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Combination of a modified block PCR and endonuclease IV-based signal amplification system for ultra-sensitive detection of low-abundance point mutations

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ABSTRACT

By combination of a modified block PCR and endonuclease IV-based signal amplification system, we have developed a novel approach for ultra-sensitive detection of point mutations. The method can effectively identify mutant target sequence immersed in a large background of wild-type sequences with abundance down to 0.03% (for C → A) and 0.005% (for C → G). This sensitivity is among the highest in comparison with other existing approaches and the operating procedures are simple and time saving. The method holds great potential for future application in clinical diagnosis and biomedical research.

1. Introduction

A point mutation is a kind of mutation that a certain nucleotide in a DNA sequence is replaced with another nucleotide. DNA point mutation may lead to the alteration of amino acids that the nucleotides code for and change the whole protein, which causes diseases [1]. Point mutations occurred in coding regions for tumor suppressor proteins are closely related to cancers [2]. Somatic KRAS mutations are found at high levels in colon cancer [3], pancreatic cancer [4] and lung cancer [5]. Mutations occurred in tumor suppressor gene p53 have been found in more than 50% of the cancer patients [6]. Therefore, sensitive detection of point mutations is of great importance. The challenge lies in that point mutations often present at very low levels in human bodies due to tumour growing [7], evolution [8], and allelic heterogeneity [9]. It requires highly sensitive detection methods that are able to identify the rare mutant sequences in a large background of single-base-different wild type sequences. The lower abundance point mutation can be differentiated, the earlier stage of diseases can be diagnosed.

Conventional methods for point mutation detection include high resolution melting (HRM) [10], sequencing [11,12], sequence specific probes [13,14], selective PCR [15–17] and enzyme discrimination [18–22]. These methods can be applied individually and they generally offer a detection limit of 1–3%. Point mutations with abundance below 1% can hardly be detected with above single method. Combination of these methods can considerably improve the sensitivity [23–29]. For example, with two-rounds of allele specific PCR (AS-PCR) [25], 0.01% mutant sequences can be differentiated. However, the method is only sensitive to a few types of mismatches and the operating procedures are time consuming. In recent years, oligonucleotide-based fluorescent probes have generated a number of promising results in both DNA detection and nuclease measurement [30–33]. By employing the newly observed discrimination ability of Endonuclease IV (Endo IV) between perfect match and single-base mismatch strands and introducing target recycling strategy and sequence specific oligonucleotide fluorescent probes into the detection of low-abundance mutation, we successfully identified mutant sequences at abundances as low as 0.01% [20,21].

In this work, we attempt to further enhance the sensitivity of point mutation detection by combination of a selective PCR with aforementioned Endo IV-based signal amplification system. Selective PCR preferentially amplifies the mutant templates over wild-type templates, thereby enriching the abundance of mutant sequences. A number of selective PCR have been developed so far, such as co-amplification at lower denaturation temperature-PCR (COLD-PCR) [16] and AS-PCR [15]. In COLD-PCR, the denaturing temperature is lowered down to a critical temperature (usually

Abbreviations: PCR, polymerase chain reaction; dNTP, deoxyribonucleoside triphosphate; UDG, Uracil-DNA Glycosylase; Endo IV, endonuclease IV; λexo, lambda exonuclease; HRM, high resolution melting; LOD, limit of detection; AS-PCR, allele specific PCR; COLD-PCR, co-amplification at lower denaturation temperature-PCR; WT, wild type; MT, mutant; AP, apurinic/apyrimidinic.

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1 °C below the melting temperature of the wild-type amplicon), so the mutant amplicon with relatively lower melting temperature denatures more easily, rendering higher amplification efficiency. However, the mechanism of COLD-PCR is still not clear and the method relies on highly precise instrument to control the temperature. AS-PCR uses a sequence specific primer with a 3′-end mismatch to the wild-type template. Taq polymerase prefers elongating at the perfect-match 3′ end, so the mutant template is amplified much more quickly. The method is convenient and sensitive, but it is not compatible with the Endo IV-based signal amplification system. As even for the pure wild-type sample, false priming and extension may produce a considerable amount of mutant products, resulting in strong background signal in later detection. Therefore, we chose block PCR to perform the first-round enrichment. The basic principle of block PCR is to add a blocker sequence which is complementary to the wild-type template to the reaction system, thus the amplification of wild-type template can be selectively inhibited. Since the positions of the blocker sequence and the primers and their chemical modifications can be flexibly designed, many different types of block PCR have been developed. Among the existing block PCR approaches, some suffer from strict requirement of high-fidelity polymerases or formation of side products, which may cause undesired positive signals in later detection [34,35]. Peptide nucleic acid clamp PCR has been reported which can avoid above limitations. However, the PNA in the final PCR product cannot be removed easily, which may interfere with the following signal amplification process [36].

Herein, we describe a modified block PCR which is not only easy to manipulate but also compatible with the following signal amplication processes. As shown in Scheme 1, the sequence of the blocker is designed to be complementary to the wild-type template, resulting in a single-base mismatched sequence to the target mutant template. The 3′ end of the blocker is phosphorylated to inhibit the polymerase extension. The blocker sequence overlaps 9 nucleotides with the reverse primer, so they will compete for hybridization with the templates. Importantly, the reverse primer is designed to be next to the allele base to prevent false priming and extension, as the target point mutation is out of the priming region. So the melting temperatures of the reverse primer/wild-type template duplex (Tm(R/WT)) and the reverse primer/mutant template duplex (Tm(R/MT)) are the same. The sequences of the blocker and the reverse primer are carefully optimized so that the Tm(R/WT) or Tm(R/MT) is lower than the melting temperature of the blocker/WT duplex (Tm(B/WT)) but higher than that of the blocker/MT duplex (Tm(B/MT)). By setting the annealing temperature around Tm(R/WT) or Tm(R/MT), the blocker will hybridize to the wild-type template, excluding the reverse primer; while the reverse primer will hybridize with the mutant template since the reverse primer is single-base mismatch with the blocker. Overall, the mutant templates are amplified exponentially while the wild-type templates are amplified linearly, thus allows selective enrichment of the target mutant templates. The PCR products are then treated with Lambda exonuclease (λ exo) to generate single-stranded sequences. The obtained reaction products are then subject to the Endo IV-based signal amplification system to further amplify the signals of the mutant sequences.

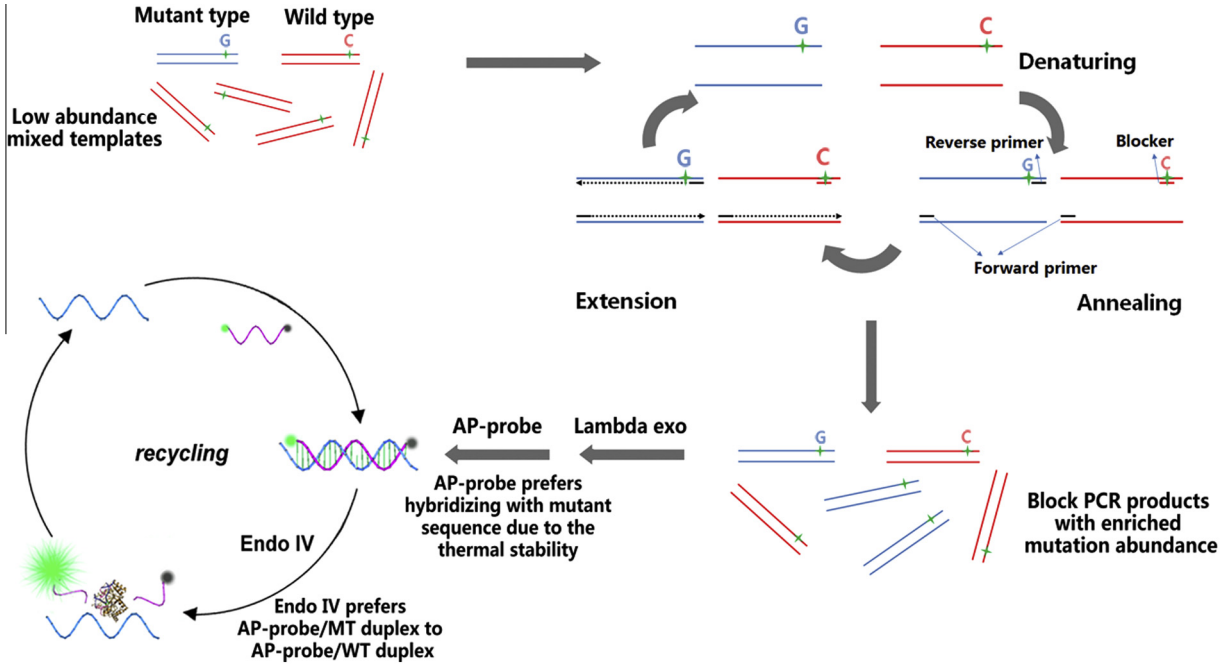
2. Materials and methods

2.1. Materials

Taq polymerase and dNTP were purchased from TIANGEN (Beijing, China). Uracil-DNA Glycosylase (UDG), Lambda exonuclease (λ exo) and ThermoPol Buffer were purchased from New England Biolabs (Ipswich, USA). Endonuclease IV (Endo IV) was purchased from Fermentas (Ontario, Canada). LC green were purchased from Biofire (Utah, USA). All the oligonucleotides (see Table 1) were synthesized by Sangon Inc. (Shanghai, China).

2.2. Determination of the melting temperatures of different DNA duplexes

To a 50 μL PCR tube containing 10 pmol of the double-stranded sequences of interest, 1 μL of LC green and 5 μL of 10 × ThermoPol



Scheme 1. Schematic illustration of the principle of the modified block PCR and its combination with Endo IV-based signal amplification system for ultra-sensitive detection of low-abundance point mutations. The green star represents the position of the point mutation. The Blocker sequence hybridizes with the perfectly matched wild-type templates in the annealing step, thus blocking the amplification of wild-type templates. After the block PCR, the abundance of point mutations is enriched and the PCR products are treated with λ exo to generate single stranded sequences. Then, AP-probes and Endo IV are added to further amplify the signals of mutant sequences.