a relevant advancement toward more versatile and cost-effective genotyping tool. The studied target was *KRAS* oncogene because the knowledge of its mutation status is nowadays used to predict which patients respond to *anti*-EGFR therapies and to prevent the development of long-term complications [15–17]. Moreover, this oncogene shows several relevant hotspots whose genotyping is helpful for therapy stratification and disease monitoring. Therefore, the novel biosensing method could support a facile, repeatable assessment of the mutational state of a given tumour in a miniaturized parallel format.

2. Experimental

2.1. Samples

DNA isolated from human cell lines SK-N-AS (ATCC CRL-2137) and HCT 116 (ATCC CCL-247) were used for the method optimization. Also, reference standards of wild-type and mutant genomic DNA (Horizon Discovery) were employed.

Twenty tumoral tissue fixed in formaldehyde and embedded in paraffin (FFPE) were collected from patients with metastatic colorectal cancer. The extraction of DNA was carried out using the QIAamp DNA Investigator kit (Qiagen) following the manufacturer's recommendations. Five blood samples (liquid biopsy) from patients with CCRm were processed as previously described [18]. MagMax cell-free DNA isolation Kit (ThermoFisher Scientific) was used for DNA extraction. The DNA amount obtained was quantified by spectrophotometry (NanoDrop 1000 Spectrophotometer) and fluorimetry (Qubit Fluorometer, Life Technologies). The average concentrations were 87 ng/μL and 1.7 ng/μL for FFPE tissue and liquid biopsy, respectively. The extracts were stored at −80 °C until processing. Target variants are listed in Table S1.

2.2. DNA amplification

PCR in blocked format. Each reaction mixture contained two region primers and a blocking oligonucleotide complementary to the wild-type variant. Table S2 lists all used oligonucleotides and Supplementary Material includes the amplification protocol.

PCR in SuperSelective format. Each reaction mixture contained DNA polymerase buffer (5x), $MgCl_2$ (2.5 mM), a mix of deoxynucleotide triphosphate (100 μM of each), digoxigenin-labeled dUTP (10 μM), upstream primer (800 nM), downstream primer (800 nM), genomic DNA (10 ng), and GoTaq Hot Start DNA polymerase (Promega, 2.5 unit). The reaction was performed under the following conditions: activation at 95 °C for 10 min and amplification (95 °C for 30 s and 60 °C for 60 s). The cycles were 45 for G12C and G12V and 40 for G12D and G13D. The diluted products (10^{-6}) were mixed and amplified using 300 nM (forward primer) and 300 nM (reverse primer). Table S3 shows oligonucleotide sequences. The thermal cycling was six cycles of 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s.

qPCR. For optimization purposes, real-time fluorescence measurements were also performed as described in Supplementary Material.

2.3. Hybridization

The DNA chips of cyclo-olefin polymer (COP, Zeonor 1060R) and polycarbonate (PC, Makrolon) were prepared following the protocols described in Supplementary Material. For products of PCR, the hybridization buffer was composed of 1 × saline sodium citrate buffer, 2.5 × Denhardt's, and 25% formamide. For products of PCR in SuperSelective format, the hybridization buffer was composed of 2 × saline sodium citrate buffer, 2.5 × Denhardt's, and 10% formamide.

A dilution of amplification products (1:10) was used and the resulting solutions were incubated in a thermal block at 96 °C for 10 min. The solutions were transferred on ice for 1 min, and 40 μL of each sample was dispensed on the corresponding matrix. The samples were incubated for 1 h at 37 °C and washed with hybridization buffer 0.1 × and 0.01 × .

2.4. Reading and data processing

Two optical detection approaches were studied, depending on the PCR labeling: fluorescence (Cy5 label) and colorimetry (digoxigenin label). For colorimetry, the protocol included a 30-min staining step. Briefly, the development reagents were a mix of 1:2500 monoclonal

anti-Dig antibody (Abcam) and 1:400 monoclonal anti-sheep-HRP antibody in PBST. Later, peroxidase substrate was added for 3 min, and the surface was washed for immersion in water and dried. A fluorescence scanner (Genepix Pro 4000 B model), a simple documental scanner (Epson Perfection 1640 model), and a smartphone (model Huawei P Smart) registered the microarray images.

Software Genepix (Axon instruments) and Image J (NCBI) were used for image analysis. Array quality was examined by studying the morphology of spots, including cross-section profiles, intensity homogeneity, diameter homogeneity, and circle shape. The analytical signals were the spot intensity minus the chip background. Experimental noise values were calculated as the standard deviation from 15 blank measurements (regions without probes). Signal-to-noise ratios (SNR) were obtained by dividing the analytical signals between noise values. The detection limits were inferred from the experimental concentration corresponding to SNR equal to 3. Assay reproducibility was estimated from replicates (intra-chip, inter-chip, and inter-day). For the identification of mutations, a discrimination factor was calculated from SNR mutant and SNR wild-type and the threshold value was 0.5. The identified genotypes were compared to the results obtained by Next Generation Sequencing studies and real-time PCR based on a commercial kit (Supplementary Material).

3. Results and discussion

3.1. DNA chip development

The initial step was activating plastic chips to generate active groups for probe immobilization. The selected approach was ultraviolet irradiation in an ozone atmosphere followed by basic hydrolysis. Thus, the photochemical cleavage of surface bonds enabled the covalent immobilization of amino-modified probes to form carboxylic groups.

Since oxygen-containing groups are responsible for the change of surface hydrophilicity, the activation efficiency was monitored from the variation of contact angle. Fig. S1 shows the effect on contact angle under different experimental conditions. The results also confirmed that the chips of PC and COP were hydrophobic in the untreated areas. In contrast, the photo-exposition and basic attack produced carboxylic groups, making both surfaces hydrophilic. The selected conditions were 5 min of UV/O_3 treatment and 10 min of basic incubation (1 M NaOH at 60 °C).

Experiments with the activated chips were conducted to test the binding capability of functionalized oligonucleotides. The selected approach was via carbodiimide reaction by spotting the primary amine probes onto the activated chips. Following a standard conjugation mechanism, the carboxyl groups of chips reacted with NH_2-functionalized probes, yielding an amide bond. Microscopy measurements confirmed that the chips maintained mechanical properties and surface homogeneity (Fig. S2). The results revealed that feature fidelity and signal intensity were maintained across the chip (Fig. S3), yielding homogenous and high signal spots on both COP and PC surfaces. The estimated immobilization density was 2–6 pmol/cm^2 (10^{12}-10^{13} molecules/cm^2), and storage of the DNA immobilized chip was more than one month at 4 °C. These features were comparable to those obtained in other oligonucleotide functionalized chips [6,10,17]. The advantage of our strategy is that the already existing technologies can strongly support the fabrication of probe-attached chips in a flexible and large-scale fashion.

The behavior of these chips as analytical platforms to transduce DNA biorecognition events was explored (Fig. 1). Low background signals confirmed the absence of significant unspecific binding. For the same probe concentration, the immobilization/biorecognition yield was higher for PC than COP, considering the signal-to-noise ratios achieved (*t*-test, p-value <0.05). Flow stability experiments (0.5–20 mL/min) in microfluidic chambers confirm the resistance of spots against washing protocols. The nature of the chip surface enabled the use of standard buffers of DNA assays, such as saline-sodium citrate or phosphate-buffered saline. Also, this experiment confirmed the potential integration of biorecognition assay in microfluidic devices. The potential advantages are miniaturization, operational simplicity, and reduction of contamination risk.

Regarding sensing features, the select oligonucleotide attachment on plastic chips was compatible with conventional optical detection. Fluorescence scanning and colorimetric sensing (scanner or smartphone camera) provided excellent chip images with high spot signals (Fig. 2). Therefore, these experiments verified the successful immobilization of the probes, the recognition of the target sequence, and versatile optical detection using lab instruments or consumer electronic devices.

3.2. Detection of blocked PCR products

The developed DNA chip was adapted for sensitively detecting blocked PCR products based on a hybridization pattern on allele-specific probes immobilized in array format. This approach shows limited novelty because it combines already-developed technologies. Nevertheless, these experiments verified the sensing capabilities of the integrated solution and their potential advantages. In conventional approaches of blocked PCR, a perfect-match single-strand oligonucleotide to wild-type DNA is added to the reaction mixture inhibiting its amplification [19]. However, unspecific amplification and the formation of primer dimers can be important limitations for liquid-phase detection methods (e.g., qPCR) because it frequently causes false positives, especially for low-abundant variants [4]. In order to avoid this problem, a sensitive and selective hybridization assay in microarray format was designed.

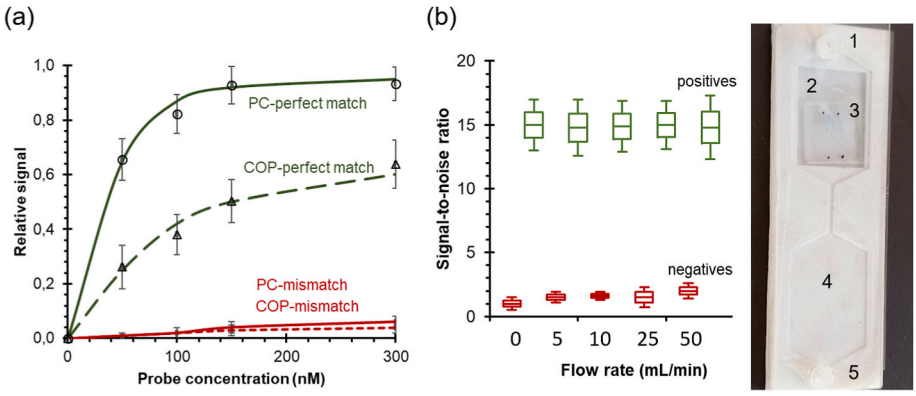
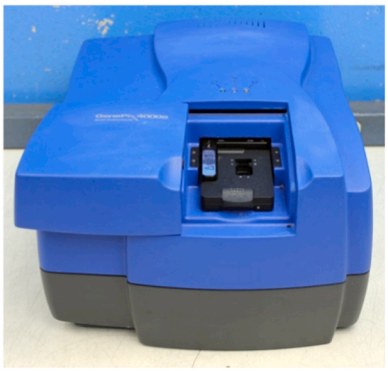
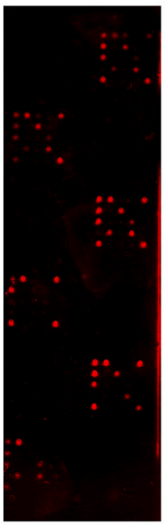


Fig. 1. Optical chip-based detection. (a) Effect of probe concentration (dispensation volume = 25 nL, incubated oligonucleotide = 40 fmol). (b) Spot intensities registered in a chip located in a microfluidic chamber. Platform dimensions: 75 × 25 × 1.5 mm. Components: 1. Input hole, 2. Chamber. 3. PC chip 12 × 12 × 0.6 mm. 4. Waste chamber. 5 Output hole. Target gene: KRAS codon 12. Probe: complementary to the native sequence. Replicates: 3.

(a)





(b)

(c)

Method	Direct	Indirect
Generated signal	Fluorescent	Colorimetric
PCR labelling	5'-end primer	5'-end primer
Marker	Cy5-fluorophore	Digoxigenin
Staining	-	Immunostaining
Staining time	0 min	40 min
Instrument	Fluorescent scanner	Scanner or smartphone
Instrument cost	> 9000 €	< 300 €
Scanning conditions	mode: 635 nm, capture resolution at 5 μm	mode: grey scale or color, capture resolution at 600 ppp
Scanning time	10 min	5 min

Fig. 2. Chip images captured depending on transduction system (a) Fluorescence: Microarray scanner. (b) Colorimetric: flatbed documental scanner or smartphone. (c) Description of detection methods. Chip: 75 × 25 mm, 12 samples per chip. Spot diameter: <150 μm.

First, the enhanced amplification of mutant variants was studied, with changing factors such as blocker concentration, primer concentration, temperature, and thermal cycle program (Fig. S4). Then, the selected blocker concentration was 100 nM (stoichiometric ratio 1:3 blocker:primer), and the annealing temperature was 65 °C for 35 cycles. The estimated inhibition factors (50–60%) demonstrated that the blocking agent prevented the elongation step of wild-type variants, enabling the preferential replication of mutant variants for a wide range of concentrations.

The products from the blocked PCR method were incubated on the chips with specific immobilized probes, designed for maximizing the hybridization of perfect-match pairs (wild-type or mutant) and hampered the coupling of mismatched products. Also, variables, such as buffer composition, time, and washing cycle, were studied to balance yield and selectivity. Adequate morphology parameters, such as spot shape, intensity homogeneity, and low background noise, were registered (Fig. S5). The features were similar to those obtained in other allele-specific DNA biochips [20]. Compared to the qPCR approach, the hybridization process increased the operation time, but the experiments confirmed a multiple detection and better identification accuracy (Fig. 3). The amplification curves showed that the non-specific types still had strong amplification signals, just with delayed cycles. However, high spot signals were clearly observed only in the presence of mutant variants. As expected in heterozygote samples, wild-type products were also detected, but with lower intensities than mutant products. Thus, each product preferentially hybridized onto the correspondent probes and failed to bind the mismatched probes.

The calculation of the quotient of SNR between mutant and wild-type (discrimination factor) allowed the accurate detection of mutant variants, as demonstrated by the excellent agreement with the reference method (sequencing). Then, the challenge of the adequate classification of oncological patients was achieved. Other key enabling features were the reduced time to results, high information per chip, high sensitivity (1000 copies of genomic DNA), and good reproducibility (>95%). The efficiency of the developed method has been demonstrated in terms of genotyping precision, rendering a DNA biochip "fit for purpose".

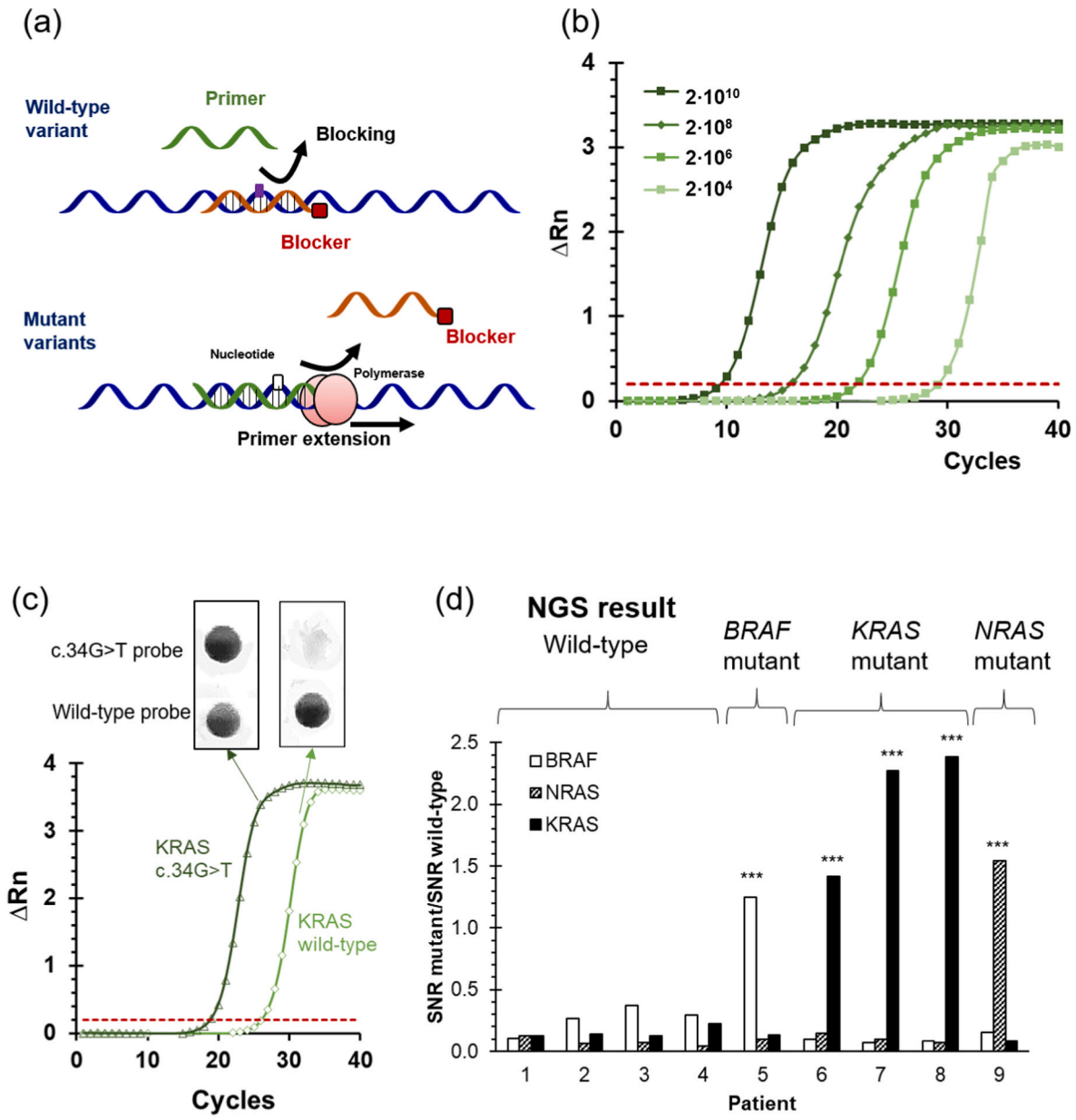


Fig. 3. Discrimination of blocked PCR products. (a) Scheme of selective amplification of mutant variants based on blocked PCR. (b) qPCR curves depending on DNA copy number (Sample: Heterozygote, c.34G > T variation). (c) Comparison of qPCR curves and chip spots obtained for the same samples: wild-type and mutant. (d) Signal-to-noise ratio between the mutant probe and wild-type for oncological patients after blocked PCR and hybridization on the developed chip. ***: *t*-test, p-value <0.001.

3.3. Detection of SuperSelective PCR products

The objective was that the developed chips combined with a more selective amplification could identify the specific nucleotide change involved in a mutational process, improving the performance over the current approaches. Given the required selectivity and sensitivity, the PCR enrichment using SuperSelective primers was chosen (Fig. 4a). However, the design of primers and probes is challenging to detect closely related mutations in multiplex assays [8,16]. The common problem is that the primer sequence must not only distinguish against wild-type sequence, but also distinguish against each of the other variants. Thus, the challenge was obtaining the correct sequences and selecting the required conditions for amplification/hybridization assay on the novel chips to detect different mutations located in the same region or codon.

The target point-nucleotide changes were in the *KRAS* gene, and the studied variations (exon 2, codon 12/13) were the most prevalent mutations (about 82% of the variations described in oncological patients) [15,17]. The primers were designed to incorporate recognition elements and intense interaction (Table S4). The first region, called the 5′-anchor sequence, hybridized strongly with the target DNA fragment, and the second, called the 3′-foot sequence, was short and mismatched with the wild-type sequence or other variants. Both regions were perfectly complementary to the mutant target sequence and physically and functionally separated by a non-coding sequence called the bridge. Also, the primers were chosen to avoid homology to either other primers or other sequences in the human genome.

Real-time PCR assays confirmed the correct amplification of each mutated DNA and a delay for the rest of the variants (wild-type and mutants), as showed threshold cycles (Ct) for each reaction (Fig. 4b, Fig. S6). The control reactions did not generate any unspecific product, such as a primer-dimer. The reactions containing mismatched templates

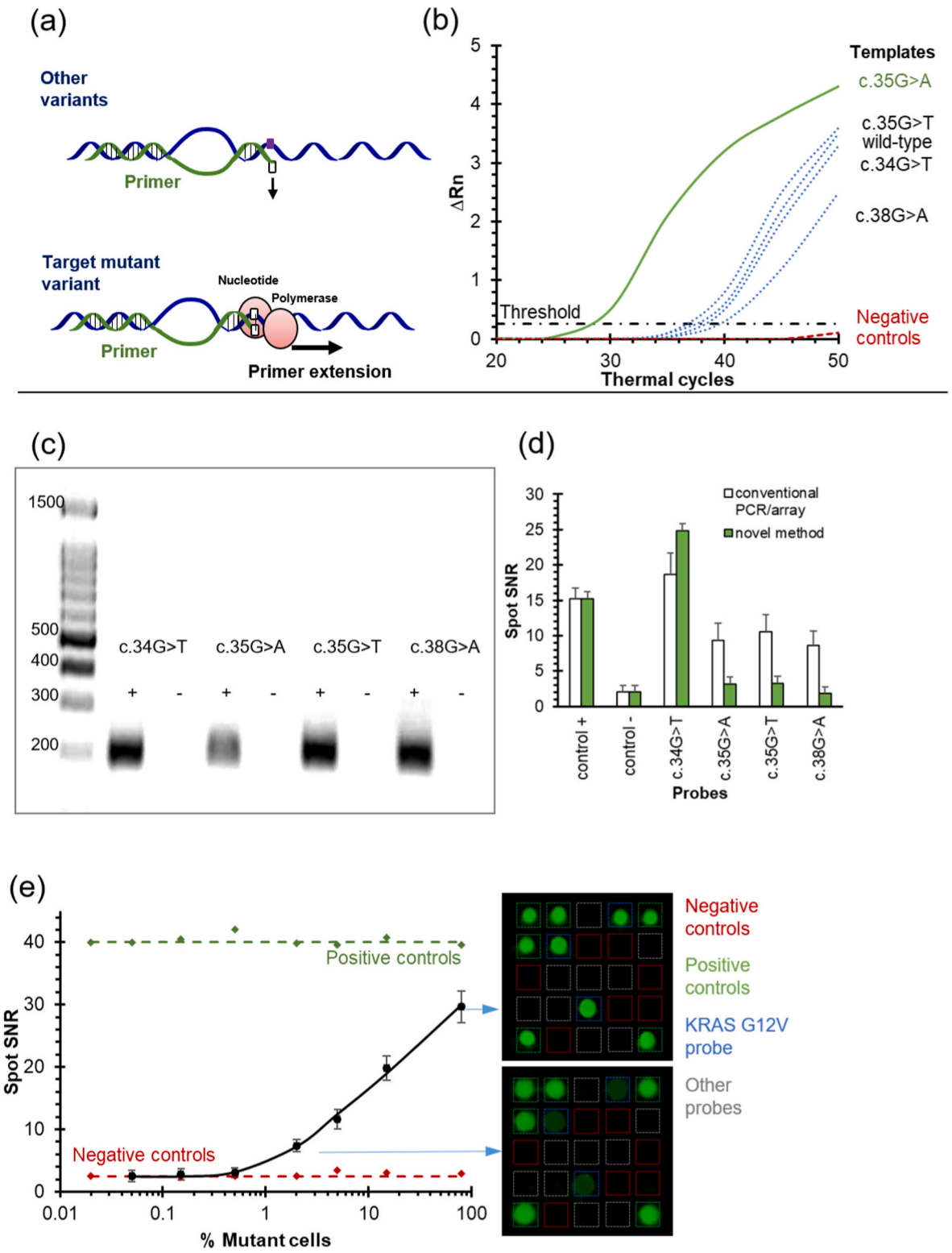


Fig. 4. PCR with SuperSelective primers: (a) Amplification mechanism. (b) qPCR curves for different DNA templates using the selective primers of c.35G > A variation. (c) Electrophoresis gel of amplified products (1.5% TAE agarose gel). (d) Spot signals compared to a conventional array method based on a PCR amplification and allele-specific probes (single-nucleotide modification). (e) Signal-to-noise ratio of spot signals depending on the mutant cell percentage (Sample: Heterozygote, c.34G > T variation).

started later because the selected structure of primers suppressed the unspecific extension from 5.6 to 11.0 cycles (Table 1).

The observed selectivity was mainly associated with a mismatch in the 3′-end nucleotide and the structure of the primer. Finally, results confirmed that the primers could form enough stable hybrids with the mutant target sequence (perfectly complementary). However, they were unlikely to form considerably weaker hybrids with all mismatched sequences. Together with these thermodynamic factors, kinetic factors encouraged the formation of perfect-match hybrids. Under annealing conditions, the hairpin structure primers led to shorter persistence times

Table 1
Average thresholds cycles (Ct), depending on templates and primers used in the amplification mixture.

Template	Primer			
	KRAS c.34G > T	KRAS c.35G > A	KRAS c.35G > T	KRAS c.38G > A
Negative control	>50	>50	>50	>50
KRAS wild-type	34.4	41.5	38.3	37.1
KRAS c.34G > T	28.8	40.0	38.0	37.7
KRAS c.35G > A	34.1	29.6	38.5	36.5
KRAS c.35G > T	34.1	39.0	29.4	36.8
KRAS c.38G > A	34.8	42.0	40.0	29.2
ΔCt	5.6 ± 0.4	11.0 ± 0.4	9.3 ± 0.4	7.8 ± 0.4

for unspecific hybrids. The estimation is in the millisecond range, unlike the conventional primers, whose mean persistence time is in the minute range [8]. It is worth emphasizing that once the primer initiated the synthesis, the replicated products incorporated the entire sequence of the primer. Accordingly, gel electrophoresis corroborated the generated product length (195 bp, Fig. 4c).

Although SuperSelective primers can be used in multiplex real-time PCR assays, there are decisive restrictions [8]. Differences in foot sequence can affect the hybrid strength (enthalpy) and the probability that the hybrid will form (entropy). As a solution, these authors proposed a fine-tuned design, adjusting the length or sequence of the bridge. Nevertheless, it requires an extensive optimization process. Our research hypothesis was that solid-phase hybridization could be the most appropriate approach for multiplexed assays. In this novel approach, the research aimed at hybridizing allele-specific PCR products and probes immobilized on the chip.

The array probes were selected considering the stability of perfect-match and mismatch complexes for all possible variants. A relevant criterion was that the probe included the target nucleotide and the bridge sequence for each target. The aim was to increase recognition yield and to reduce non-specific interactions because this region is only present in the target product. In fact, the optimization experiments showed that the hybridization required less restrictive conditions than the previously presented method based on blocked PCR. The composition of the hybridization buffer and washing solutions contained less formamide percentage, indicating that the non-specifically bound DNA was easily removed and only kept perfectly matched hybrids.

The incubation of products from the PCR with SuperSelective primers on the chips with specific immobilized probes led to accurate hybridization patterns (Fig. 4d). The discrimination ratios and, consequently, the assay selectivity, were higher than those obtained using a conventional PCR/allele-specific array [7,14] or the new array-based blocked PCR method. The better results can be explained by considering the probe sequences and the thermodynamic analysis of perfect-match pairs against the mismatched products. In conventional PCR/allele-specific array and blocked PCR, the probes for a specific mutation changed in a single nucleotide respect to the rest of allele variants (mutant or wild-types). While in PCR with SuperSelective primers, each probe had a specific sequence, considering target nucleotide and bridge. Thus, the number of different nucleotides of a certain mutant probe with respect to the rest of the variants was 13–14. The estimated variation of Gibbs energy was higher for perfect-match pairs and with a melting temperature 20–40 °C higher (Table S5). Also, the analytical performance was excellent since the achieved precision was 5–12%, the working template amount was 2500 copies/μL, and the sensitivity was 1% mutant in total DNA (Fig. 4e). These values determined from human cell lines met the needs of clinical application.

The current approach showed several advantages over existing methods that use SuperSelective primers and fluorescence measurement during the amplification. In a qPCR instrument, a meaningful Ct value was observed for the target mutant variant, but the rest of the variants were also amplified, hindering the interpretation. Also, the multiplexing capability is limited by the number of detection channels and compatible fluorophores. Instead, array-based detection offers an efficient solution. Even increasing the operational time, the generated pattern is easily interpreted, more selective, and the primer labelling is simple. Moreover, the required equipment is only a simpler thermocycler and a smartphone as the alternative to a multi-channel qPCR instrument. In conclusion, the novel modification of SuperSelective PCR, combined with a solid-phase hybridization, significantly improves the existing methods for mutation identification (Table S6).

3.4. Patient genotyping

Recognition patterns based on the complementary probes immobilized in an array format on the chip provided unequivocal genotype discrimination. For that, the probe design considered the specific recognition of construction (anchor, bridge, and foot); meanwhile, the length and sequences were tailored for a fast-stable hybridization in array format (multiplex assay). Control probes guaranteed the assay quality. The chip array layout (i.e., center-to-center distance, spot diameter, number of replicates) was designed based on the surface wettability and the resolution of any array reader. The initial experiments addressed determining the performances using DNA from cell lines (cancerous and wild-type cells), hybridizing the PCR products at low temperatures (37 °C), and short times (1 h). Excellent chip images were registered, even using low-cost imagining systems like a document scanner. Different hybridization patterns were obtained depending on patient genotype (Fig. S7). It is relevant to emphasize the absence of unspecific background, thanks to the composition of the chosen hybridization buffer and the medium polarity of the chip surface. According to the recorded signal-to-noise ratios, the method was highly selective since only the probes complementary to the hybridized product showed a high spot intensity. At the same time, the values in the rest of the spots were practically nil. Discrimination factors (signal quotient between target and other variants) ranged from 5 to 30.

The patients were classified considering the mean SNR value per each probe in the correspondent arrays and the results compared to those obtained using the NGS technique (Fig. 5). The clinical specificity was 100%, the sensitivity was 77%, and the areas under the ROC curve were 0.57, 1.00, 0.80, and 1.00 for mutations G12C (c.34G > T), G12D (c.35G > A), G12V (c.35G > T), and G13D (c.38G > A), respectively. A detailed analysis (Table S7) showed that all cases without a mutation in the targeted locus were correctly classified (cases = 87). Also, positive cases were satisfactorily detected and identified (cases = 10). However, one case of G12C-mutation and two of G12V-mutation were not detected. As the array technique showed a worse detection limit than the NGS technique, these false negatives were interpreted due to the low number of mutant cells. The effect of sample nature was ruled out because of the discrepancies observed in PTFE tissues (1 case) and liquid biopsies (2 cases). Therefore, these results confirmed its potential for supporting the selective identification of single-nucleotide variations in complex samples.

Regarding the impact, the application scope of our genotyping tool is high because the analytical performances were comparable to other diagnostic tools for detecting the mutational status of oncogenes based on qPCR [19,21], silicon array chips [10], magnetic devices [22], lab-on-chip platforms [23], electrochemical sensors [24–26], and optical sensing devices [6]. The novel method showed good sensitivity, only overcome by digital PCR technology [27]. The main advantages were the multiplexing capability, competitive cost of materials, and simplicity of use.

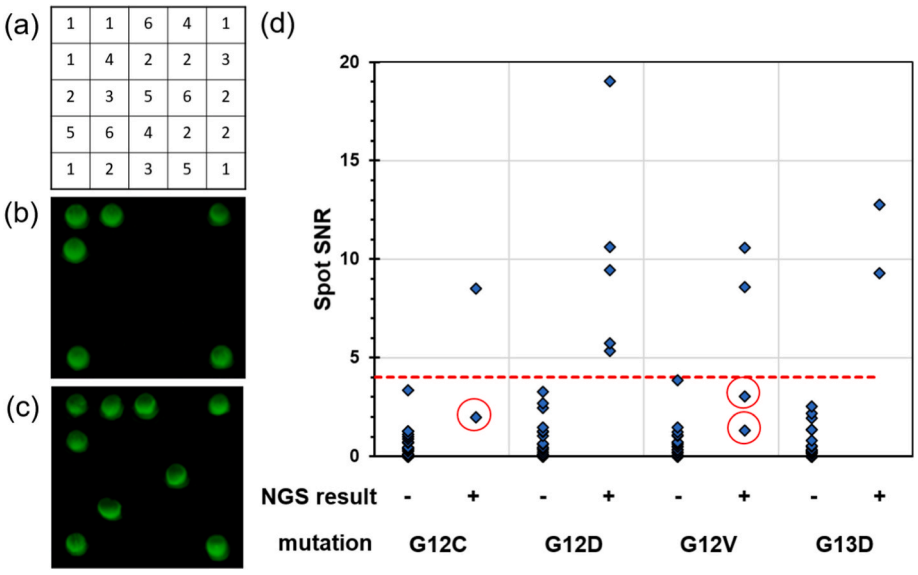


Fig. 5. *KRAS* genotyping from biopsy samples in protein format annotation: (a) Probe layout: 1: positive controls, 2: negative controls, 3: G12C, 4: G12D, 5: G12V, 6: G13D. (b) Fluorescent array image of wild-type patient. (c) Fluorescent array image of G13D-mutant patient (d) Signal-to-noise ratio of spot signals. NGS technique: (−) Patients with negative results. (+) Patients with positive results. Red dashed line: threshold limit for array-based diagnosis.

4. Conclusions

Identifying specific mutations in genomic DNA is essential for the personalized treatment of a broad spectrum of diseases and disorders. Although high-sensitive techniques are currently available, they show limited features for a sustainable clinical practice. Efficient medical care requires new promising technologies able to provide a fast-cheap answer about the best medical therapy and predict its effects, especially considering multiple biomarkers. This research contributes to the relevant challenge of adopting new diagnostic strategies which are easy to use, with lower cost, increased accuracy, and reproducibility of results.

The results confirm the potential benefits of soft photoactivation applied to thermoplastics as analytical substrates. Beyond the obvious advantages of being relatively inexpensive, scalable, and compatible with mass production techniques and methods used to manufacture DNA arrays, we believe this capability will enable new biochips with a unique approach to monitoring cancer patients.

A reliable application in the molecular diagnostic area has been demonstrated, determining the mutational status of a critical oncogene such as *KRAS*. The chips have been successfully validated for mutational analysis (blocked PCR variant). Nevertheless, the main novelty of the presented research is identifying the specific mutation as the result of developing an array-based approach for detecting products from SuperSelective PCR variant. Moreover, the prototype detects up to multiple variants in a single experiment. Although the hybridization step is time-consuming, the discrimination patterns are more evident than those registered by qPCR approaches or arrays supported on allele-specific probes. Our biosensing method has proved easy to use, more versatile for multiple mutations located in the same gene, and cheaper than conventional methods supported on bulky instruments.

Some recommendations for the successful extension to other hot-spots have been included. The most important factors are a correct design of involved oligonucleotides and a fine selection of the hybridization process, including the chip activation, functionalization, and handling. Another of the following research stages can be the assay miniaturization in a single chip, i.e., a microfluidic chip fabricated using thermoplastic substrates. Results are especially suited for developing miniaturized biosensor systems comprising optical detection schemes due to their easy processing and excellent optical properties, i.e., high transparency and low autofluorescence. The developed method also offers immediate opportunities to simplify genotyping into the clinical routine and to improve patient management with shorter turnaround times.

CRediT authorship contribution statement

Luis A. Tortajada-Genaro: Conceptualization, Data curation, Writing – review & editing, Supervision. **Ana Lazaro:** Investigation. **Sara Martorell:** Investigation. **Angel Maquieira:** Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aca.2023.341343.

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