Martorell, S.; Maquieira Catala, A.; Tortajada-Genaro, LA. (2022). A genosensor for detecting single-point mutations in dendron chips after blocked recombinase polymerase amplification. The Analyst. 147(10):2180-2188. https://doi.org/10.1039/d2an00160h

ARTICLE

# Genosensor for detecting single-point mutations in dendron chip after blocked recombinase polymerase amplification

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

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A biosensing system was developed to accurately detect a single nucleotide change in the target organism genome, integrating a selective isothermal amplification and a sensitive dendron-mediated DNA hybridization assay in array format. The novelty arises from the coupling reactions of dendron and its use as a crosslinker. The allele-specific probes were oligonucleotide-dendron conjugates prepared by fast, clean click-chemistry (thiol-yne reaction) and coupled onto the photo-activated surface of polycarbonate substrates (carbodiimide reaction). The output was forest-array chips with multipoint-site crosslinkers and compatible with current microarray fabrication technologies. The products of blocked recombinase polymerase amplification (blocked RPA), formed at 37°C, were hybridized with attached probes for specific nucleotide genotyping. The developed approach exhibited a sensitive recognition of DNA variants compared to chips based on linear crosslinkers (10-100 folds), showing excellent analytical performances for planar chip and fluidic formats. The methodology was successfully applied to detect H1047R mutation in *PIK3CA* gene (c.3140A>G) from clinical samples of human cancer tissues, being the results consistent with sequencing techniques. The colorimetric biosensing method was reliable, versatile, low cost, sensitive (detection limit genomic DNA: 0.02 ng/µL), and specific (accuracy >95%).

## Introduction

The identification of mutations has an important role in the correct diagnosis/prognosis in human cancers and other diseases.[1] Although methods such as qPCR or sequencing are employed, innovative point-of-care testing is needed to expand testing capacity.[2] In this context, approaches based on isothermal amplification techniques are a powerful alternative due to excellent operational conditions for integrated devices.[3] Compared to PCR-based assays, recombinase polymerase amplification (RPA) is faster (20-40 min), works at a lower temperature (35-42°C), simplifying the assay and the required equipment.[4] RPA has been integrated into microfluidic devices,[5] microarray-based biosensors,[6] and lateral flow assay.[7] Thus, RPA is a potential candidate for exploiting methods with affordable, easy-to-use, fast, robust, and equipment-free solutions in decentralized laboratories and low-resource environments.

For the detection of single-point mutations, some RPA-based approaches have been described, such as RPA-microarray hybridization,[8] real-time RPA,[9] and zip-RPA.[10] A relevant variant, called blocked-RPA, was developed for selective amplification of particular DNA variants based on the enrichment of minority alleles.[11] The principle is the addition of perfect-match oligonucleotide to the native allele in the reaction mixture favours the replication of mutant variants. The method shows advantages compared to blocked-PCR[12] or isothermal variants regarding the number and cost of required reagents. However, maximizing the assay sensitivity is a pending challenge when the concentration of mutant cells in human samples is scarce compared to the native cells. Herein, we present an enhanced array-based method to reach the detection limit required to detect single-point mutations. The research aims to conjugate dendritic molecules with allele-specific probes to obtain chips that detect blocked-RPA products in a sensitive, versatile, multiplex, and easy-to-read mode.

In the last years, several authors have exploited the capability of dendrimers and dendrons to generate multi-attaching sites on surfaces, such as gold and glass.[13] Also, there are examples in plastics such as PDMS or PMMA.[14,15] The general approach is the chemical modification of these supports using dendrons to control surface wettability, probe spacing, or improve bioreactivity.[16-18]

A novelty of our study is the dendron-mediated immobilization strategy for developing a forest-array DNA biosensor onto a polycarbonate chip through the focal point. In this orientation of the dendron, the surface is hyperbranched, showing multiple peripheral groups. Moreover, the DNA probe-dendron conjugates can be photochemically bounded by smart click chemistry and immobilized in an activated polymer. So, the aim was that the attachment of the DNA probe takes place in a few minutes or even seconds with high yields.

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Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

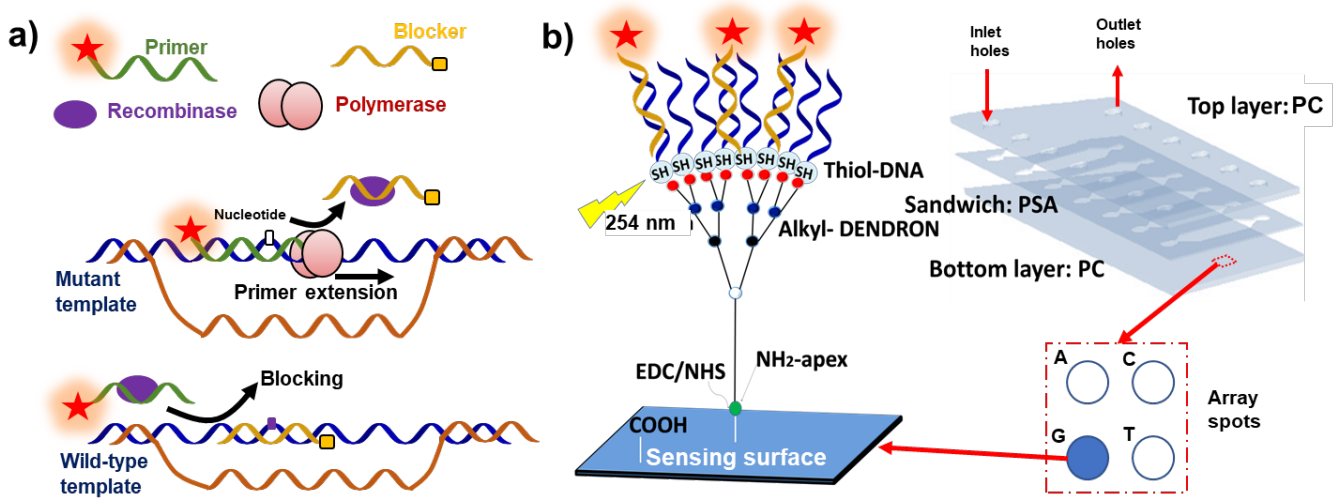



**Fig. 1.** Assay scheme for the genotyping of single-point mutations. a) blocked-RPA mechanism for allele-specific enrichment at constant low temperature. b) Selective hybridization to immobilized dendron-mediated probes onto a multi-chamber plastic chip.

It does not require additional reagents, catalysts, or solvents and it is clean due to the absence of subproducts. [19-20] This kind of DNA chip functionalization can exhibit prominent advantages for the fabrication of microchip-based analysis systems, including high automation, short assay time, and low reagent consumption.[21]

Therefore, our research hypothesis was that the accurate discrimination of single-point mutations is possible by performing a blocked isothermal amplification, a later hybridization in a dendron-mediated array of allele-specific probes (Fig. 1). Also, we explored efficient techniques for creating microscale systems compatible with colorimetric detection and focused on simple DNA diagnostic tools.

## Results and discussion

### Immobilization of DNA-probe/dendron conjugates

The first challenge was to obtain reproducible forest-like nanostructures on planar chips. So, highly-branched crosslinkers, such as dendrons, were conjugated to specific DNA probes before its immobilization onto activated PC surfaces (Fig. S1). The selected strategy was a click-chemistry reaction between alkyl-hyperbranched dendrons and thiol-oligonucleotides by irradiating at 254 nm. The thiol-yne reaction was triggered by UV photons and involved the radical addition of the sulphur atom to the unsaturation, generating a C-S bond. Thus, DNA-dendron hybrids were immobilized based on the coupling of $NH_2$-apex and the carboxylic groups of the chip surface by a carbodiimide reaction.

The effectivity of the dendron-mediated immobilization was studied, performing hybridization assays between attached probes and perfect-match oligonucleotides (Fig. 2). Experiments indicated that robust signals were obtained with a stoichiometric ratio of 1:10 (dendron:probe), achieving a fast photo-activated coupling (30 s). Also, the steric interactions of the bulky dendrons controlled the density of anchor molecules bound to the surface. The result was the reliable attachment of probes onto the chip surface under mild reaction conditions.

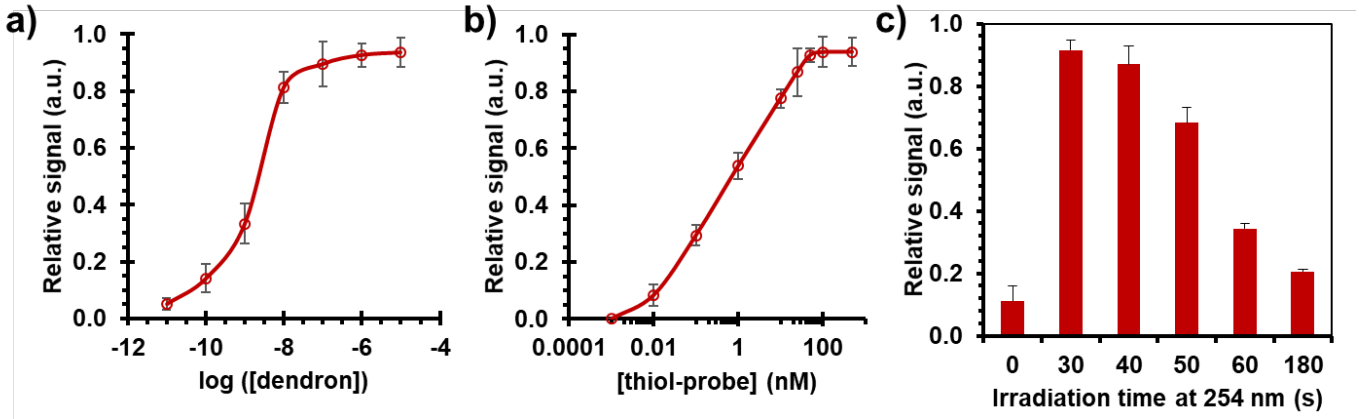



**Fig. 2.** Formation of DNA-probe/dendron conjugates: a) Effect of dendron concentration using 100 nM of the thiol-functionalized probe. b) Effect of the concentration of thiol-functionalized probe at $10^{-8}$ M of alkyne-dendron. c) Effect of irradiation time. Signals correspond to a chip after a hybridization assay of a labelled oligonucleotide complementary to the probe.

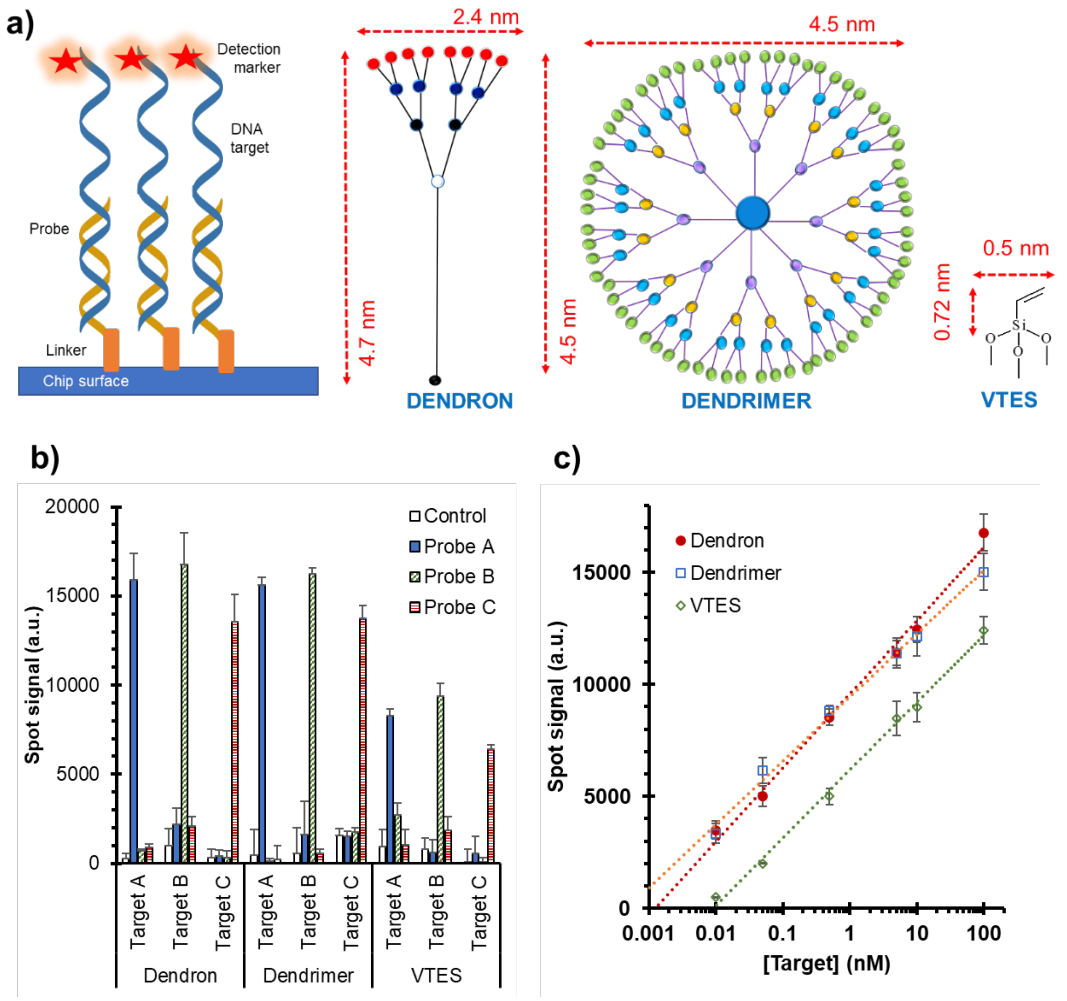



**Fig. 3.** Comparison of immobilization strategies using dendron, dendrimer, and VTES as linkers. a) Scheme of functionalized surface (vertical view): dendron with 8 alkyl-branches (3D-surface), dendrimer with 64 carboxylic-branches (3D-surface), and VTES silane as a crosslinker for a one-point attachment (2D-linear surface). b) Spot signal for selective hybridization assay with complementary and no-complementary targets. c) Spot intensities using 100 nM complementary oligo depending on the target concentration.

**Comparison with other immobilization strategies**

Dendron-based immobilization (8 terminal groups) was compared to those based on the dendrimer (64 terminal groups) and VTES (1 terminal group) approaches. Detailed information about the preparation of these chips is included as Supplementary Material (Fig. S2 and S3). The experiment consisted of incubating three DNA targets onto chips with complementary and no-complementary probes (Fig. 3).

Although all platforms discriminated 100% of the targets, forest surfaces exhibited higher responses than the VTES platform. Moreover, a calibration assay was performed, hybridizing the targets (up to 100 nM) with the complementary target.

Similar responses were obtained for both dendron and dendrimer platforms, while lower signals were observed for silane-modified chips, about 25% lower. The detection limit was found at 0.005 and 0.05 nM for 3D and 2D surfaces, respectively.

The interpretation of the results required considering the disposable sites for recognizing target molecules and their accessibility. While the silane-modified chip only has one-point sites for covalent binding of probes, dendrons and dendrimers-modified chips exhibit many sites.[14,18,20] Moreover, the flexibility of the branches, the reduction of the steric hindrance, and the greater distance surface-probe promoted higher efficiency in terms of immobilization and hybridization in forest surfaces.

The number of available sites per surface area explained the similar behaviour between dendron-based and dendrimer-based chips. Considering the terminal groups, the potential number of probes conjugated to dendrimers is higher than dendrons, being 64 and 8, respectively. However, the spatial distribution of probes is different. The reported dimensions of both architectures[22] indicated that 2.4-3.5 molecules of dendron (G3 diameter 2.4-2.9 nm) occupied the same area as one dendrimer (G3.5 diameter 4.5 nm). Moreover, the high-immobilization density and steric effects also limited the accessibility to the bottom side of dendrimers for the target oligonucleotides. Therefore, the number of available probes

was lower than immobilized probes in a dendrimer-mediated chip.

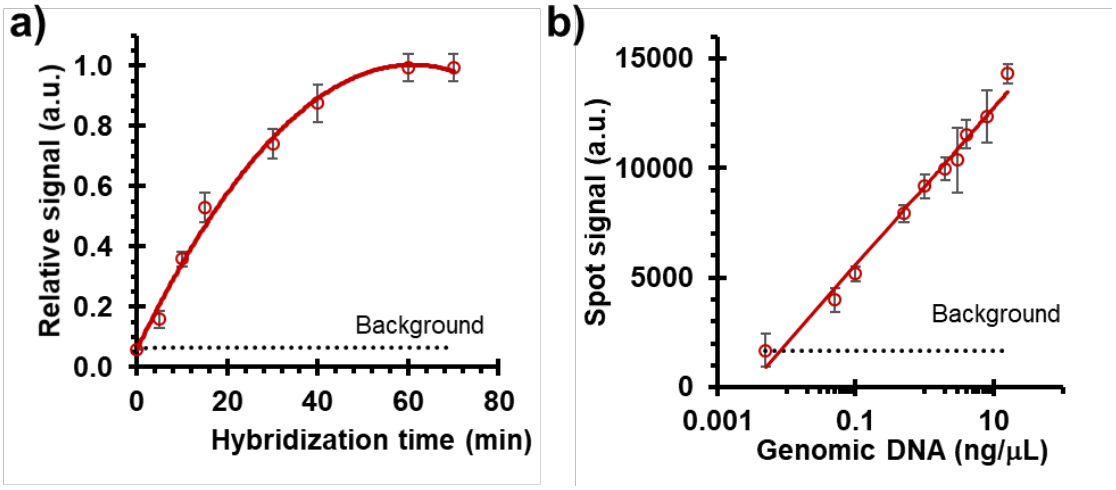



**Fig. 4**. Detection of amplification products from RPA amplification using dendron-mediated chip: a) Influence of hybridization time in registered signal. b) Calibration curve of genomic DNA hybridized on the dendron-mediated array.

Thus, an equal number of available sites for DNA hybridization was achieved, showing similar responses for both platforms. But the dendron-mediated approach provided several advantages over the dendrimer-mediated chip described in this paper and our previous study (Table S2). Briefly, the synthesis of probe-conjugates was straightforward and faster (30 s) than carbodiimide coupling (30 min). Second, the required oligo amount was lower to show similar spot intensity. Third, the different coupling reactions minimized the unspecific immobilization of probes onto the activated surface, improving background signal.

**Hybridization of blocked-RPA products**

For the specific detection of mutations in forest-array polymer chips, we first set up the selective isothermal amplification of the target region and its hybridization. For that, genomic DNA mixtures of wild-type and mutant human genome were added to blocked-RPA reaction solution under different experimental conditions (Fig. S4 and S5). Although RPA amplification assay can operate at varying temperatures (35-45ºC) and reaction times (20-60 min), the selected conditions of blocked-RPA were founded at 37°C and 40 min, agreeing with previous studies.[18] The key element was the blocker oligonucleotide and the thermodynamic stability of its template hybrids. With the right design, the base pair mismatch prevented the stable formation of the mutant template/blocker hybrid. So, the annealing/extension of primer was feasible, leading to an effective exponential replication of mutant DNA. While for the wild-type template, the selected oligonucleotide facilitated a higher blocker binding than the primer binding.[11,23] Also, the addition of blocker oligonucleotide exhibited a dose-dependent suppression of wild-type product signal, as expected. These results demonstrated that the competition between the upstream primer and the blocker reduced the percentage of the amplified wild-type allele, enabling the selective discrimination of the specific mutation.

The amplification yields agreed with those obtained using blocked-PCR. But, under isothermal conditions, the test required less time and did not need of a thermocycler.

Regarding the hybridization conditions on the dendron-mediated array, the recognition process achieved the half-value and the maximum after 20 min and 60 min, respectively (Fig. 4a). Compared to single-strand oligonucleotides, the selective recognition of RPA products by dendron-mediated probes was achieved using a restrictive hybridization buffer, as recommended for these biosensing assays.[11] But, no special condition was required to use blocked RPA against regular RPA reactions.[6] The calibration curve showed that a concentration of 8 ng/μL produced the saturation signal, and the detection limit was 0.02 ng/μL of genomic DNA (Fig. 4b). The analysis of genomic DNA mixtures led to clear recognition patterns, even for solutions containing a low amount of mutant DNA (Fig. S6). These features are excellent for DNA-based applications.[6,9,13]

**Assay integration**

Chips with microfluidic channels were developed to automate and miniaturize the assay (Fig. 5). As a proof-of-concept, the pump-free platform was made from simple, inexpensive plastic materials and adhesives, enabling the parallel hybridization reactions of seven samples per chip. This prototype (20 μL volume) halved the required amounts of blocked-RPA product, developing solutions and washing solutions. The chip also prevented the contamination of the sample. Parallel experiments conditions implemented on fluidic and no-fluidic chips also showed comparable responses. Therefore, both assay formats showed a reliable detection of specific mutations from human cells, fulfilling the requirements for integrated approaches.[24]

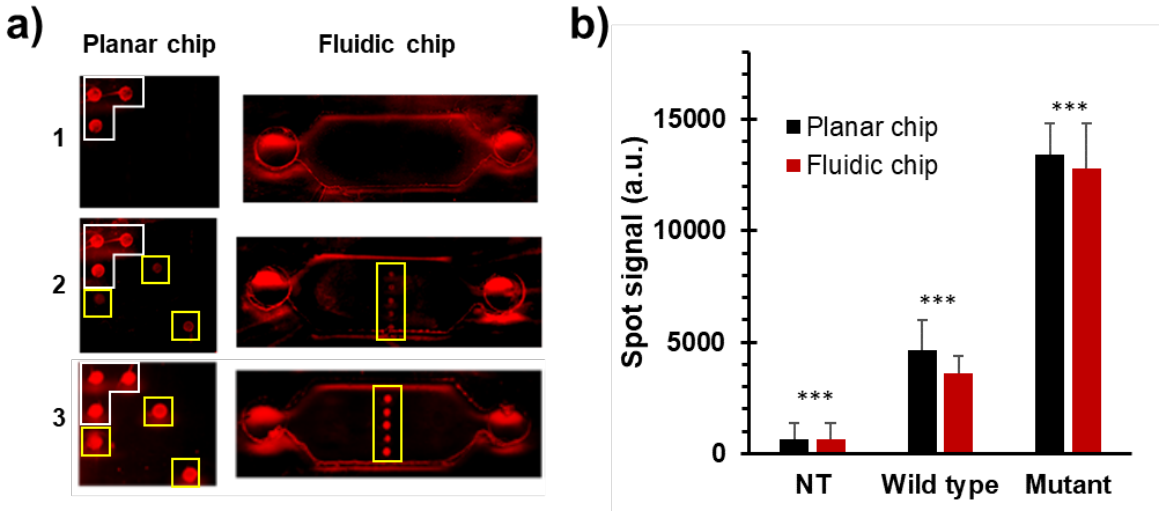



**Fig. 5**. Detection of p.H1047R PIK3CA mutation after blocked-RPA amplification: a) Array images of planar and fluidic-chamber chips for negative control (1), mutant sample at 4 ng/μL (2), mutant sample at 4 ng/μL (3). Colour code: white for control spots; yellow for p.H1047R probe. b) Spot signal of comparative formats for mutation discrimination assay.

**Application to clinical samples**

As a proof-of-concept, the genotyping of single-point mutation in the *PIK3CA* gene (phosphoinositide-3-kinase, catalytic alpha polypeptide) was studied. The chosen hotspot variation was *H1047R* mutation that lies within the kinase domain and results in increased phosphorylation of protein kinases, growth factor-independent cell survival, and transformation in cell culture.[25,26] Thus, we studied the selective and sensitive detection of this single-nucleotide variation from heterogeneous pathological samples aimed at the early diagnosis, individualized therapy, and prognosis.

The developed method was applied to DNA from formalin-fixed paraffin-embedded tissues. Spot intensities from patient samples were less reproducible than the values obtained for cell lines. Nevertheless, as the recognition pattern observed was clear, patients were classified as wild-type or mutant c.3140A>G (Fig. 6 and Fig. S7). Those results agreed with the reference method, next-generation sequencing. The displayed results demonstrated the sensing capabilities to detect the mutated alleles in heterozygous tissue specimens of human cancers.

The analytical performances were comparable to those reported using selective isothermal approaches such as rolling circle amplification [27] or loop-mediated isothermal amplification.[28,29] The advantages are that blocked-RPA/dendron-mediated chip requires less time, fewer oligonucleotides per reaction, and lower working temperature.

## Experimental

**Reagents**

Polycarbonate substrate (PC, Makrolon®) (dimensions 75 mm × 25 mm) and pressure-sensitive adhesive PSA (ARcare 90445, AdhesiveResearch) were used for the fabrication of chips. Polyester bis-MPA dendron, 8 acetylene, 1NHBoc core (generation 3), N-hydroxysuccinimide (NHS), 2-(N-morpholino) ethane sulfonic acid (MES), 1-ethyl-3-(3-dimethyl aminopropyl) carbodiimide (EDC), and bovine serum albumin were purchased from Sigma-Aldrich.

The deprotection reaction of dendron was an acidic hydrolysis. For that, the amino-protected dendron (10 mg/mL) in 10% methanol was mixed with hydrochloric acid at 3 M for 3 hours under end-over-rotation. The solution was neutralized with sodium hydroxide at 1.5 M.

The reagents used for genomic DNA extraction were PureLink extraction kits (Invitrogen) for culture cells and GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) for patient samples. DNA amplification kits were DNA Amplitool kit (Biotools, Spain) for PCR and TwistAmp Basic RPA kit (TwistDx, UK) for RPA. The array printing solution was EDC at 50 mM and NHS at 50 mM in MES 0.1 M. The hybridization buffer was saline-sodium citrate buffer (pH 7.0), containing 150 mM sodium chloride, 15 mM sodium citrate, and formamide 25%.

The hybridization washing solution was composed of NaCl at 15 mM and trisodium citrate at 1.5 mM. The developing buffer (pH 7.4) was Tween 20 (0.05%) in phosphate-buffered saline containing 137 mM NaCl, 12 mM phosphate, and 2.7 mM KCl.

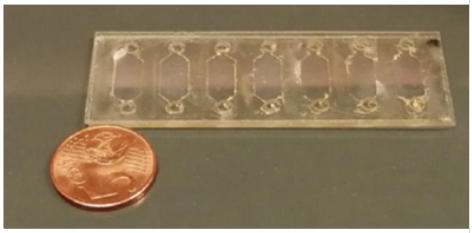
Monoclonal anti-digoxigenin antibody and monoclonal antisheep-HRP antibody were supplied by Abcam. The HRP-substrate was 3,3',5,5'-tetramethylbenzidine solution (ep(HS)TMB-mA, SDT Reagents). DNA oligomers were provided by Eurofins (Table SI.1).

**Fabrication of planar array chip**

The synthesis of DNA-probe/dendron conjugates was based on a click-photochemical reaction. Thiol-yne coupling of alkyne-dendron at 5 nM with the thiol-DNA probe at 50 nM, was carried out irradiating UV light at 254 nm for 30 s. The storage of conjugates was at 4ºC until use.

The activation of PC substrate was plasma UV/ozone oxidation (10 min) and incubation with sodium hydroxide to generate carboxylic acid groups (30 min, 60ºC).[11]

a)



b)

| | RSD intra-assay | RSD inter-assay |
|---|---|---|
| Cell lines | 4-9% | 9-12 % |
| Patients | 3-10% | 10-15% |

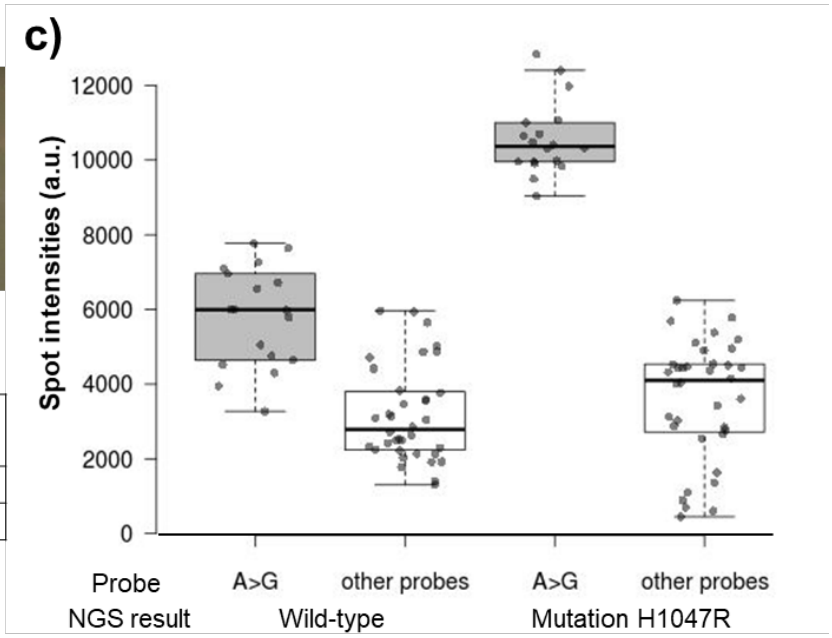



**Fig. 6.** Analysis of patient samples based on blocked-RPA and allele-selective hybridization on dendron-mediated chips. a) Image of chamber chip. b) Results of reproducibility study. RSD: relative standard deviation. c) Spot signal for oncological patients grouped depending on the result of next-generation sequencing (NGS) for target locus in *PIK3CA* gene (codon 1047, nucleotide 3140).

Then, dendron-DNA conjugates were spotted onto the carboxylic surface by non-contact printing (drop volume 30 nL, room temperature, and 70 % humidity) (AD 1500 Biodot). Seven arrays of thiol-allele-specific probes conjugated with alkyl dendrons were printed per chip, including replicates of target probe and controls. The chips were incubated at room temperature for 1 h to induce the carbodiimide reaction. After washing and drying with air flushing, they were stored at 4°C.

**Fabrication of fluidic array chip**

The Autodesk inventor (CAD-3D) model was used to design a seven-chamber chip with the dimensions of 75 mm x 25 mm x 2.03 mm and a volume of 20 μL per chamber (Fig. 1). The micro-cavity chip consisted of two PC sheets (1.0 mm thickness) and an intermediate PSA layer. DNA-dendron hybrids were immobilized on the bottom PC layer, as described above. The layout was line-test-arrays of probes, including six replicates per target. PSA layer was cut with Grapfhtec Pro equipment (force: 18 units; velocity: 20 units), creating chambers (0.5x1 $cm^2$). Input and output holes (diameter 200 μm) were milled on top PC layer by milling machine (CCD/ATC model, Bungard Elektronik). The microstructures were aligned and sealed carefully. Quality controls confirmed the absence of leaks or air bubbles.

**Samples and DNA extraction**

Metastatic colorectal cancer DNA samples pathologically confirmed were obtained from formalin-fixed paraffin-embedded (FFPE) biopsy tissues of patients from the Oncologic Service of Hospital Clínico Universitario La Fe (Valencia, Spain). For optimization, genomic DNA human cells (SK-N-AS, ATCC CRL-2137 and HCT 116, ATCC CCL-247) and buccal cells were also used. The concentration of the DNA extracts (ng/μL) was determined by spectrophotometry using NanoDrop 2000c and by fluorimetry using Qubit dsDNA HS Assay Kit (ThermoFisher Scientific).

**Isothermal amplification**

The reaction mixtures for blocked-RPA were prepared with rehydrated buffer, 14 mM of magnesium acetate, 240 nM of upstream primer, 240 nM of downstream digoxigenin-labeled primer, 50 nM of blocking oligonucleotide, 4 ng of genomic DNA, and the enzyme pellet. The heating system was a simple laboratory oven (Memmert) at 37°C for 40 min (Fig. 1b).

**Hybridization assay and detection**

Amplification products hybridized to the selective DNA probes-conjugates immobilized on chips. Blocked-RPA products (2 μL) were mixed with the hybridization buffer (18 μL) and heated (95 °C, 5 min). Then, the solutions were dispensed onto sensing areas and incubated at 37 °C for 60 min. The arrays were rinsed with progressive dilutions of hybridization washing buffer. The chip staining was an immunoreaction using a solution of anti-digoxigenin antibody (1:2500) and HRP-labelled secondary antibody (1:400) in developing buffer for 30 min. After the colorimetric substrate was dispensed, a dark blue solid deposit was formed in positive spots after 3 min. The reading of chips by an office scanner (Epson Perfection 1640SU) produced monochromatic images (Tagged Image File Format, colour depth 16-bit, scale 0–65535). The optical intensity signals of each spot (diameter 230 μm) and chip background were quantified (Image J software, NCBI).

All experiments were performed in accordance with the relevant national regulations and institution policies, followed the Declaration of Helsinki, and approved by the ethics committee (LAFE2015/096).

## Conclusions

Personalized medicine requires diagnostic tools that support clinical decisions based on specific DNA variants. Focused on this objective, the presented research describes a colorimetric approach that avoids the limitations of expensive technologies, such as sequencers. Our study has demonstrated that detecting single-point DNA mutations is feasible by combining a powerful isothermal amplification and hybridization assay on a dendron-mediated chip.

High selectivity is reached thanks to the blocked-RPA, which promotes the enrichment of minority alleles at 37 ºC in 40 min, using only three oligonucleotides, and avoiding expensive, bulky equipment. The high sensitivity results from an enhanced hybridization assay, taking profit from 3D-functionalized plastic substrates. The scientific interest of the present research is also related to the approached coupling strategies. (i) The conjugates of DNA-probe and dendrons have been prepared by a fast and clean click-chemistry coupling. (ii) The conjugates bounded on the thermoplastic substrate through the focal point. (iii) The hyperbranched peripherical groups of dendron and their flexible structuration reduced steric impediments and the surface-probe distance. These features enabled the effective recognition of amplification products and improved the performances of the hybridization assay compared to linear functionalized surfaces or other 3D surfaces. Furthermore, the experiments performed by simple prototypes (planar and microfluidic chips) can support the development of advanced microfluidic platforms fabricated on thermoplastics.

Following the methodology for detecting H1047R mutation **in** *PIK3CA* oncogene, we achieved the single-point genotyping in heterogeneous human samples even with a higher concentration of wild-type genomic DNA. Therefore, this DNA-sensing method could contribute to a new generation of biomedical devices analyses for biomarker-guided therapies.

## Conflicts of interest

There are no conflicts to declare.

## Author contributions

S. Martorell: Investigation, Writing; A. Maquieira: Supervision; L.A. Tortajada: Conceptualization, Methodology, Data curation, and Writing.

## Acknowledgements

The MINECO Project PID2019-110713RB-I00 and Generalitat Valenciana PROMETEO/2020/094.

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