Martorell-Tejedor, S.; Tortajada-Genaro, LA.; Maquieira Catala, A. (27-1). Magnetic concentration of allele-specific products from recombinase polymerase amplification. Analytica Chimica Acta. 1092:49-56. https://doi.org/10.1016/j.aca.2019.10.006

# Magnetic concentration of allele-specific products from recombinase polymerase amplification

Sara Martorell,[a,b] Luis A. Tortajada-Genaro,[*,a,b,c] and Ángel Maquieira[*,a,b,c]

a Instituto Interuniversitario de Investigación de Reconocimiento Molecular y Desarrollo Tecnológico (IDM), Universitat Politècnica de València-Universitat de València, Camino de Vera s/n, E46022 Valencia, Spain. E-mail: luitorge@qim.upv.es, amaquieira@qim.upv.es

b Unidad Mixta UPV-La Fe, Nanomedicine and Sensors, IIS La Fe, Valencia, Spain

c Departamento de Química, Universitat Politècnica de València Valencia, Spain

## Abstract

The studied challenge is the specific detection of low-abundant genomic variants that differ by a single nucleotide from the wild type. The combination of blocked recombinase polymerase amplification (RPA) and selective capture by probes immobilised on magnetic-core particles integrated into a flow system is presented. The sensing principle was demonstrated as the effective concentration-detection of the specific generated products was achieved. The analytical performance of resulting assay was successfully compared to PCR-based methods or array formats, providing faster effective detection of the selective products. As proof of concept, the single-nucleotide substitutions of the *KRAS* gene at codon 12 were studied in chip with parallel microchambers and permanent magnets. The blocked RPA products (generated at 37 °C) from tumour biopsies (extracted DNA 4 ng) provided a specific fluorescent bead-line that depends on the present mutation. The results agree with those reported by next-generation sequencing and provide new opportunities for *in vitro* diagnostic and personalised medicine.

**Keywords:** isothermal amplification; magnetic beads; in-chip hybridization; DNA genotyping; optical biosensing

## 1. Introduction

Molecular technologies has advanced the identification of the genomic diversity and their role in human diseases [1]. Implementing a personalised approach into patient care needs fast-response methods that are cost-effective and compatible with low-resource settings to solve the limitations of technically skilled personnel and specialized infrastructures. Early diagnostics reduces disease effects and health-care expenses. A conventional methodology relies on the selective recognition of the sequence-specific oligonucleotides on active electrodes or passive planar chip surfaces, and subsequent signal transduction in integrated microsystems [2]. Another successful approach is based on oligonucleotide-coated particles, which involves fewer technical requirements, a bigger surface area, easy chemical modification and effective mixing with samples [3,4]. Micro-sized magnetic beads have been the most extensively applied in diagnostic chips as the external magnetic field (electro-magnets, coils or permanent magnets) controls their movement [4,5]. The selective magnet-induced isolation of the molecules captured to particles improves the assay selectivity and sensitivity [6]. Furthermore, these assays reduce both volumes of reagents and samples and cuts the time required for target molecules to diffuse to the interrogating probes [7]. Their use has enabled well-established protocols for the simplified extraction, amplification and detection of nucleic acids. Thus the microfluidic systems yield a platform that facilitates the rapid and automated DNA analyses [8,9].

One common limitation of these methods is that the need for a DNA amplification step to achieve a copy number big enough to be detected. The consequences are complex platforms for DNA-based diagnostic tools with a high demand for the auxiliary instrumentation. To overcome some of these drawbacks, several isothermal techniques have been developed, and their integration into microsystems or portable devices has improved nucleic acid-based on-site assays [10]. Probe-conjugated magnetic beads have recognised specific templates from clinical sample lysates and are later amplified by either loop-mediated isothermal amplification (LAMP) [11] or rolling circle amplification (RCA) [12]. Regarding to detection, promising assays combine a linear RCA-type amplification and later particle aggregation [13]. However, other enzymatic methods can potentially perform better (e.g. high yield, exponential reaction kinetics, lower temperature). Recombinase polymerase amplification (RPA) is an unexplored candidate, despite being one of most sensitive and selective isothermal techniques, with excellent operational conditions for integrated devices [14].

We herein firstly report research that focused on the selective capture of RPA products onto probe-conjugated microbeads confined within a microfluidic channel and using a permanent magnet. This challenge spans from the impact on the hybridisation kinetics and to the assay integration, and includes the dynamics during magnetic-concentration. Due to the amplification mechanism, the nature of the RPA mixture is complex and an influence on the detection of the generated products compared to PCR or other isothermal techniques was expected, as reported previously [15].

The aim of the presented approach is the concentration of allele-specific products from RPA using magnetic beads for the sensitive identification of single-point mutations. The detection of these variations in the genome is important because they have been associated with disease progression and treatment efficiency [16]. Although some examples describe the discrimination of single-nucleotide mismatches based on allele-specific hybridisation to probes anchored on magnetic beads [8,17,18], they all are restricted by the PCR amplification technique. For achieving the challenge of specific discrimination, an affordable fast-response method was developed, based on blocked isothermal amplification, bead-based hybridization, magnetic concentration and optical detection. Our empirical goal was to establish the principles and experimental conditions for the differential capture and detection of the RPA products in a microfluidic system. In case of a correct discrimination of single-base variants, patients could be classified in the adequate group and the suitable treatment could be applied as personalised medicine.

## 2. Experimental

### 2.1 Chip assembly

The micro-system was composed of a supporting platform and microfluidic chips that enable the simultaneous analysis of eight samples. The 3D-printted platform, designed using the CAD-3D Autodesk inventor (Autodesk, USA), was manufactured in polylactic acid (PLA, RS Components, UK). The printing technology was fused filament fabrication (The Ultimaker Cura 2, Ultimaker, Netherlands). The printing parameters were: nozzle 0.4 mm; layer height 0.1 mm; infill density 100%; print speed 60 mm/s; travel speed 120 mm/s; print temperature 210 °C and bed temperature 60 °C. Eight neodymium magnets (dimensions 2.2×0.5×5 mm, vertical force 0.1 kg, slip resistance 0.02 kg) were integrated. The microfluidic chip, manufactured in cyclic olefin polymer, contained eight 20 µL-rhombic chamber reactors (ChipShop, Germany). The dimensions of each

chamber were 4 mm×13.8 mm×0.35 mm. The chip was aligned to the platform by placing the magnets below the middle of the chip.

## 2.2 Target

As proof of concept, the method was developed to analyse hotspots in the *KRAS* gene. Single-base substitutions of this oncogene are extensively investigated as genetic markers in human cancer pathogenesis and therapies [19]. We specifically identified the most frequent mutations (codon 12), which were p.G12C (c.34G>T, rs121913530), p.G12S (c.34G>A, rs121913530) and p.G12V (c.35G>T, rs121913529). Primer and probes were designed by considering the thermodynamic parameters of recognition process. Bioinformatic tools (OligoCalc software, Northwestern University, USA) reported that the selected sequences were unlikely to form internal hairpin structures, self-dimers or heterodimers. The used oligonucleotides were supplied by Eurofins (Germany).

## 2.3 Conjugation of particles

A set of magnetic-core particles coated with probes (wild-type and mutants p.G12C, p.G12S and p.G12V) were prepared. The selected particles were Dynabeads MyOne carboxylic acid (Invitrogen, NY, USA). Beads ($7.12\times10^8$) were washed with 2-(N-morpholino)-ethanesulfonic acid buffer (MES, 100 mM, pH 4.8) (Sigma-Aldrich) for 30 min (2 times) and resuspended. A total of 0.6 nmol amino-modified probe ($NH_2$-$C_6$-$T_5$-AGTTGGAGCTNGTGGCGTAGG, N = A, C, T or G) in MES buffer with ethyl carbodiimide hydrochloride (1.25 M) was added and incubated (overnight, room temperature). Then beads were washed 3 times with Tris-Tween buffer (1 M of Tris 1 M, 10 % of Tween 20, pH 8) and suspended in Tris-EDTA buffer (1 M of Tris, 100 mM of EDTA, pH 8). Functionalisation efficiency was quantified by determining the probe excess in relation to its initial concentration (absorbance at $\lambda$=260 nm).

## 2.4 DNA extraction and amplification

For the optimisation assays, two cell cultures were used, cell line SK-N-AS (ATCC, wild-type for *KRAS* codon 12) and cell line HCT 116 (ATCC, c.38G>A), from a primary carcinoma tissue. For method validation, healthy and oncological patients were recruited

for the present study according to ethics regulations. QIAcube robotic workstation (Qiagen, Germany) extracted genomic DNA from 5-µm thick formalin-fixed paraffin-embedded tissue sections. The used reagents were mini kit FFPE Qiagen. The concentration of the DNA extracts (ng $\mu L^{-1}$) was obtained by spectrophotometry using NanoDrop 2000c (ThermoFisher Sci., USA), and by fluorimetry using Qubit dsDNA HS Assay Kit (ThermoFisher Sci.).

The reagents used for the genomic DNA amplification came in the TwistAmp Basic RPA kit (TwistDx, UK). The mixtures (50 µL) for the blocked isothermal amplification were prepared with the enzyme pellet in rehydrated buffer, 480 nM of magnesium acetate, 480 nM of upstream primer and downstream primer, 70 nM of blocking agent, 4 ng of genomic DNA, 0.01 mM aminoally-dUTP-Cy5. The employed heating system was an Eppendorf Thermomixer with MTP adapter (300 rpm, Eppendorf), operating at 37 °C for 40 min. Also, real time thermocycler (TS2, Qiagen) was used for the optimisation assay. The reaction mechanism is described in Fig. SI.1.

## 2.5 In-chip assay

The amplification products (2 µL) were mixed with 18 µL of hybridisation buffer (1x saline sodium citrate buffer, 30% formamide), containing beads conjugated with each specific probe (final concentration $7.12\times10^5$ beads $\mu L^{-1}$). Solutions were loaded to chip by a pressure-driven flow (20 µL, < 3 s) (Fig. SI.2). Incubations were run at 95 °C for 10 min (denaturation) and at 37 °C for 30 min (hybridisation). Then, the magnets retained the magnetic particles inside each reaction chamber to enable the total removal of the supernatant. The reactor was washed with 20 µL of 0.01% SSC buffer (SSC 1×: 150 mM sodium chloride-15 mM trisodium citrate pH 7.3).

A surface fluorescence reader imaged the bead-line in each reactor. This detector was equipped with a high sensitivity charge coupled device camera (Retiga EXi, Qimaging Inc., Canada), with light emitting diodes (Toshiba TLOH157P, Japan) and filters ($\lambda_{ex}$ 635 nm, $\lambda_{em}$ 670 nm). Intensities were also compared to those obtained by fluorescence microscopy (Leica DCF 3000G, Germany). Image processing (segmentation and quality assurance) was performed by reporting the bead-line and background intensities (mean and standard deviation).

A discrimination factor was calculated to assign each patient to a genetic population (wild-type, p.G12C, p.G12S or p.G12V). Specifically, the factor for a given population

was the relative response of the corresponding probe-conjugated bead in relation to all the beads. The patient was assigned to the group with the highest discrimination factor.

2.6 Reference methods

Hybridisation on chip (colorimetric chip detection). The RPA products were detected and identified by a hybridisation assay on rectangular slides (25 mm×75 mm). The protocol is described in a previous paper [20]. Briefly, amine-DNA probes were immobilised on a modified plastic chip. The format was four arrays per chip and four replicates per target.

The amplified products (5 µL) were mixed with 45 µL of hybridisation buffer, heated (95 °C, 5 min) and dispensed onto sensing arrays. After incubation (37 °C, 60 min), the arrays were rinsed with progressive dilutions of hybridisation washing buffer. After performing the colorimetric staining chip, the results were read.

*Sequencing.* Somatic mutations detection was performed by the Ion Torrent PGM technology (ThermoFisher Scientific). The Oncomine Solid Tumor DNA kit enabled the simultaneously analysis of hotspots mutations in 22 genes (included *KRAS* gene). A multiplex PCR amplification of 10 ng of genomic DNA generated the DNA barcoded libraries. The data from the sequencing runs were aligned to the hg19 human reference genome and variant calling.

## 3. RESULTS AND DISCUSSION

### 3.1 Bead capture and concentration of RPA products

After an isothermal DNA amplification, the specific isolation of the products was studied, based on the selective hybridisation onto beads and their magnetic concentration. A relevant variable was the probe amount immobilised onto the magnetic-core particles. Conjugation was performed to obtain optimal surface-probe density to maximise hybridisation efficiency (probe density about $10^{12}$ molecules $cm^{-2}$). The selected relationship between the 5'-amino-labelled probes and the carboxylic-functionalized particles (1 µm diameter) was $8.4\times10^{-19}$ moles per particle. Thus, an important of probe excess was needed compared to the particle surface area ($3.14\times10^{-8}$ $cm^2$).

The number of probe-conjugated particles and the washing protocol are relevant parameters for the hybridisation of DNA to the bead-immobilised probes confined within

a microfluidic channel [6,7,9]. Specific experiments were performed to establish the best assay conditions. Fig. 1 shows that by increasing the bead number, the signal and the area occupied by the magnetic particles in the reaction chamber were, respectively, more intensive and bigger until saturation. The selected amount was $14.2 \times 10^6$ of probe-conjugated beads for a rhombic chamber chip of 20 μL. A stringent hybridisation buffer (low ionic strength buffer, high formamide content) favoured the perfect-match bonds and reduced non-specific interactions. By increasing the number of wash cycles, the percentage of the captured beads was lower. Also, the linear decreases in the bead-line signal were observed for both single-strand DNA and RPA product, being higher for the double-strand DNA. The results revealed a loss of particles despite of magnet action and a major denaturation for the amplification products, respectively. Thus the method succeeded in controlling the hybridisation and washing conditions, and yielded a final concentration factor that ranged between 1:8 and 1:10.

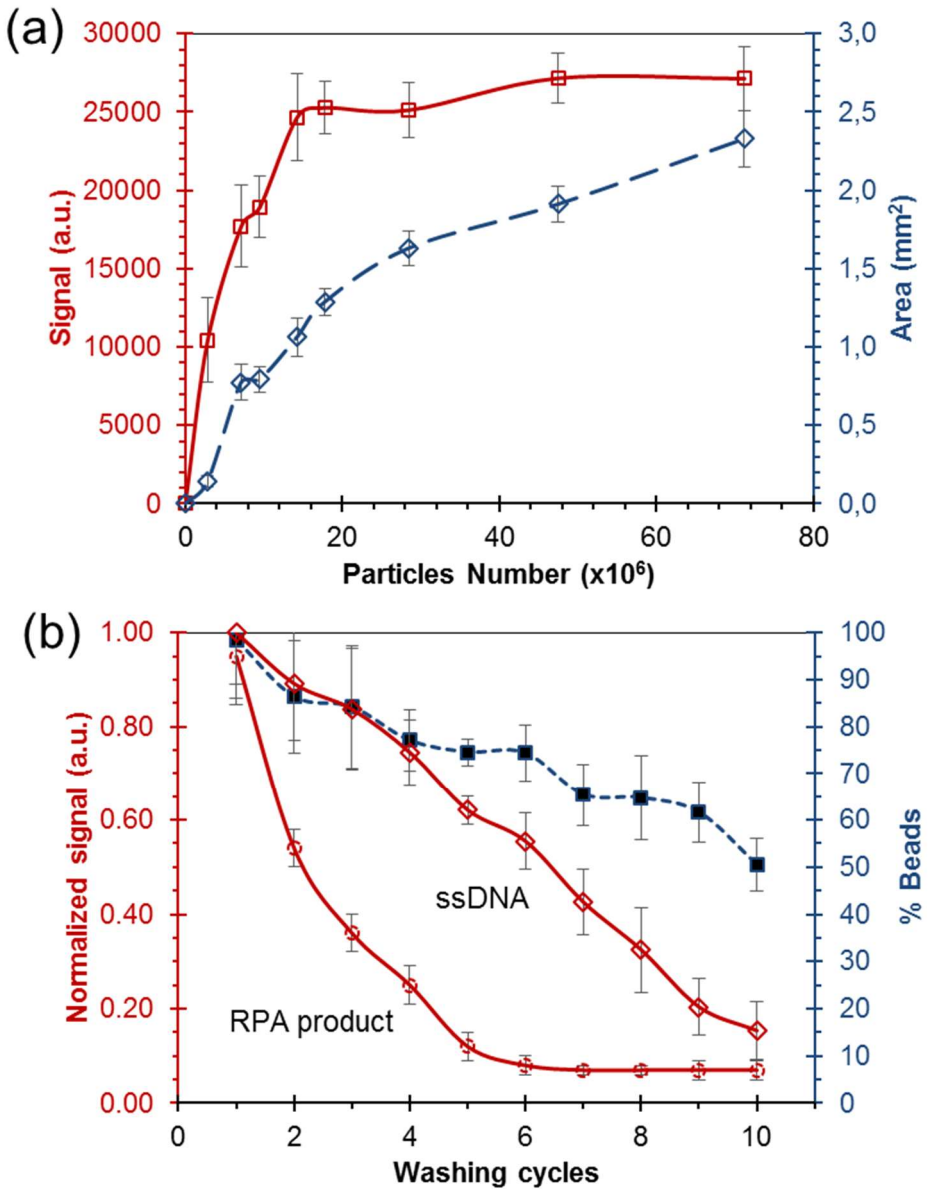



*Fig. 1 (a) Effect of the number of particles on the hybridisation reaction. (b) Effect of wash cycle on the hybridisation reaction. Replicates = 3.*

Under those experimental conditions, the hybrids RPA product/probe were formed at low temperature (37 °C, about 20°C lower than the melting temperatures) for a short period (30 min). After applying the magnetic field and washing, the particle aggregation was induced and a packed bead-line was observed with a width of about 0.8 mm (Fig. SI.3). Null and low responses, comparable to those obtained for an empty microchamber, were observed for the probe-free particles and the non-complementary probe-conjugated particles, respectively. Thus, the method was reliable because significant fluorescent

responses were recorded only in the microchambers with the particles conjugated with the complementary probes. Replicate experiments also confirmed that the method was reproducible, obtaining a relative standard deviation of 15-20 %. Regarding the sensitivity, the estimated detection limit was 250 copies of genomic DNA from biopsy tissue.

### 3.2 Comparison with PCR-based method

The proposed assay demonstrated a relevant potential for detecting DNA templates and for being integrated onto point-of-care genetic platforms. The following experiments were focused on the performances of the isothermal and the PCR-based approach [5,7,9]. The results showed that the capture yields (>90 %) were similar between RPA products and PCR products, but the magnetic-induced concentration differed depending on the employed amplification technique (Fig. 2). The movement of beads with PCR products to the magnet zone was effective at 15 s, while the beads with RPA products needed 60 s and external flushing. This behaviour was related to the differences of reaction composition of both enzymatic DNA amplifications. The RPA formulation contained a high-molecular-weight polyethylene glycol (PEG) and crowing agents, used to facilitate isothermal amplification and to reduce the mixing effects of thermal convection during the reaction run at low temperatures [14]. However, these components negatively affected the bead-based assay. In high-viscosity fluids like RPA media, the balance between the drag (viscous resistance) and magnetic force leaded to a lower particle velocities. Some groups of beads were aggregated through the microfluidic channel, slowing down the bead-concentration towards the magnet. Nevertheless, the magnetic-field-induced concentration of the RPA products was robust and effective in terms of the final amount of beads over the magnet.

Other analytical performances, such as reproducibility, sensitivity and hybridization selectivity were also similar. As expected, the main advantages of RPA derived from their isothermal nature, such faster response (i.e. amplification 30 min RPA vs. 90 min PCR) and minimal auxiliary instruments (i.e. unnecessary thermal cycler).

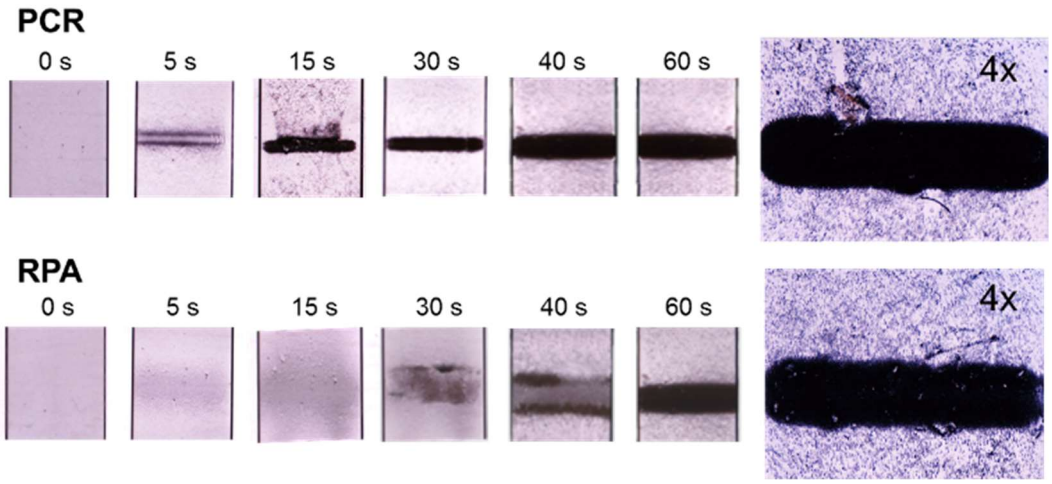



*Fig. 2 Sequential micrography of the magnetic-field-induced bead concentration into the microfluidic chip, depending on the used amplification technique. Magnification factor: 10×. Target:* wild-type variants of *KRAS* gene.

### 3.3 Comparison with the planar chip format

The performances of homogeneous hybridisation on the magnetic particles in the microfluidic chamber chip was also compared against a conventional approach based on a heterogeneous format (array in chip) [2,20].

The hybridisation kinetics of RPA products at 37 °C were examined (Fig. 3a). A saturation-type curve was obtained at the 30-minutes incubation time for the assay based on magnetic beads in the microfluidic device, with 60 min for planar chip assays. The observed behaviours matched to the Langmuir model for DNA hybridisation [21], described by the following equation:

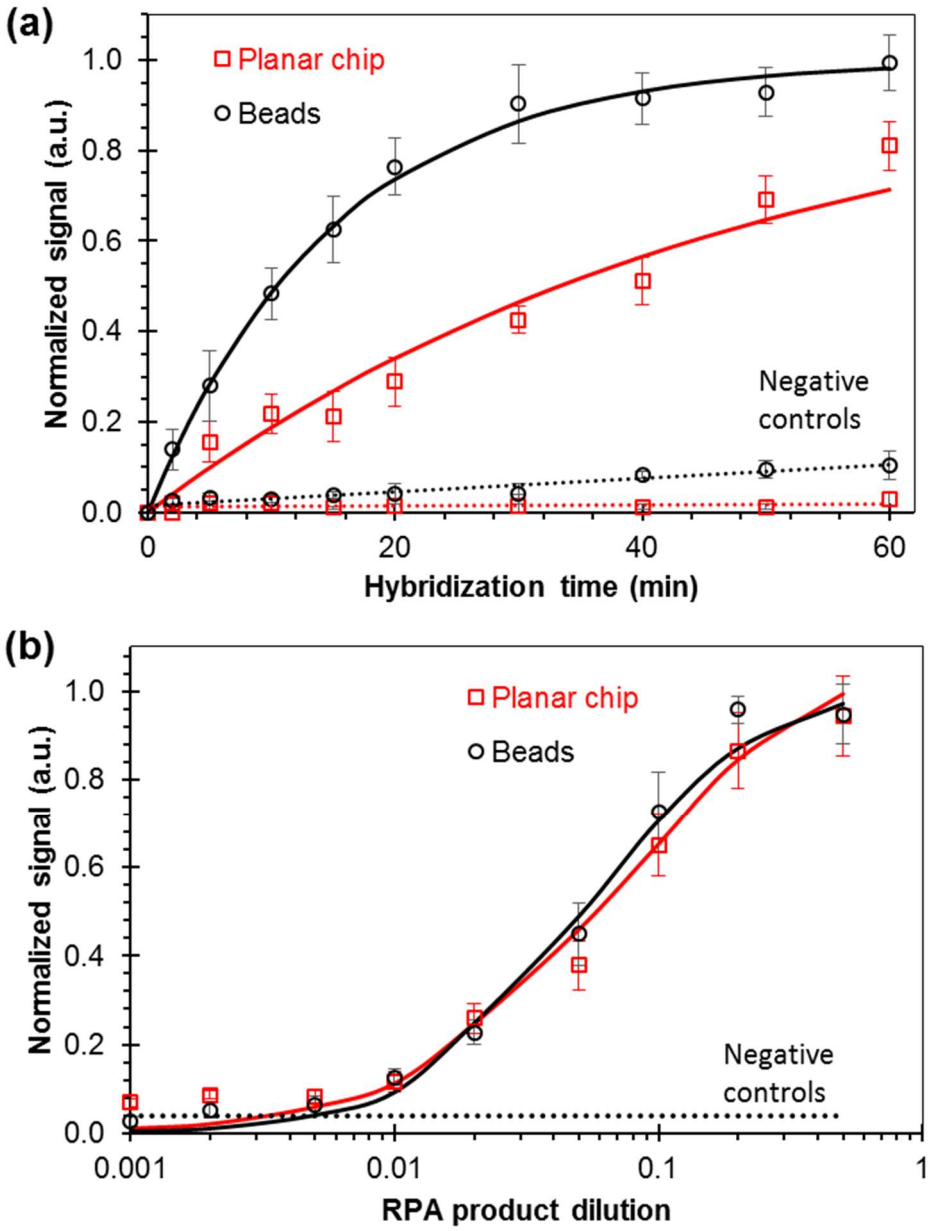
$$x(t) = x_{eq}[1 - \exp\left(-\frac{t}{\tau}\right)]$$

where x(t) is the time dependent hybridisation fraction, $x_{eq}$ the equilibrium hybridisation fraction and $\tau$ is related to the hybridisation rate constant (including the denaturation correction).

The estimated half-life times ($\tau$-values) were 48.2 min for the planar chip format and 14.8 min for the bead-based format with regression coefficients of 0.960 and 0.997, respectively. Thus, the hybridisation process of the RPA amplification products was faster with the probes immobilised on beads than on chip. The cause of these differences were examined. The estimated immobilization densities of specific probes were similar,

being 1-5 pmol $cm^{-2}$ for magnetic beads and 2-6 pmol $cm^{-2}$ for plastic chip. Also, the number of binding sites, calculated from the active areas in both approaches, was comparable. However, there are important differences in the reaction nature. In a planar chip, the transportation process of target molecule for the bulky solution to the surface must be considered, delaying the time required until the saturation. A homogeneous format (i.e. solid-liquid interface) would need much longer time compared to the heterogeneous format (i.e. liquid) [22].

For DNA-based diagnostic or prognostic, tests need to display high sensitivity because numerous applications involve the detection of the target sequence present in small copy numbers. To explore this detection capability, dilution experiments were performed, using the amplification products obtained from genomic DNA. Fig. 3b shows that the data fitted to a logistic response curve (regression coefficients 0.990-0.993). The limits of detection, defined as the target amount and statistically differed from the negative controls, were a dilution factor of RPA products about 1:200. These results agreed with the lower hybridisation yields expected for the amplification products by considering the competiveness between the probe and the complementary strand for the same target strand [5,7,15]. Assay reproducibility, obtained from replicate experiments and expressed as relative standard deviation, ranged between 5% and 15%. The calibration curves were similar for the homogeneous and heterogeneous formats (equation parameters, t-test p-value >0.05, F-test p-value >0.05), which confirmed the feasibility of the developed analytical approach.




293

294 *Fig. 3 Comparison of planar chip and bead formats. a) Effect of hybridisation time on*
295 *fluorescence signal. Fitting curve:* $y = a \cdot (1-e^{-x/b})$*. b) Influence of the number of copies on*
296 *fluorescence intensity. Incubation time: 60 min for the planar chip and 30 min for beads.*
297 *Fitting curve:* $y = L/(1 + a \cdot e^{-b*x})$*.*

298

299 **3.4 Enrichment of mutant variants**

300 As proof of concept, the RPA-particle concentration method was applied in the
301 challenging filed of the detection of sequence changes. In case of single nucleotide
302 variations, a high-selective amplification approach prior to the detection was required

303 because the mutant variants must be identified in a wild-type genomic DNA background.
304 A novel method for the preferential amplification of minority mutations before the
305 magnetic-based concentration was studied, called blocked RPA [20]. The hypothesis
306 was that the enrichment of these variants against the wild-type strand could induced,
307 based on the addition of a blocking oligonucleotide to the RPA reaction solution,
308 improving the latter on-bead hybridization.

309 The first stage was to select a wild-type blocker following a design criteria based on an
310 RPA mechanism and from the calculated thermodynamic properties. In order to reduce
311 the amplification in the primer annealing step, one restriction was for the blocker
312 candidates to partially overlap the upstream primer (clamp effect). This design avoids
313 the strand-displacing activity of the polymerase used in RPA (Bsu polymerase) if the
314 primer was bond to the template.

315 Moreover, the position of the hotspot in the blocking agent sequence drastically varied
316 the estimated stability of the mismatched hybrids. A central position allowed a variation
317 in free energy to lower between 1.5 and 1.7 $\times$ $10^5$ J $mol^{-1}$ for the single-nucleotide
318 mutations (Fig. 4). According to these values, the blocker would compete with the
319 upstream primer for the same site in the wild-type strand. The DNA extracts from
320 control/mutant cell lines were amplified in the presence of the blocker (0-400 nm) by the
321 PCR and RPA approaches in solution format. If the blocking agent was absent, there
322 were no differences between the yield amplification of the wild-type and the mutant
323 templates ($10^7$-$10^8$, test t: p=1.0). By increasing the concentration, the end-point
324 responses decreased in both mixtures. The experimental data were fitted to a four-
325 parameter logistic equation:

326 $$Signal = d + \frac{a - d}{1 + \left(\frac{[bloq]}{c}\right)^b}$$

327 where [bloq] is the blocking agent concentration, d is the background signal, a is the
328 signal for the lack of the blocking oligonucleotide, c is related to the concentration at the
329 inflection point and b is related to curve steepness. The c-parameter was related to the
330 effective blocking concentration that reduced the signal to half. The values were 46±7
331 and 160±30 for the wild-type and mutant templates, respectively. Thus adding the
332 blocker led to a selective reduction in amplification, and showed that the blocker was
333 preferentially hybridised onto the wild-type strand than onto the mutant. The selected
334 concentration was 70 nm (stoichiometric ratio of 1:7 compared to the upstream primer)

because the biggest differences were observed at this concentration. The experiments also confirmed that the chain terminator, dideoxycytidine included in the 3'-end blocker, avoided its undesired extension by polymerase action.

These results were confirmed from the kinetics measurements in vials (Fig. 4). When the blocking agent was absent, a similar behaviour was observed for the wild-type and mutant solutions. Positive signals were observed after 5-10 min, and the maximum difference was achieved after 40 min of amplification. The addition of the oligonucleotide blocker preferentially inhibited the primer extension on the wild-type strands (a 40 % reduction) compared to the mutant variants (a 7% reduction).

**(a)**

| | $\Delta G$ (J mol$^{-1}$) |
|---|---|
| Wild-type | $-1.08\times10^{5}$ |
| p.G12C | $-0.91\times10^{5}$ |
| p.G12S | $-0.94\times10^{5}$ |
| p.G12R | $-0.93\times10^{5}$ |

**(b)**

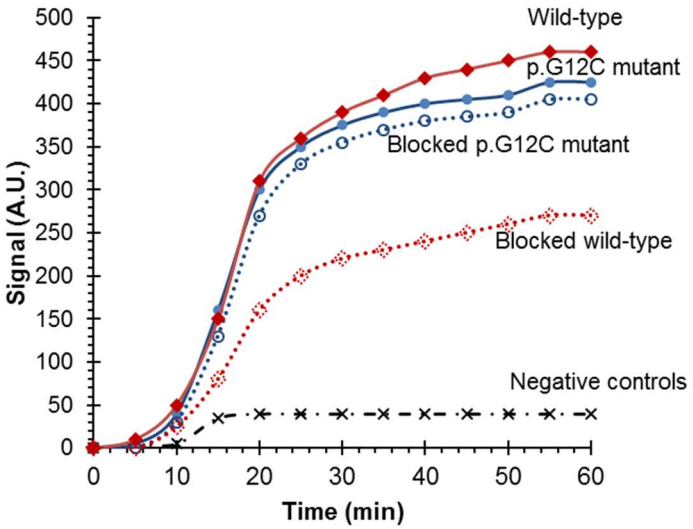



**Fig. 4** Blocked RPA of *KRAS* hotspot: (a) Variation of the free energy associated with the formation of DNA duplexes (blocker-template) at 37ºC. (b) Real-time RPA amplification in the solution format using DNA extracts from the wild-type (◊), mutant (O) and negative control (×) samples in the presence of blocking agent at 0 nM (continuous line) and 70 nM (discontinuous line).

The versatility of method was examined, performing the enrichment of the mutant variants in three hotspots. The method was applied for the allele discrimination of *KRAS* (codon 12), *PIK3CA* (exon 9) and *PIK3CA* (exon 20). The reported data (gel electrophoresis, real-time fluorescence and solid-phase hybridization) confirmed that blocked RPA is a reliable enrichment method, because wild-type DNA amplification was minimized while mutant DNA would be promoted to be amplified (Fig. SI.4 and 5). The products from blocked RPA were effectively isolated using magnetic-core particles

coated with specific probes. A selective detection was achieved (Fig. SI.6), because the mutant variants provided a fluorescent bead-line with a higher signal-to-noise ratio than the wild-type variant (t-test, p-value <0.05).

The detection capability was quantified by mixing products from the mutant and wild-type genomic DNA incubated with both types of magnetic beads. To better evaluate cross-reactivity, a double labelling experiment was performed. The labelling fluorophores of dUTPs were 5-FAM and Cy5 for the wild-type and mutant RPA mixtures, respectively. Significant fluorescent responses were obtained only in the assay when the bead was conjugated to the complementary allele specific probe (Fig. 5). A relationship between the responses and mutation percentage was clearly established (Fig. SI.7), and demonstrated that it was possible to detect a small amount of mutant strands even in the presence of large amounts of the wild-type gene.

### 3.5 Bead-based identification of the mutant variants

The discrimination among several variants that only differ in a single nucleotide requires a selective recognition and isolation. Thus the ability of selectively identifying each specific mutation for a particular hotspot was investigated (*KRAS* targets: wild-type, p.G12C, p.G12S, p.G12R). Blocked RPA products were aliquoted to different reactors of the microfluidic chip that contained specific probe-conjugated particles. So, the discriminating elements were spatially separated, but integrated into a single chip (parallel assay). Under the selected conditions, each variant provided a high response in the bead-line of the corresponding probe, whereas the responses associated with the other probes were significantly low (t-test, p<0.005). The comparison made with the heterogeneous assay confirmed the correct formation of a perfect-match hybrid between the RPA product and one specific probe (Fig. 6a). Therefore, these results demonstrated a selective capture, an effective concentration and, consequently, an unequivocal identification (Fig. 6b).

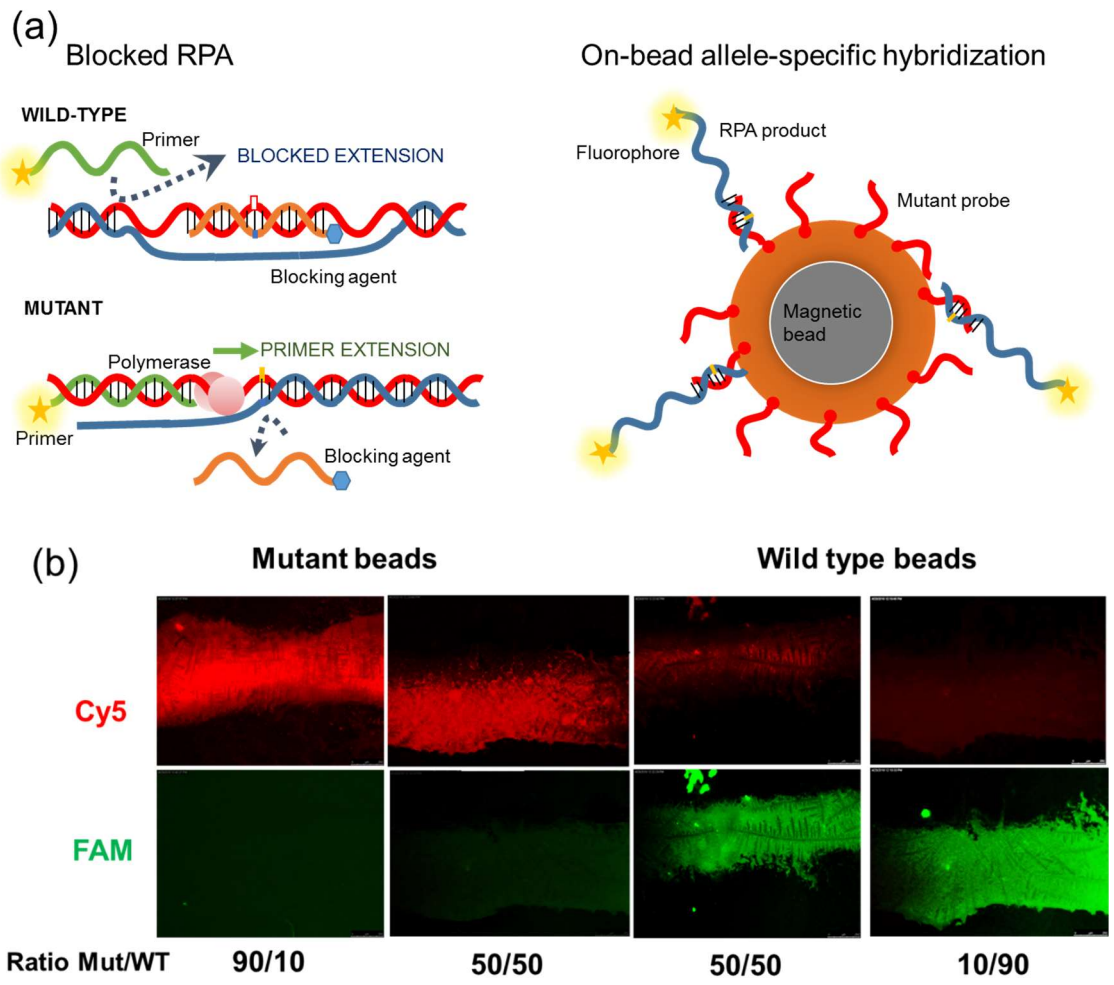
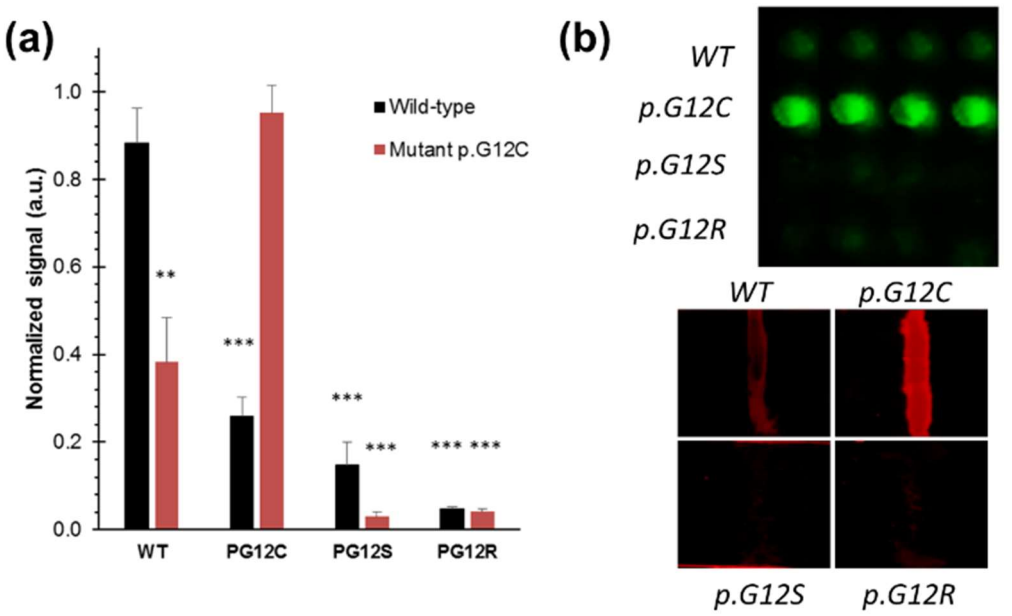



*Fig. 5. Mutation discrimination based on the blocked isothermal amplification and the allele-specific capture and concentration to the probe-conjugated magnetic particles. (a) Reaction schemes. (b) Fluorescence images for Cy5 and FAM channels obtained by a three-variable experimental design: KRAS gene p.G12C mutant/wild-type ratio (M/WT), labelling method (Cy5-dUTPs: mutant RPA and FAM-dUTPs: wild-type RPA) and probe-conjugated bead nature. Magnification factor: 10×. Genomic DNA: 1,333 copies.*

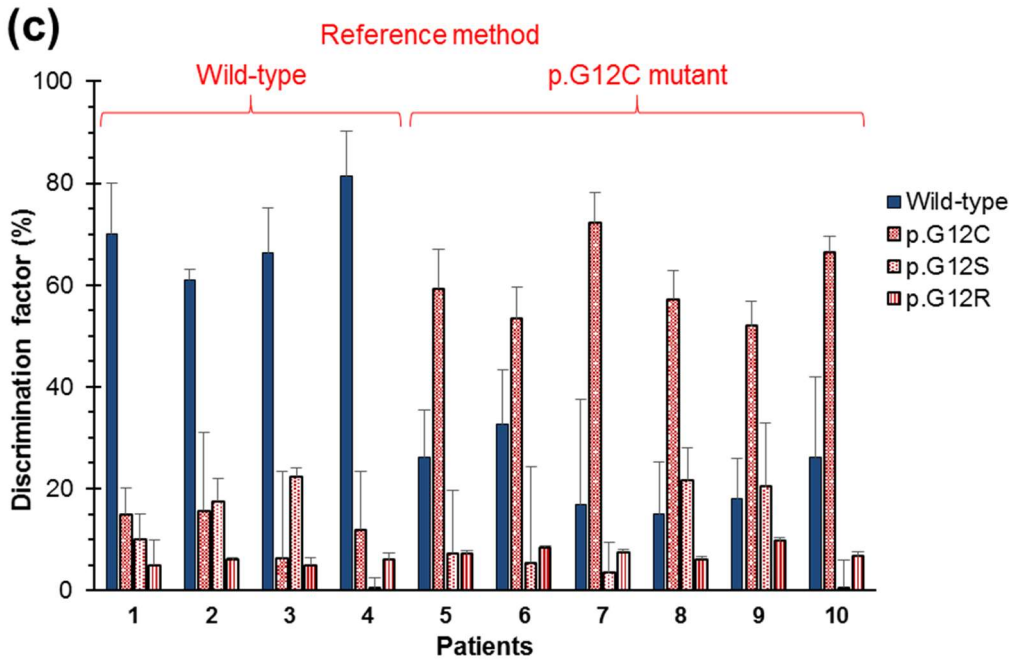
In a blind-assay, the developed method was applied to analyse the samples from the tumour biopsies (genomic DNA from formalin-fixed paraffin-embedded tissues). On-chip assay was parallelised for mutation detection and identification in a simple assembly (Fig. SI.8). Compared to the results obtained using the cell cultures, the inter-assay reproducibility for the tissue samples was about 2-folds worse (relative standard deviation: 20-25%) and non-specific responses were higher (30 %). Nevertheless, the population assignation was achieved by calculating of a discrimination factor based on

401 the relative responses for all the probe-conjugated beads (Fig. 6c). Four samples were
402 classified as wild-type and six samples as p.G12C mutant. These genetic variant
403 assignations agreed with the results reported by the reference method (next-generation
404 sequencing). Therefore, the hybridisation of the RPA products from complex biological
405 samples onto probe-conjugated microbeads confined within a microfluidic channel was
406 satisfactorily demonstrated.

407 Several advantages have been identified compared to similar approaches for
408 determining the mutational status of clinically relevant hotspots [6,23–25]. First, the
409 amplification at a low constant temperature (37 °C, 60 min) allowed the analysis time
410 and material resources to be reduced. Second, magnetic-induced formats are most
411 useful for developing miniaturised sensors with enhanced assay sensitivity and
412 exceptional selectivity (e.g. low-amount of DNA template, high concentration factor for
413 detection, exhaustive washing). Third, there are several compatible detection principles
414 for the cost-effective measurement of biosensing responses by enabling point-of-care
415 genetic analyses [26-27]. The developed system is aimed to fulfil the ASSURED
416 requirements described by the World Health Organization: Affordable, Sensitive,
417 Specific, User-friendly, Rapid & robust, Equipment-free, and Delivered.




418




419

420 *Fig. 6. a) Normalised signal obtained for the probe-conjugated beads for a wild-type*
421 *sample and a mutant sample (p.G12C). **: p-value < 0.005, ***: p-value < 0.0005. b)*
422 *Image obtained for a mutant sample (p.G12C). Top: Planar chip format. Bottom: Bead*
423 *format. c) Analysis of patient samples. Discrimination factor for each genetic variant was*
424 *calculated from the bead-line responses. Samples were grouped based on next-*
425 *generation-sequencing results.*

426

## Conclusions

In the last few decades, major progresses has been made in the DNA-based tests by applying probe-coated magnetic particles in microfluidic chips. One important pending challenge is to improve analytical performances for widespread exploitation, such as sensitivity, selectivity, portability and fast-response. Our research addresses the problem by integrating an isothermal amplification technique, which fulfils the demanded requirements with excellent capabilities. The assay is performed at constant temperature, with fewer needs for auxiliary instruments and by keeping the reaction yields of the conventional PCR-based approach or other isothermal techniques.

While similar published techniques have been applied to detect large regions (tens of nucleotides) or variants in synthetic templates, collected evidence has endorsed that the approach here developed is able to recognise low-abundant variants of single-nucleotide mutations in DNA from tumour tissues.

As our results have been generated using a simple prototype, more efforts are required to achieve a fully-integrated device. Nevertheless, the study has demonstrated its efficiency, reliability and sensitivity, as well as it has provided insights into the development of innovative fast cost-effective DNA diagnostic platforms to support the clinical treatment.

## Acknowledgements

The authors acknowledge the financial support received from EU FEDER, the Spanish Ministry of Economy and Competitiveness (MINECO project CTQ2016-75749-R and Technical Support Personnel PTA- 2016).

## References


[1] L.E. MacConaill, Existing and emerging technologies for tumor genomic profiling, J. Clin. Oncol. 31 (2013) 1815–1824. doi:10.1200/JCO.2012.46.5948.

[2] O.Y.F. Henry, C.K. O'Sullivan, Rapid DNA hybridization in microfluidics, TrAC - Trends Anal. Chem. 33 (2012) 9–22. doi:10.1016/j.trac.2011.09.014.

[3] J.P. Lafleur, A. Jönsson, S. Senkbeil, J.P. Kutter, Recent advances in lab-on-a-chip for biosensing applications, Biosens. Bioelectron. 76 (2016) 213–233. doi:10.1016/j.bios.2015.08.003.

[4] T. Jamshaid, E.T.T. Neto, M.M. Eissa, N. Zine, M.H. Kunita, A.E. El-Salhi, A. Elaissari, Magnetic particles: From preparation to lab-on-a-chip, biosensors, microsystems and microfluidics applications, TrAC - Trends Anal. Chem. 79 (2016) 344–362. doi:10.1016/j.trac.2015.10.022.

[5] S. Rödiger, C. Liebsch, C. Schmidt, W. Lehmann, U. Resch-Genger, U. Schedler, P. Schierack, Nucleic acid detection based on the use of microbeads: A review, Microchim. Acta. 181 (2014) 1151–1168. doi:10.1007/s00604-014-1243-4.

[6] T. Tangchaikeeree, D. Polpanich, A. Elaissari, K. Jangpatarapongsa, Magnetic particles for in vitro molecular diagnosis: From sample preparation to integration into microsystems, Colloids Surfaces B Biointerfaces. 158 (2017) 1–8. doi:10.1016/j.colsurfb.2017.06.024.

[7] A. Van Reenen, A.M. De Jong, J.M.J. Den Toonder, M.W.J. Prins, Integrated lab-on-chip biosensing systems based on magnetic particle actuation-a comprehensive review, Lab Chip. 14 (2014) 1966–1986. doi:10.1039/c3lc51454d.

[8] S. Wang, Y. Sun, W. Gan, Y. Liu, G. Xiang, D. Wang, L. Wang, J. Cheng, P. Liu, An automated microfluidic system for single-stranded DNA preparation and magnetic bead-based microarray analysis, Biomicrofluidics. 9 (2015) 1–17. doi:10.1063/1.4914024.

[9] V.F. Cardoso, A. Francesko, C. Ribeiro, M. Bañobre-López, P. Martins, S. Lanceros-Mendez, Advances in Magnetic Nanoparticles for Biomedical Applications, Adv. Healthc. Mater. 7 (2017) 1700845. doi:10.1002/adhm.201700845.

[10] Y. Zhao, F. Chen, Q. Li, L. Wang, C. Fan, Isothermal Amplification of Nucleic Acids, Chem. Rev. 115 (2015) 12491–12545. doi:10.1021/acs.chemrev.5b00428.

[11] C.H. Wang, K.Y. Lien, J.J. Wu, G. Bin Lee, A magnetic bead-based assay for the rapid detection of methicillin- resistant Staphylococcus aureus by using a microfluidic system with integrated loop-mediated isothermal amplification, Lab Chip. 11 (2011) 1521–1531. doi:10.1039/c0lc00430h.

[12] M. Kühnemund, D. Witters, M. Nilsson, J. Lammertyn, Circle-to-circle amplification on a digital microfluidic chip for amplified single molecule detection, Lab Chip. 14 (2014) 2983–2992. doi:10.1039/c4lc00348a.

[13] C. Lin, Y. Zhang, X. Zhou, B. Yao, Q. Fang, Naked-eye detection of nucleic acids through rolling circle amplification and magnetic particle mediated aggregation, Biosens. Bioelectron. 47 (2013) 515–519. doi:10.1016/j.bios.2013.03.056.

[14] I.M. Lobato, C.K. O'Sullivan, Recombinase polymerase amplification: Basics, applications and recent advances, TrAC - Trends Anal. Chem. 98 (2018) 19–35. doi:10.1016/j.trac.2017.10.015.

[15] S. Santiago-Felipe, L.A. Tortajada-Genaro, R. Puchades, Á. Maquieira, Parallel solid-phase isothermal amplification and detection of multiple DNA targets in microliter-sized wells of a digital versatile disc, Microchim. Acta. 183 (2016) 1195–1202. doi:10.1007/s00604-016-1745-3.

[16] N. Dey, C. Williams, B. Leyland-Jones, P. De, Mutation matters in precision medicine: A future to believe in, Cancer Treat. Rev. 55 (2017) 136–149. doi:10.1016/J.CTRV.2017.03.002.

[17] H.S. Sloane, K.A. Kelly, J.P. Landers, Rapid KRAS Mutation Detection via Hybridization-Induced Aggregation of Microbeads, Anal. Chem. 87 (2015) 10275–10282. doi:10.1021/acs.analchem.5b01876.

[18] Y.M. Chang, S.T. Ding, E.C. Lin, L.A. Wang, Y.W. Lu, A microfluidic chip for rapid single nucleotide polymorphism (SNP) genotyping using primer extension on microbeads, Sensors Actuators, B Chem. 246 (2017) 215–224. doi:10.1016/j.snb.2017.01.160.

[19] A.I. Phipps, D.D. Buchanan, K.W. Makar, A.K. Win, J.A. Baron, N.M. Lindor, J.D. Potter, P.A. Newcomb, KRAS-mutation status in relation to colorectal cancer survival: The joint impact of correlated tumour markers, Br. J. Cancer. 108 (2013) 1757–1764. doi:10.1038/bjc.2013.118.

[20] S. Martorell, S. Palanca, Á. Maquieira, L.A. Tortajada-Genaro, Blocked

514 recombinase polymerase amplification for mutation analysis of PIK3CA gene,
515 Anal. Biochem. 544 (2018) 49–56. doi:10.1016/j.ab.2017.12.013.

516 [21] A. Halperin, A. Buhot, E.B. Zhulina, Hybridization isotherms of DNA microarrays
517 and the quantification of mutation studies, Clin. Chem. 50 (2004) 2254–2262.
518 doi:10.1373/clinchem.2004.037226.

519 [22] Y. Gao, L.K. Wolf, R.M. Georgiadis, Secondary structure effects on DNA
520 hybridization kinetics: A solution versus surface comparison, Nucleic Acids Res.
521 34 (2006) 3370–3377. doi:10.1093/nar/gkl422.

522 [23] W. Shen, Y. Tian, T. Ran, Z. Gao, Genotyping and quantification techniques for
523 single-nucleotide polymorphisms, TrAC - Trends Anal. Chem. 69 (2015) 1–13.
524 doi:10.1016/j.trac.2015.03.008.

525 [24] E.S. Yamanaka, L.A. Tortajada-Genaro, N. Pastor, Á. Maquieira, Polymorphism
526 genotyping based on loop-mediated isothermal amplification and smartphone
527 detection, Biosens. Bioelectron. 109 (2018) 177–183.
528 doi:10.1016/j.bios.2018.03.008.

529 [25] I. Hernández-Neuta, I. Pereiro, A. Ahlford, D. Ferraro, Q. Zhang, J.L. Viovy, S.
530 Descroix, M. Nilsson, Microfluidic magnetic fluidized bed for DNA analysis in
531 continuous flow mode, Biosens. Bioelectron. 102 (2018) 531–539.
532 doi:10.1016/j.bios.2017.11.064.

533 [26] E.S. Yamanaka, L.A. Tortajada-Genaro, A. Maquieira, Low-cost genotyping method
534 based on allele-specific recombinase polymerase amplification and colorimetric
535 microarray detection. Microchimica Acta, 184 (2017) 1453-1462.

536 [27] A. Lázaro, E.S. Yamanaka, A. Maquieira, L.A. Tortajada-Genaro, Allele-specific
537 ligation and recombinase polymerase amplification for the detection of single
538 nucleotide polymorphisms. Sensors Actuators, B Chem. 298 (2019).126877.