Chapter 3

Design of Oligonucleotides for Allele-Specific Amplification Based on PCR and Isothermal Techniques

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Abstract

Single-nucleotide variations have been associated to various genetic diseases, variations on drug efficiency, and differences in cancer prognostics. The detection of these changes in nucleic acid sequences from patient samples is particularly useful for accurate diagnosis, therapeutics, and disease management. A reliable allele-specific amplification is still an important challenge for molecular-based diagnostic technologies. In the last years, allele-specific primers have been designed for promoting the enrichment of certain variants, based on a higher stability of primer/template duplexes. Also, several methods are based on the addition of a blocking oligonucleotide that prevent the amplification of a specific variant, enabling that other DNA variants can be observed. In this context, genotyping methods based on isothermal amplification techniques are increasing, especially those assays aimed to be deployed at point-of-care applications. The correct selection of target sequences is crucial for reaching the required analytical performances, in terms of reaction time, amplification yield, and selectivity. The present chapter describes the design criteria for the selection of primers and blockers for relevant PCR approaches and novel isothermal strategies. Several successful examples are provided in order to highlight the main design restrictions and the potential to be extended to other applications.

Key words Primer design, Single-nucleotide mutations, PCR, Isothermal amplification, Allele-specific technique, DNA biosensing

1 Introduction

The growing advances in DNA sequencing have enabled a deep knowledge about the genome of several species, finding the main genetic variations and their frequencies [1]. There are sequence alterations inherited from parents (germline) or acquired over the life of an individual (somatic). The changes in the nucleotide sequence may or may not cause phenotypic changes, the latter being the principal driver of diseases. Specially, the mutations are crucial for all living organism because they are permanent alterations in the sequence of genomic DNA, modifying its integrity, stability, and functionality. A change in the structure and function of coded protein can produce some catastrophic disorders and

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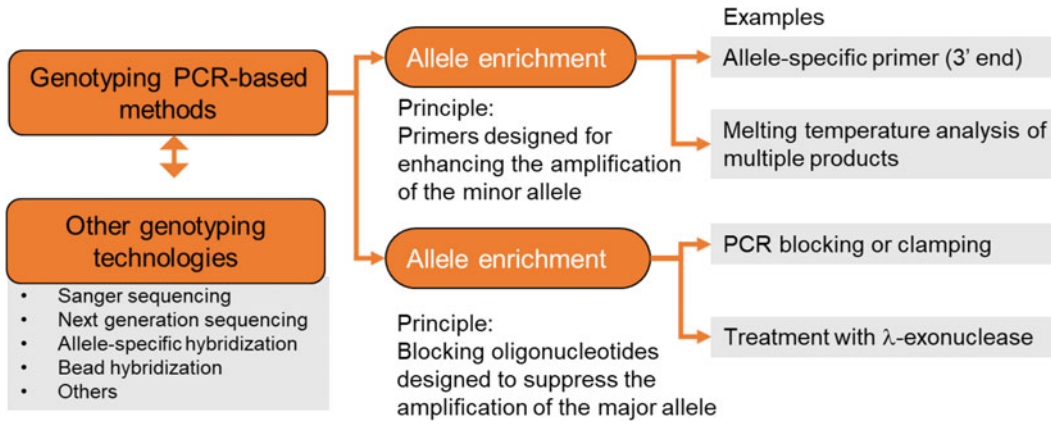


Fig. 1 Classification of allele-specific PCR-based methods

abnormalities. Mutations can result from unintentional errors, during cell division, DNA repair, and replication or as the result of environmental factors. Genome analyses have identified sequence variants related to disease susceptibility, treatment efficacy, and adverse drug responses [2].

Accurate genotyping of single-nucleotide variants (polymorphisms or mutations) is playing an important role in the development of advanced molecular-based solutions, including personalized medicine. For instance, allele-specific methods allow health professionals to accurately diagnose a disease and prescribe appropriate treatment specific to each individual or a target population. An overview of the main genotyping technologies is given in Fig. 1. Nowadays, sequencing techniques are considered the gold standard for low frequency allele detection in high-throughput format. However, their high cost and time-consuming limit their massive use. Thus, the PCR-based detection of genetic markers is still interesting, especially for those to be deployed in sustainable health systems and scenarios aiming at point-of-care detection. The objective is to implement methods with high capability for detection and quantitation, simultaneously providing good mutation sensitivity, high mutation multiplexing, fast turnaround, and low reagent and instrument cost [3].

A variety of PCR-based methods have been developed to enhance detection of genetic variants, being classified in allele enrichment and allele suppression. However, the extension of an existing method to new target locus can show problems due to their complexity. In some cases, amplification errors are caused by mispriming, because the design of primer is one of the most crucial factors affecting the success and quality of any amplification-based method [4]. In fact, the selection of oligonucleotides for the discrimination of DNA variants is more difficult than conventional PCR-based methods. Hence, there is a need for a correct choice of

involved oligonucleotides, in order to develop efficient methods. In the present chapter, the criteria to find the candidate primers and blockers for allele-specific assays are revised.

2 General Requirements

2.1 In Silico Design

For general PCR-methods, automated algorithms, software or specialized websites are available for primer design. Some of them are able to consider the presence of a polymorphism in the input sequence or in primers, Primer3 Plus being the most popular free primer design software [5, 6]. However, few of them are useful for the specific objective of allele-selective amplification. Thus, the optimal sequences are often done manually using a sequence alignment program and checking the properties of possible sequences.

The selection of oligonucleotides usually follows a common initial protocol. Once the target variation has been defined, the next step is to find potential primers regions on the corresponding gene sequences. The design algorithm starts by considering the default requirements, widely described in the literature [4, 7, 8]. The general parameters to check are related to the primers and to the amplicon such as melting temperature, length, GC content, self-complementary, primer-dimer and hairpin formation, degree of degeneracy, end stability, and end specificity. Table 1 shows the main restrictions for designing primers in any PCR technique. A general recommendation is the selection of short products, because they amplify more effectively. Also, stable secondary structures, such as hairpins, tetraplex structures associated to poly-C or poly-G regions, must be avoided because they can interfere the annealing and extension steps. In multiplexed assay, the risk of unbalanced amplification or primer-dimers is high. Additional design

Table 1
Default design restrictions applied for the primer design

	Requisite	Parameter	Recommended interval
Primers	Selectivity	Length	16–25 nucleotides
		T_m	55–65 °C
	High amplification efficiency	ΔT_m primers	<5 °C
	Avoid stable secondary structures	GC%	35–75%
		Self-annealing	Avoid
		Base degeneration	Avoid
	Avoid secondary products		
Products	Avoid stable secondary structures	Length	50–150 pb

T_m: Melting temperature

restrictions must be incorporated, such as similar melting temperatures of primers and the absence of cross-reactivity.

Depending on the allele-specific method, the design algorithms progressively include additional requirements for achieving the required selectivity [9, 10]. So, several candidates are removed because they are not adequate for the detection of DNA variants. The following sections describe the specific restrictions depending on the discrimination method.

2.2 Primer Validation

The output information from design algorithm is a set of oligonucleotides (primers and blocker) per each locus and the working temperatures obtained from thermodynamic calculations. The next step is the confirmation experiments, performing assays to control samples. Several quantification methods of PCR products are available, depending on the number of DNA variants simultaneously analyzed. The first category is individual allelic methods including end-point approaches, single channel fluorescence qPCR, and most biosensing methods. The second category comprises methods based on multiple channel fluorescence qPCR and allele selective PCR combined to capillary electrophoresis or DNA microarrays.

In spite of the efforts for selecting proper sequences, several errors can be observed. Primer-dimers, mismatched regions, or other nonspecific amplification products could be generated. Sometimes, the resulting methods suffer from limited accuracy for differentiating single nucleotides and low (or none) multiplexing capability.

3 Primer Design for Allele Enrichment in PCR-Based Methods

3.1 Method Principle

Several PCR-based techniques have aimed on enhancing the amplification of the minor allele by the design of primers specific to a unique target variant (Fig. 2). The activity of polymerase is mainly focused on the duplication of the perfect-match strand, eluding the mismatched sequences. After several amplification cycles, the number of copies are several orders higher than the rest of the initial present alleles, enabling their detection. Obviously, an erroneous annealing of perfect-match primer to other alleles has a drastic consequence on assay selectivity, since these undesired variants would be amplified in a high yield. Allele-specific PCR has the advantage of single-nucleotide discrimination with no additional probes or enzymes required.

3.2 Design Considerations

The main restriction for designing an allele-specific primer is the position of primer respect to the DNA variant. The forward (or reverse) primer is selected, considering the 3′-end nucleotide matches to a single targeted variation in the template sequence.

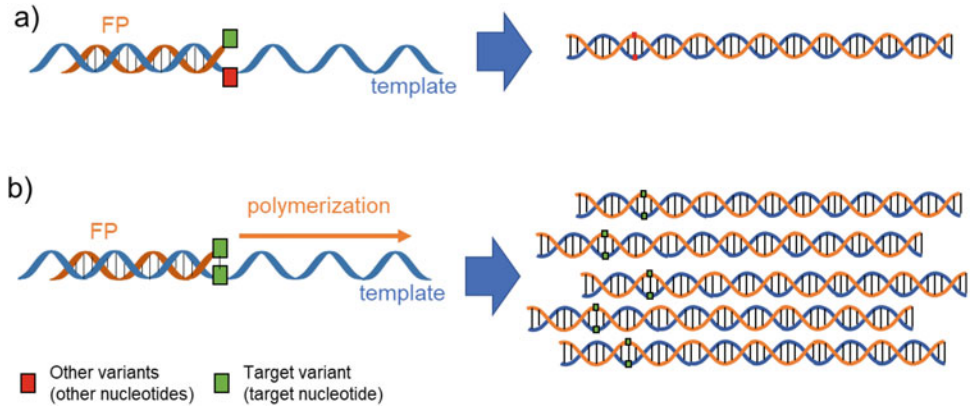


Fig. 2 Scheme of an allele enrichment approach based on the use of allele-selective primers: (**a**) Absence of target template: null (or low) amplification efficiency. (**b**) Presence of target allele: high amplification efficiency. Mechanism principle: Perfect annealing between primer and target template

Thus, the complex primer-template is more stable than the rest of mismatch hybrids. The reverse (or forward) primer is chosen, applying the general restrictions of PCR design.

Compared to conventional PCR, the number of candidate primers are lower. The theoretical pairs of the allele-specific primers to be used in a single assay are tens. In a multiplex assay (genotyping of several loci at time), the number of possible combinations can achieve hundreds or thousands. Nevertheless, few software tools are available for primer design in the genotyping field. The WASP tool is a primer design program applied specifically to allele-selective PCR [11]. Other remarkable program is BatchPrimer3, based on the Primer3algorithm, which incorporates a specific module to choose the best primer pairs for genotyping [12]. Recently, our research group developed a novel design workflow for generating the oligonucleotides [10]. The innovative algorithm, called Multi-SNP, supports the simplest approach, e.g., single reaction per variant, to the most complex alternatives, e.g., multiplex amplification (Table 2). The workflow was developed on the basis on the choice of the oligonucleotide sets available to satisfactorily amplify/detect the given variants. If a specific module is activated, the output can include the zip-code for each primer and probe sequences per each targeted single-nucleotide polymorphism (SNP), useful information for universal microarray detection.

3.3 Enhanced Approaches

A number of strategies have been proposed to improve the specificity and reliability of DNA primers in this technique. An interesting approach is the use of nucleotide analogs to facilitate base conversion at 3′ mismatched sites [13]. The most extended solution is called the amplification refractory mutation system [14]. The inclusion of deliberate mismatch at the penultimate base of primers enhances the reaction selectivity, because the complexes between

Table 2
Modules used for designing primer for allele enrichment approach (multi-SNP software [10])

	Input information	Analysis	Output information
Basic	• Sequences (FASTA) • Primer design constrains	• Pre-selection of forward and reverse primers (Single module) • Selection of primer sets (Multiple module)	• Selected primer sets • Oligonucleotide parameters • Recommended PCR conditions
Advanced	Database of Zip-code sequences	• Selection of zip-codes (Zipcode module) • Selection of intentional mismatches	

primer and mismatched strands are less stable. Depending on the template sequence of wild-type and mutant variants, a specific base is recommended for producing the highest destabilizing effect [15]. Table 3 summarizes the possible cases and the recommended mismatch in the primer sequence. According to the design algorithm, allele-specific primers are chosen considering the 3′-end nucleotide and the recommended destabilizing penultimate base. Performing the amplification reactions using respective primers, the result is that consecutive mismatches at the two terminal bases prevent the undesired extension of mismatch alleles, enriching the target variant.

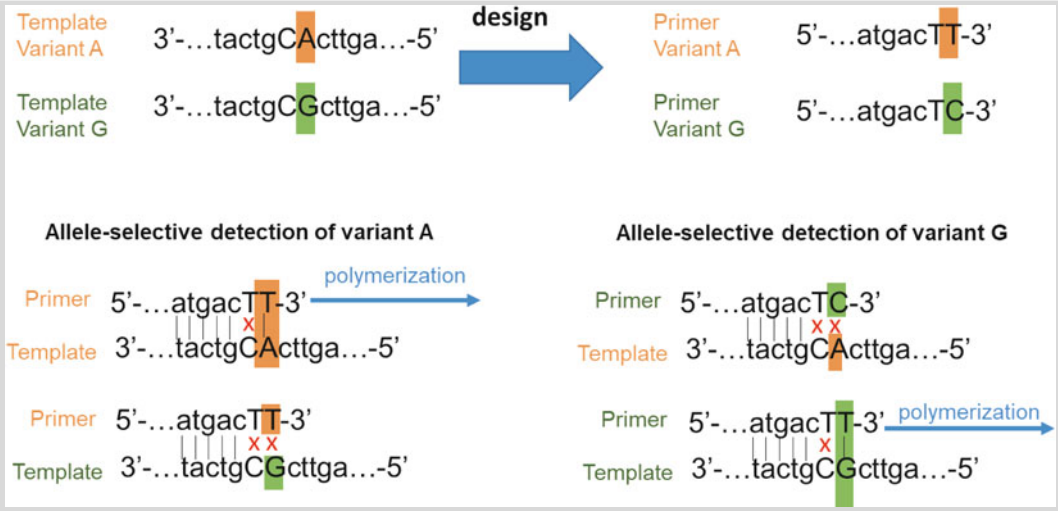
4 Primer Design for Allele Suppression in PCR-Based Methods

4.1 Method Principle

The strategy of several PCR-based techniques is focused on the wild-type variant. In many clinical samples, such liquid biopsies and certain solid tissues, the copy number of mutated DNA is extremely low. Consequently, the undesired amplification of the abundant wild-type template generally masks the generated copies of target mutant. Thus, the approach is to reduce or eliminate the formation of wild-type copies, enabling the efficient amplification of minority alleles. These methods, called blocked PCR or clamp PCR, introduce a reagent in solution to specifically block amplification of wild-type DNA [16]. Figure 3 shows the general allele suppression mechanism for the detection of a single-nucleotide mutation. The formation of blocker-template hybrid inhibits the DNA polymerization, making easier the duplication a detection of other sequences.

Table 3
Suggested mismatch at penultimate base of primer sequence and destabilizing effect for enhancing allele-specific amplification, depending on template sequence. An example for the variation A/G is also represented

	Anterior base of template (3′–5′)				Mismatch of template	Effect
	A	C	G	T		
Destabilizing mismatch at penultimate base of primer	G	T	A	C	A/G	Strong
	G	T	A	C	C/T	Strong
	G	T	A	C	T/T	Strong
	G	T	A	C	C/C	Strong
	C	A	T	G	T/G	Medium
	T	A	C	G	A/C	Medium
	G	G	A	A	A/A	Weak
	G	G	A	A	G/G	Weak
	–	–	–	–	A/T	None
	–	–	–	–	G/C	None



4.2 Design Considerations

Both primers are blockers are the critical components, as their properties control the exquisite specificity and sensitivity that make this method uniquely powerful. The blocker can be designed to bind DNA template depending on the chosen primer region. In case of overlapping design, a competition between primer and blocker is established to generate a duplex with DNA template at the same region. A stable blocker/template complex favors the blocker annealing against the primer, so wild-type replication is directly avoided. In case of not overlapping design, both primer and blocker bind to the DNA template in different regions. But the presence of stable blocker/template complex in downstream sense inhibits elongation step by halting the polymerase activity. Other

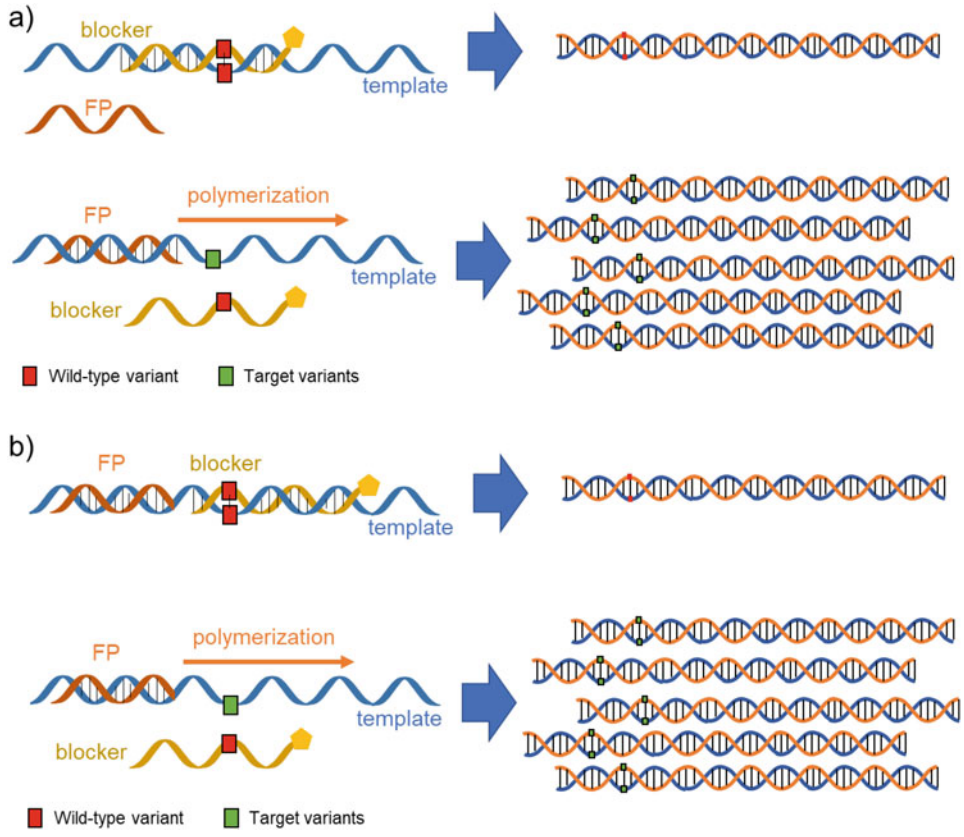


Fig. 3 Scheme of an allele suppression approach based on the use of blockers: (**a**) Overlapping approach. (**b**) Not overlapping approach

important requirement is that the blocker cannot function as primer as they are intrinsically resistant to the DNA-specific enzymatic activity. A common solution is the selection of 5-′-functionalized oligonucleotides, because the presence of an inert group prevents its extension [17].

Regarding the sequence selection, a recommendation is to apply the algorithm used for probes in methods such as qPCR or hybridization assays in array format [4]. Constrictions are related to avoid cross-hybridization between primers and blocker, or secondary structures that reduce the annealing or amplification yield. Nevertheless, a proper selection is more critical in blocked PCR. An incomplete blocking on amplification of the clamped DNA can produce an excessive copy number, resulting in unspecific detection signal. An incorrect blocker sequence can yield a total inhibition of amplification, including target variants. Thus, the goal is to maximize the stability difference between allele complexes. Generally, the best option is that the blocker contains perfect-match nucleotide for the studied variant at a central position due to a high

destabilization and the reduction of the melting temperature higher than 3–5 °C. Thereby, the sequence effect controls the selective suppression of a specific allele, generally wild-type.

Ideally, the working temperature can be estimated on the basis on thermodynamic calculations, considering the melting temperatures. The inhibiting effect can be obtained from the associated differences in melting temperatures between blocked template and the rest of variants combined to the number of copies available to be replicate. Generally, empirical optimization is also required because there are many interactions possible among DNA species that could influence melting temperature of DNA/blocker complex [3].

4.3 Enhanced Approaches

An improvement of assay performances is achieved if the melting temperature of a perfectly matched blocker-DNA duplex is much higher than that of a perfectly matched DNA-DNA duplex of the same length. A solution is the use of nucleic acid analogous. Published blockers include peptide nucleic acids (PNAs), locked nucleic acids (LNAs), and some bridged nucleic acids [16, 18]. In PNA clamp methods, the resulting hybrid PNA-nucleic acid duplexes exhibit high thermal stability. PNA/DNA interactions are commonly more stable than the corresponding DNA/DNA duplex minimizing undesired amplification, being 1 °C per base pair. On the other hand, the erroneous mismatched duplexes are less stable than the corresponding DNA/DNA duplex of the same sequence, maximizing the duplication of target alleles [19]. The incorporation of LNA, chemical modified nucleoside, also enhances the clamp effect, and consequently the specific single-nucleotide recognition [20, 21].

5 Primer Design in Isothermal Methods

5.1 General Considerations

PCR-based techniques are widely used for genotyping purposes, but they have several drawbacks, leading to specificity and amplification yield variations depending on the reaction conditions [22]. The main limitation is the need for strict temperature control to avoid irreproducible enzymatic activity. Furthermore, more than 90% of the total time is due to temperature cycling rather than to the polymerization process. These disadvantages, coupled to the compulsory requirements of specific equipment, such as thermocyclers with high-volume, mass, and energetic properties, limit their massive use in several scenarios, such as low resources settings, or decentralized laboratories.

In the last decades, several enzymatic reactions to DNA replication at a constant temperature have been proposed as amplification alternatives. Some of these isothermal approaches provided excellent performances for biosensing [23, 24]. The use of certain

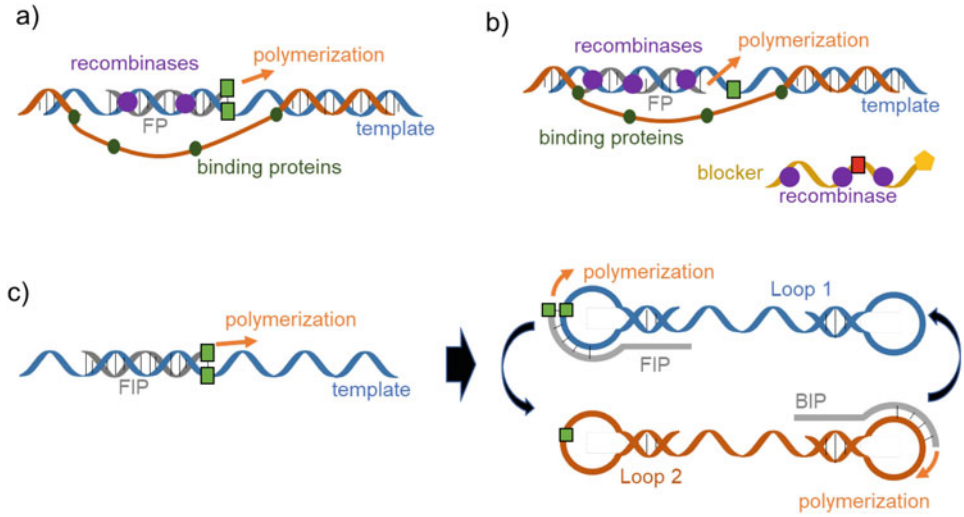


Fig. 4 Scheme of isothermal amplification methods: (**a**) allele-specific RPA, (**b**) blocked RPA, (**c**) allele-specific LAMP (3′-format)

enzyme (or combination of enzymes) avoids heating the double-stranded nucleic acid for template separation, and consequently the use of a thermocycler for heating/cooling cycles. Then, DNA assays are performed using only a simple heating system, making the integration of point-of-care technologies easier.

In the field of low-cost genotyping, several assays have been suggested for allele-specific discrimination. They include variants based on recombinase polymerase amplification (RPA) [25], loop-mediated amplification isothermal (LAMP) [26], and rolling circle amplification (RCA) [27].

The primers are chosen depending on the specific requirements associated to each amplification mechanism (Fig. 4). The most common method for designing LAMP primers is to use PrimerExplorer from Eiken Chemical Co. Ltd. Other examples of specific software are PrimedRPA for RPA [28] and LAVA for LAMP [29]. For allele selective enrichment or suppression, the specific factors are also required.

5.2 Allele-Specific RPA

RPA is an isothermal technique that replicates DNA supported in the action of several enzymes and binding proteins for stabilizing primer-template complexes and opened strands. Then, the recombinase (T4 uvsX) recognizes targeted DNA templates and specific primers at a high affinity and catalyzes subsequent homologous pairing and strand exchange. Bsu polymerase extends the recognized oligonucleotide, duplicating the template strand (Fig. 4a).

The DNA polymerization requires two primers (forward and reverse), selected following similar criteria to allele-specific PCR approach, although longer oligonucleotides are recommended for

certain template sequences [24]. In the allele-specific format, the 3′-end nucleotide is chosen, depending on the allele to be enriched. The polymerase mainly produces the elongation of the perfect-annealed primer/template, at a low working temperature (37 °C). Furthermore, the Pol I large fragment (Bsu polymerase) lacks exonuclease activity (3′→5′) that may modify the target nucleotide. Therefore, the presence of mismatches on their 3′-extreme to hamper the nonspecific reaction due to the combined action of two enzymes.

5.3 Blocked RPA

The mechanism of blocked RPA involves a variation of recombinase action respect to the standard RPA. The blocker modifies the formation of recombinase/primer/polymerase complex, because there is a competition between primer and blocker for binding to the opened template strand (Fig. 4b).

Basically, the blocker is selected considering that (1) the mutation position is at a central position, (2) melting temperature should be higher to both primers (upstream and downstream primers), and (3) a 3′-end modification is required to inhibit the extension. Also, thermodynamic criteria are considered to induce more stable complexes for the binding site in DNA template at a working temperature of 37 °C.

5.4 Allele-Specific LAMP

Conventional LAMP is the most extensively studied isothermal amplification technique. The main advantages over other approaches are its high yield, good tolerance to inhibitors, short time, and compatibility with several detection principles. The conventional approach relies on four primers to recognize six different sequences of the target DNA, which also leads to very high specificity. The action of a highly strand-displacing DNA polymerase (Bst polymerase) generates large amounts of dumbbell-like structures under isothermal conditions (60–65 °C).

For allele-specific amplification, two approaches have been described, based on (1) the annealing selectivity of allele-specific inner primers (3′AS-LAMP) and (2) the selective DNA synthesis from a dumbbell-like starting structure (5′ AS-LAMP). The key feature is the position of single-nucleotide variation in the primer sequence (Fig. 4c). For 3′ AS-LAMP format, the polymorphism was located at the 3′-end of the forward inner primer, leading to allele-specific primers and a reverse inner primer common to both reaction mixtures. In the 5′ AS-LAMP format, the polymorphism was located at the 5′-end of both forward inner primer and backward inner primer. Therefore, the selection is performed applying the general requirements of LAMP design and an additional constraint related to the alignment of allele-specific primers to the target variant.

6 Examples of Applications

Hundreds of applications based on allele-specific PCR have been described in the last 30 years, covering a wide range of research fields [13–15, 30]. These techniques have been widely applied to establish clear discrimination factors and identify populations with a given polymorphism. A relevant interest is the profiling of variants with low frequencies in fields such as cancer prognosis. These methods also facilitate point of care or field-use nucleic acid testing for other clinical applications in which rare variants exhibit disproportionate impact, such as antibiotic-resistant subpopulations of infectious diseases. The discrimination of SNPs supporting pharmacogenetics or fetal diseases is other relevant area.

The amplification assay has been integrated with different detection events, including from conventional gel electrophoresis to simple lateral flow systems. For instance, Kompetitive Allele-Specific PCR (KASP) is one of the uniplex SNP genotyping platforms, and has evolved to be a global benchmark technology, particularly in crop improvement [31]. In this chapter, the study was focused on methods with high-performances and/or capabilities to be implemented in point-of-care systems. Figure 5 shows several examples of applications based on low-cost detection or biosensing.

6.1 Example 1

The performance of Multi-SNP algorithm supporting allele-specific PCR methods was evaluated for the simultaneous genotyping of three SNPs [10]. The assayed polymorphisms were related to immunosuppressive drugs administered after solid organ transplantation. Oligonucleotide candidates were confirmed by discriminating homozygote and heterozygote populations using a fluorescence solution method and two colorimetric microarray methods on polycarbonate chips. Also, the genotyping of specific SNPs associated to attention-deficit hyperactivity disorder was achieved [32]. Buccal swab and blood samples taken from patients and controls were analyzed by allele-specific amplification. The simultaneous discrimination of three SNPs was successfully achieved, providing excellent analytical performances.

6.2 Example 2

The strategy of allele-specific RPA has been applied to the genotyping of four SNPs related to the treatment of tobacco addiction [33]. In this study, the assay was performed in a chip platform containing 100 wells, manufactured with a 3D printer. The primers were selected to produce important changes in free energy between perfect-matched and mismatched primer/template duplexes. Results also showed that the LNA primers improved allelic discrimination compared to conventional primers.

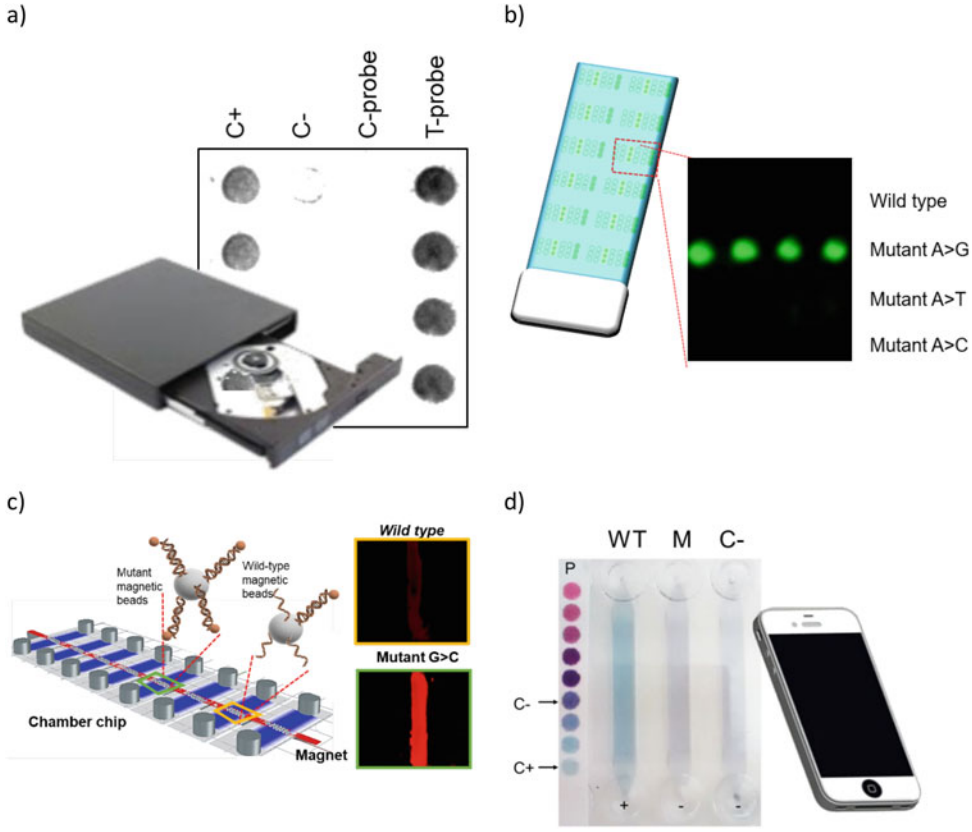


Fig. 5 Examples of allele-specific amplification performed in point-of-care systems: (**a**) Allele-specific PCR combined to hybridization assay and compact disc detection [10]. (**b**) Allele-specific RPA combined to hybridization assay and low-cost colorimetric detection [33]. (**c**) Blocked-RPA combined to magnetic bead hybridization inside of a chamber chip [34]. (**d**) Allele-specific LAMP in 5′ AS-LAMP format combined to microfluid chip assay and smartphone detection [35]

6.3 Example 3

The first method based on blocked-RPA approach aimed the enrichment of mutated templates in heterogeneous specimens as tumor tissues [34]. The amplification was combined to a fast hybridization assay performed in microarrsay format on plastic chip and colorimetric detection. The detection of mutations in *PIK3CA* oncogene was achieved operating at 37 °C during 40 min. The assignations agreed with those provided by next-generation sequencing. In a second study, the combination of blocked-RPA and selective capture by probes immobilized on magnetic-core particles integrated into a flow system was presented [35]. As proof of concept, the single-nucleotide substitutions of the *KRAS* oncogene were studied in chip with parallel microchambers and permanent magnets. The blocked RPA products (generated at 37 °C) from tumor biopsies provided a specific fluorescent bead-line that depended on the present mutation.

6.4 Example 4

Several studies have confirmed the successful discrimination of allele-specific LAMP. Screening of SNPs typing of the *CYP2C19* gene from untreated blood samples with minimal user operation was achieved [36]. The assay was developed inside an integrated microcapillary containing preloaded reagents and DNA extraction card. Specific point mutations in the dhfr-ts gene have been identified to resistant phenotypes from lysates of the malaria parasite [37]. Both methods (3′AS-LAMP and 5′AS-LAMP) were compared and applied to human samples to genotype an SNP biomarker (rs1954787) located in the *GRIK4* gene and related to the treatment response to antidepressant drugs [38]. The discrimination methods were successfully combined to on-chip hybridization and integrated into user-friendly optical devices (smartphone). A recent paper describes a novel modification for improving selectivity of 3′AS-LAMP approach [39]. An artificial mismatch into the third position from the 3′ terminus of the probes prevented false-positive extension reaction of the probes.

6.5 Example 5

Allele-specific RCA was introduced for the detection of point mutations [40]. In the assay, an allele-discriminating padlock probe (3′-end) was designed for targeting the sequence. A circular template generated by enzymatic ligation upon the recognition of a point mutation on the oncogene could be amplified isothermally by Phi29 DNA polymerase. Blocked LAMP was proposed, incorporating of PNA-modified blocker in the reaction mixture of LAMP [41]. The mixture contained four primary primers for the target regions on the *KRAS* gene and a clamping PNA probe that was complementary to the wild- type sequence. The method discriminated cDNA from four kinds of pancreatic carcinoma cell lines with or without the point mutation.

7 Conclusions

Genetic testing has an increasing interest in several fields, such as pharmacogenomics, noninvasive cancer recurrence monitoring, and pathogen strain identification. During the last decades, genotyping PCR-based methods contribute to provide valuable information about DNA variants. The challenge has been to develop methods with high selectivity and sensitivity, independently on allele abundance. In this way, the role of primer design is crucial in order to generate the expected products, avoiding nonspecific products, and incorrect genotyping results. A proper selection of design tools may reduce the required resources by lowering the chances of failed experimentation.

An ideal algorithm must be designed to support methods based on individual or multiple allelic detections. However, an important conclusion is that there are few examples of design software for

allele-specific amplification and few of them offer the capacity to discriminate different mutant subtypes. This chapter reviews the key points of design algorithms and the main requirements for both allele enrichment and allele suppression approaches.

Isothermal methods provide excellent future perspectives, especially in conditions where the needs of molecular analysis methods are growing, such as personalized medicine and targeted treatment of animals or plants. Since they are conducted at constant temperature, there is no time loss resulting from the temperature changes that are necessary for conventional PCR methods. Therefore, isothermal-based methods are potentially more cost-effective than conventional ones. The published applications by RPA, LAMP, or RCA variants have demonstrated to be useful even for minimally equipped laboratories.

Compared to other genotyping technologies, PCR-based methods, and specially the isothermal variants, are extraordinarily useful for developing point-of-care testing. In consequence, a fast cost-effective clinical decision should be provided in areas such as diagnosis, treatment choice and monitoring, and prognosis.

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