Enhanced asymmetric blocked qPCR method for affordable detection of point mutations in KRAS oncogene

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ABSTRACT

An accurate genetic diagnostic is key for adequate patient management and the suitability of healthcare systems. The scientific challenge lies in developing methods to discriminate those patients with certain genetic variations present in tumor cells at low-concentrations.

We report a method called enhanced asymmetric blocked qPCR (EAB-qPCR) that promotes the blocker annealing against the primer-template hybrid controlling thermal cycling and reaction conditions with nonmodified oligonucleotides.

Real-time fluorescent amplification curves of wild-type alleles were delayed by about eight cycles for EAB-qPCR, compared to conventional blocked qPCR approaches. This method reduced the amplification of native DNA variants (blocking percentage 99.7%) and enabled the effective enrichment of low-level DNA mutations. Excellent performance was estimated for the detection of mutated alleles in sensitivity (up to 0.5% mutant/total DNA) and reproducibility terms, with a relative standard deviation below 2.8%. The method was successfully applied to the mutational analysis of metastatic colorectal carcinoma from biopsied tissues. The determined single-nucleotide mutations in the *KRAS* oncogene (codon 12-13) totally agreed with those obtained from next-generation sequencing.

EAB-qPCR is an accurate cheap method and can be easily incorporated into daily routine to detect mutant alleles. Hence these features are especially interesting to facilitate the diagnosis and prognosis of several clinical diseases.

Keywords: bioanalytical methods; allele-selective qPCR; *KRAS* oncogene; mutation genotyping; DNA variant detection

Introduction

In the precision medicine era, the detection of minority alleles is crucial because it may affect clinical decisions in the fields of cancer, prenatal diagnosis, or infectious diseases [1, 2]. In fact the ability to distinguish single-nucleotide mutations is becoming essential for selecting correct treatment according to patients' individual characteristics [3]. However given the heterogeneous nature of tumors, the mutated DNA from cancer cells must be detected when non mutated DNA from normal cells are abundant and present [4]. One relevant example is the genotyping of mutations in the *KRAS* oncogene, before the treatment based on monoclonal antibodies such as cetuximab and panitumumab. Wild-type patients better respond to antibody-based therapeutic medicines and have higher survival rates [5, 6].

The detection of mutated variants when excess wild-type DNA is present requires high-performance assays. Thus routine applications in diagnostics require accurate, selective, easy-to-implement and cost-effective techniques [7]. To date, the most useful methods for detecting single-nucleotide mutations can be classified into two categories; sequencing methods and minority allele enrichment strategies [8]. The main advantage of sequencing methods is they identify the specific mutation, although Sanger sequencing shows limited sensitivity, a high contamination risk and low throughput [9]. Likewise, the expense associated with pyrosequencing and next-generation sequencing (NGS) techniques is currently high for instruments (up to €10^5) and for running costs (up to €10^3) [7]. In several clinical scenarios, PCR methods for enriching minority alleles are the key alternative [10, 11]. The first approaches were allele-specific PCR [12], amplification refractory mutation system PCR (ARMS) [13] and restriction fragment length polymorphism PCR (RFLP) [14]. Droplet digital PCR (ddPCR) offers very high selectivity (10^{-3} to 10^{-8}), although ddPCR is still time-consuming and expensive (instrument up to €10^5 and up to €20 per sample) [15].

In recent years, considerable research has focused on methods supported by standard qPCR equipment because it is frequently available in laboratories thanks to its robustness, affordable price and general reagents. In this category, an interesting method is co-amplification that operates at lower denaturation temperature PCR (COLD-PCR) [16]. Despite its high sensitivity, mutation enrichment depends on the sequence context and, thus, certain mutations in a DNA sequence may be more difficult to detect than others [17]. Another strategy is based on the improvement of the blocked qPCR method by incorporating modified oligonucleotides, such as peptide nucleic acid (PNA), locked nucleic acid (LNA) and LNA/DNA chimeras [18, 19]. Their function selectively inhibits the amplification of wild-type sequences to produce a selective hybrid between the target and the blocker [20, 21]. However, these modifications are expensive.

Modified PCR methods has been described to avoid the plateau phase of PCR and to improve amplification specificity [22]. A relevant method is the linear one after exponential PCR (LATE-PCR) because the preferential enrichment of mutant sequences is achieved applying a specific reaction sequence [23]. The stages include a linear pre-amplification (4 steps, 10 cycles), the conversion of single-strand DNA into double-strand DNA (4 steps, a few cycles) and the exponential amplification of mutants (4 steps, 50 cycles). In each stage, the preferential hybridization of the blocker onto the wild-type template strand is improved because the reaction is paused at the optimal annealing temperature of the blocker. The main drawbacks are the large number of amplification cycles and the stringent working conditions required to open the stem of the blocker. Nowadays novel assay principles to improve the reliability of PCR-based methods are still necessary for extensive use [24].

Herein a novel approach, called enhanced asymmetric blocked qPCR (EAB-qPCR), is reported. The combination of asymmetric PCR with a specific blocking agent and the addition of a new thermal cycling stage enriches minority DNA variants. Blocker annealing is favored by minimizing nonspecific recognition and maximizing the inhibition of perfect-match amplification.

In this way, EAB-qPCR was designed for the discrimination of one base pair mismatch to enable the detection of mutant variants.

Materials and Methods

Target. Single-nucleotide mutations in the *KRAS* gene (codons 12-13) were selected as the model given their high prevalence and clinical significance [6]. Nucleotide sequences were obtained from the National Biotechnology Information Center database (NCBI Gene 3845). Specific primers and blockers were designed for the EAB-qPCR method, as described in Supplementary Information (Table SI.1 and SI.2). All the oligonucleotides, purified by HPLC, were ordered from Eurofins Genomics (Germany).

Cell lines and patients. Human SK-N-AS cells with a wild-type variant for the target region and HCT116 cells with mutant c.38G>A (*KRAS* p.G13D) were purchased from the American Type Culture Collection (ATCC, USA) and were used for method optimization purposes. Formalin-fixed paraffin-embedded (FFPE) biopsy tissues were obtained from the Oncological Service of the Hospital Clínico Universitario La Fe (Spain). Carcinomas were sampled in the infiltrating area of the growth, avoiding the necrotic center. Tissues corresponded to 20 patients with metastatic colorectal cancer who had been pathologically confirmed. Samples were fixed in less than 24 h and stored at 4ºC until DNA extraction. All the experimental protocols were conducted according to the ethics and the Declaration of Helsinki, including informed consents obtained from each patient.

DNA extraction. The genomic DNA of the cell lines was extracted using the PureLink Genomic DNA kit (Invitrogen, USA). For the genomics of the metastatic colorectal cancer samples, extraction was performed by the QIAamp DNA Investigator kit (Qiagen, Germany). The quality

and concentration of the extracted DNA (ng/µL) were determined by spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific, USA). Extracts were stored at -20 °C until processed.

EAB-qPCR method. Reactions were performed in a total volume of 12.5 µL containing 1x TB Green Premix Ex Taq (Takara, Gallini, Spain), 1x ROX reference dye II (Takara, Gallini, Spain), 300 nM of the forward primer, 150 nM of the reverse primer, 150 nM of the blocker agent and 1 µL of each DNA extract (4 ng/µL, equivalent to 1300 copies). The reagents were loaded in 96-well microplates (Axygen PCR, Fischer Scientific, Spain), covered with ultra-pressure sealing film (Thermo Fisher Scientific, USA). Amplification and detection were carried out by the ViiA 7 Real-Time PCR System instrument (Applied Biosystem, USA). Thermal cycling was: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles of amplification of 1 s at 95 °C (denaturation), 60 s at 65 °C (blocker annealing) and 35 s at 55 °C (primer annealing and extension, fluorescence acquisition). Reactions were run in duplicate and the experiment included one negative control and no template control. Optionally, a melting curve analysis was acquired from 60 °C to 95 °C at a thermal transition rate of 0.5 °C per second.

Data interpretation. The data were analyzed with software included in a ViiA 7 Real-Time PCR System. The C_q value, defined as the cycle number at which a significant increase in fluorescence is detected, was recorded. The detection threshold was set at $\Delta Rn = 0.2$, calculated from the signal increment between both fluorophores. For genotyping, a discrimination factor was calculated as the delay of the wild-type amplification in relation to mutant amplification ($\Delta C_q = C_{q,\text{wild-type}} - C_{q,\text{mutant}}$). For the discrimination of patients, a logic gate was defined on the basis of quantification C_q ($C_{q\ \text{cut-off}}$). Samples were declared as mutants or wild type if the measured C_q was lower or higher than 25, respectively.

Reference methods. Conventional blocked qPCR and Next Generation Sequencing were also applied to tumor samples, see Supplementary Material.

Results

Principle of selective enrichment EAB-qPCR. Fig. 1 presents the scheme of the EAB-qPCR mechanism, enabling the enrichment of minority alleles, including the discrimination of mutant variants, and even alteration only involves a single-nucleotide change. The method can be considered an enhanced variant of blocked-qPCR based on promoting the wild-type template/blocker hybrid against the template/primer hybrid. After DNA denaturation occurs, an intermediate step is included for the selective annealing of the blocker to the wild-type template. With the correct selection of reaction conditions, the base pair mismatch between the blocker and mutant DNA suffices to prevent the formation of the mutant template/blocker hybrid. In the next reaction step, the annealing of the primer to the template is targeted. The primer elongation of the blocked sequence by polymerase would not occur, whilst the effective exponential replication of mutant DNA is possible. This effect is enhanced under asymmetric conditions because the residual undesired production of the complementary strand reduces (linear growth). In the qPCR plot, the expected result is a delay in amplification curves and. Minority strands are specifically detected (low C_q), despite the initial presence of wild-type alleles in high proportions.

Selective enrichment method. The reaction conditions of the EAB-qPCR method were examined, studying the selective enrichment of the mutant *KRAS* variants. For correct genotyping, the discrimination factor (ΔC_q) was chosen as selection criteria. The preliminary experiments ended with high amplification yields, obtained when the primer annealing/extension step was constant at 55 °C and 35 s (Fig SI.1). Excellent results were obtained with a simple 3'-

end capped oligonucleotide, which avoided using expensive molecules, such as peptide nucleic acids (PNA) and locked nucleic acids (LNA) among others [18].

Concerning the blocker annealing step, a wide operational window (temperature and time) was established from the estimated stability of the blocker/template and primer/template hybrid and the compatible conditions with the later elongation action of polymerase. Temperature variation (56-70 °C) gave a maximum curve value in the measured ΔC_q (Fig. 2a). The discrimination factors suggested that low temperatures did not avoid the undesired primer annealing in the template strand, while high temperatures produced unstable hybrids for both the primer and blocker. These results proved that competition between the primer and blocker for the template strands could be modulated. The time effect (5-80 s) fitted a saturation curve (Fig. 2b), as expected when considering the conventional hybridization kinetics between two perfect-match oligonucleotides [25]. The greater discrimination took place at 65 ºC and 60 s.

In order to improve enrichment, the blocker concentration was varied (Fig. 2c). At low concentrations, the amplification of all the variants was similar ($\Delta C_q < 1$). By increasing the amount, the quantification cycle (C_q) was nearly constant for mutants and higher for the wild type. The maximum difference without significantly reducing amplification yields was achieved at 150 nM, and corresponded to half the reverse primer concentration. Therefore, adequate concentration selection favored the enrichment of mutant alleles.

Different stoichiometric ratios between primers were also studied to improve the discriminant effect (Fig. 2d). When lowering the reverse primer concentration, the amplification delay of the native variant increased (higher C_q). The shift of the wild-type curve can be interpreted based

on the residual availability of the template strands to be replicated. Although the blocker was bound to the anti-sense native strand, the sense strand was still available. The maximum

discrimination (ΔC_q) was reached using 150 nM of the reverse primer and 300 nM of the forward primer (ratio 1:2). These conditions reduced the linear residual amplification of the native variant.

Comparison to conventional blocked qPCR. The enhanced genotyping capability of the EAB-qPCR method was experimentally confirmed. Conventional blocked qPCR was chosen as a control because it allowed to evaluate the effect of the blocker hybridization in the amplification yield and the assay selectivity, keeping all other conditions unchanged. The amplification curves showed the blocker annealing step favored the inhibition of the wild-type allele more than the mutated variants by displacing curves to higher cycles (Fig. SI.2). Discrimination capability was also compared to conventional blocked qPCR under symmetric and asymmetric conditions. Both methods resulted in a long wild-type curve delay (Fig. 3) and the calculated discrimination factors (ΔC_q) were 0.6 and 3.1 for conventional blocked qPCR approaches, compared to 7.9 for EAB-qPCR. Thus, our novel method more effectively inhibited the replication of the wild-type allele. Also, the blocking percentage was estimated from the delay data and the amplification efficiency equation (Table SI.3). Although mutant strands were also recognized (up to 15%), the blocker mainly hybridized to the wild-type strands, being blocking percentage 43.7-90.7% and 99.7% for blocked qPCR and EAB-qPCR, respectively.

Analytical performances. The amplification efficiency of the EAB-qPCR method was evaluated from serial dilutions of the mutant template (c.34G>T). Fig. 4a shows a quantitative response according to the template copies. The measured C_q values matched a linear behavior from 20 to 2 x 10^{10} copies per reaction, with a slope of -3.21 and a regression coefficient of 0.995 (Fig. 4b). From the calibration slope, good amplification efficiency was estimated with 102.6%.

194 Comparable results were observed for conventional blocked qPCR (Fig. SI.3a), and the
195 calculated values were -3.26, 0.995 and 104.8%, respectively.

196 Reproducibility was determined from triplicate assays and expressed as relative standard
197 deviation, with values going from 2.2% to 2.8%. The high consistency among the parallel results
198 confirmed the robustness of our proposed method.

199 Enrichment capability was estimated from the mixtures of the mutant (*KRAS* c.34G>T) and wild-
200 type DNA, and total DNA remained at 10^7 copies. By lowering the mutant percentage, a longer
201 amplification delay was recorded for EAB-qPCR (Fig. 4c) and the curve displacement was the
202 equivalent to a reduction in the initial template copies. As expected, the quantification detection
203 cycles showed a linear correlation with the logarithm of the mutant percentage (Fig. 4d). The
204 estimated detection limits were 1.5% for blocked qPCR and 0.5% for EAB-qPCR. Therefore, the
205 novel method provided 3-fold better enrichment capability.

206

207 **Application: detection of mutant variants in clinical samples.** The capability of EAB-qPCR
208 as a diagnostic tool in metastatic colon cancer was examined. In a double-blind study, biopsy
209 tumor tissues were classified depending on *KRAS* genotype by EAB-qPCR and two reference
210 methods (conventional blocked qPCR and NGS).

211 In all the patients, the EAB-qPCR provided a positive response, although conservation (formalin-
212 fixed and paraffin-embedded) could lead to DNA degradation (Fig. 5a). Likewise, replicate
213 assays yielded precise results (C_q variation below 0.8). Two groups of amplification curves were
214 distinguished: one with an average C_q of 23.2, another with an average C_q of 31.6 (average
215 delay of 8.4 cycles). By defining $C_{q,cut\text{-}off}$, a binary classification criterion was applied for
216 genotyping purposes. The DNA samples with C_q over 25 were classified as wild type, whereas
217 the samples with C_q below 25 were considered mutants (Fig. 5b). Therefore, there were 11 wild-

type patients 11 (55%) and nine *KRAS* mutants (45%). According to the oncologic guidelines, only those patients assigned to the wild-type group would be good candidates to receive monoclonal antibody therapy. On the contrary, the mutated group should develop resistance and present shorter progression-free survival [26].

These results were compared to those obtained by conventional blocked qPCR. In this method, the delay of wild-type curves was shorter (average delay of 2.6 cycles) and the classification window for genotyping, defined between two patient groups, was narrower (Fig. 5c and 5c). Thus, uncontrolled variations in the DNA template amount might lead to false-positive or false-negative assignations. In fact, the conventional method yielded an uncertain identification with two patients' samples.

Accuracy was estimated by independently sequencing patients' samples by applying NGS (Table SI.4). A total agreement of the assigned mutant genotypes validated the developed method. The estimated clinical sensitivity and selectivity were 100%. Regardless of the mutation's type and position, EAB-qPCR was capable of detecting all the studied variants in codon 12 and 13 of the *KRAS* oncogene (c.35G>A, c.34G>T, c.35G>T and c.35G>C). The mutation percentage in biopsied tissue samples correlated with the measured C_q values, estimating a detection limit about 0.05 % (Fig. SI.4). Therefore, EAB-qPCR can be considered a reliable method, although the large wild-type DNA amount in tumor tissue can hinder the detection of mutant alleles.

Discussion

In the last decades, various qPCR-based methods have been reported for the detection of single-nucleotide changes [8, 10, 11]. A common drawback of blocked approaches is guaranteeing effective primer/blocker competence [27]. The developed method, called EAB-

qPCR, shows excellent amplification performances for low-abundant mutant variants in complex samples. The novelty involves the combination of asymmetric qPCR, a blocking agent and a proper thermal cycling. Our strategy minimizes undesired DNA replications because the blocker's recognition process is promoted by controlling the thermodynamic conditions. For EAB-qPCR method, only three wild-type strands per 1000 copies escaped from the blocker's action, yielding a high selective enrichment of minority alleles.

Among the current genotyping techniques, EAB-qPCR can be classified as a high-moderate sensitive method for mutational analyses (0.1–1%) [7], and was only overcame by ddPCR technology [15] and ice-COLD-PCR [28] (0.001-0.1%). As the main difference lies in the thermal cycle, operational EAB-qPCR features were similar to other PCR-based methods, such as instrument (e.g. fluorescent thermal cycler), auxiliary equipment or material [29]. The assay cost is lower (about 2.5 € per assay) than the approaches that use modified oligonucleotides as blocking agents (i.e. PNA, LNA) [20, 21] or fluorescent markers (i.e. COLD-PCR) [4, 17]. In addition, the oligonucleotide design is easier to be implemented than COLD-PCR approaches. Compared to ARMS-PCR, our approach avoids the use of allele specific primers, that require a laborious process for optimization, and improves the detection capability because ARMS-PCR reports false positives when the mutant content is below 1% [30].

Concerning to assay time, EAB-qPCR was generally slightly longer than other qPCR variants (1 min per cycle) and shorter than LATE-PCR (20 cycles less) [23]. Similar sample requirements were estimated given the quality and amount of DNA (4 ng/µL, equivalent to 1300 copies). EAB-qPCR did not entail substantial additional requisites compared to qPCR-based genetic testing that is currently performed routinely in laboratories [24, 31]. However, the discrimination capability of EAB-qPCR was several times higher than several PCR approaches and, consequently, enhanced enrichment extends potential clinical applications. Therefore, most of

current genotyping techniques are expensive, tedious and complex, and require specialized techniques compared to EAB-qPCR.

Achieved sensitivity (~~up to~~ 0.5~~1~~% mutant percentage) and reliability (high accuracy and reproducibility) enabled the detection of single-nucleotide mutations in clinical human tissues (solid biopsies), as we demonstrated with colorectal cancer subjects. In fact, the amplification efficiency in paraffin-embedded biopsied tissues from patients had not been hampered by some interfering factors, such as presence of inhibitors. The validation study performed by NGS evidenced that our method can detect mutant alleles in tissues, even those with low percentages of tumor cells. The accurate discrimination was achieved independently on the kind of mutated base. In most of clinical scenarios, this detection capability is enough to choose the proper treatment or patient classification. Using SYBR Green as a detection dye makes EAB-qPCR simple and universal for the detection of single-base mutations. For the determination of the specific genotype, EAB-qPCR can be improved combining it with Taqman probes (or similar probes) or adding discrimination steps, such as fast hybridization assays [32], although these approaches would increment the method complexity.

The amplification performances of EAB-qPCR proved that reliability can be applied to more situations where minority alleles can be detected. Furthermore, extending this method to detect other DNA alterations is relatively easy. One potential example is prenatal diagnosis because the enrichment of fetal DNA sequences in the presence of excess maternal DNA requires sensitive solutions. In the infectious diseases field, the detection of a few copy numbers of microorganisms is crucial. For that, the requirements are clearly identified. Primers should be chosen for a selective amplification of the target region with a high amplification yield following the standard design algorithms for qPCR methods based on thermodynamic data (e.g. GC percentage, length, melting temperature, absence of secondary structures). The other requirements relate to the blocker. First, the blocker must strongly hybridize to the native

template (wide variation in free energy, ΔG). To minimize the undesired inhibition of mutant variants, mutations must be in a central position given a greater destabilization of mismatched complexes (low ΔG). Second, the blocker/template hybrid must be stabler than the primer/template hybrid to establish the intermediate step of the thermal cycle. Third, the blocker should partially overlap the forward primer. This clamp strategy induces greater competition at the binding site by destabilizing the formation of primer/blocker/template complexes. Fourth, the 3'-end must be functionalized to avoid blocker elongation by polymerase activity during the thermal cycling. In short, the EAB-qPCR method requires a blocker oligonucleotide with stronger hybrids for the wild-type template than the mutant template ($\Delta G_{Blocker,wild\text{-}type} > \Delta G_{Blocker,mutant}$).

In conclusion, EAB-qPCR turned out to be an accurate cost-effective approach for extensive use in clinical laboratory settings, because the assay is performed with oligonucleotide without modifications such as LNA or PNA. Indeed, we demonstrated that EAB-qPCR enables an accurate profiling of DNA variants to make genomic analyses more affordable and economical. Therefore, the proposed strategy has the potential to become a powerful biosensing tool to support patient prognosis and classifications in appropriates population groups for diagnostics or for receiving personalized treatment.

Declarations

Conflict of interest. The authors declare that they have no conflict of interest.

Ethical approval.

Research involving human subjects complied with all relevant national regulations, institution policies and is in accordance with the tenets of the Helsinki Declaration (as revised in 2013), and has been approved by the authors' Institutional Review Board. Informed consent was obtained from all individuals included in this study.

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Figure 1. Scheme of the mechanism of the EAB-qPCR method applied to a wild-type allele (left) and mutant alleles (right). The unfilled white squares correspond to the different mutant genotypes that may exist H: A, C or T.

Figure 2. Discrimination effect depending on the EAB-qPCR conditions. a) temperature, b) time, c) blocker concentration, and d) stoichiometric ratios between primers (forward:reverse). Target: the *KRAS* gene (codon 12-13). Mutant: p.G13D (c.38G>A). Template: Cell lines at 10^5 copies.

Figure 3. Amplification curves: (a) conventional blocked qPCR in symmetric format, (b) conventional blocked qPCR in asymmetric format and (c) EAB-qPCR. (d) Percentage of blocking estimated from efficiency calculations. WT: wild-type template. MUT: mutant template. Target: the *KRAS* gene (codon 12-13). Template: Cell lines at 10^7 copies. Mutant: p.G13D (c.38G>A).

Figure 4. Assay sensitivity of EAB-qPCR. a) Effect of DNA copy number for the mutant template. b) Correlation between C_q and DNA copy number. c) Effect of the mutant template %. d) Correlation between C_q and the mutant template percentage. Mutant: *KRAS* c.34G>T.

Figure 5. Mutational analysis of cancer patients. a) Amplification curves of EAB-qPCR. b) Discrimination map of EAB-qPCR. c) Amplification curves of conventional blocked qPCR. d) Discrimination map of conventional blocked qPCR. Specific mutant variants were determined by Ion Torrent sequencing technology.