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Isothermal amplification detection of nucleic acids by a double-nicked beacon

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ABSTRACT

Isothermal and rapid amplification detection of nucleic acids is an important technology in environmental monitoring, foodborne pathogen detection, and point-of-care clinical diagnostics. Here we have developed a novel method of isothermal signal amplification for single-stranded DNA (ssDNA) detection. The ssDNA target could be used as an initiator, coupled with a double-nicked molecular beacon, to originate amplification cycles, achieving cascade signal amplification. In addition, the method showed good specificity and strong anti-jamming capability. Overall, it is a one-pot and isothermal strand displacement amplification method without the requirement of a stepwise procedure, which greatly simplifies the experimental procedure and decreases the probability of contamination of samples. With its advantages, the method would be very useful to detect nucleic acids in point-of-care or field use.

The development of efficient methods for highly sensitive and rapid detection of sequence-specific nucleic acid is essential for the early diagnosis of serious diseases. Nucleic acid amplification detection is one of the most valuable tools in application-oriented fields such as diagnosis of diseases, genetic disorders, and genetic traits [1–3]. Polymerase chain reaction (PCR) provides a general protocol for the amplified detection of DNA. However, thermal cycling of the PCR technique imposes instrumental constraints and a long test cycle, which limits its application in the field of nucleic acid rapid detection [4]. To circumvent the limitations of traditional PCR in amplification detection, recent research has turned toward the isothermal amplification methods [2,5–9] such as nucleic acid sequence-based amplification (NASBA) [10], loop-mediated amplification (LAMP) [11], helicase-dependent amplification (HDA) [12,13], rolling circle amplification (RCA) [14–19], and strand displacement amplification (SDA) [20–22]. These techniques offer those developing point-of-care testing (POCT) diagnostic platforms [23–25] a powerful tool to amplify nucleic acids with no need for additional thermal cycling equipment for repeated heating and cooling processes [5,23]. In 2014, Shi and coworkers devised a double-nicked beacon-mediated isothermal amplification (BAMP) reaction for short oligonucleotides (miRNA Let-7a) [26]. The BAMP system produces a simple, rapid, and cascade nonlinear amplification detection method. However, the detection target of BAMP must have free 3′ end, which limits its further application.

Here, we present a novel isothermal nucleic acid amplification technology based on a double-nicked beacon. This method expands the detection target of the BAMP method and can detect the target with or without a free 3′ end. In addition, this method is one-pot mode and easy to operate. On account of these reasons, this method would be useful for the efficient detection of simple portable detection devices for POCT.

Materials and methods

Materials and reagents

All nucleic acids (Table 1) were designed by using NUPACK software (http://www.nupack.org) and synthesized by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd, Shanghai, China. The Klenow fragment polymerase (exo^{-}, 5 U/μl),

Abbreviations used: PCR, polymerase chain reaction; POCT, point-of-care testing; BAMP, beacon-mediated isothermal amplification; dNTP, deoxynucleoside triphosphate; rDNA, ribosomal DNA; ssDNA, single-stranded DNA.

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Table 1
Sequences of nucleic acids used in this work.

Nucleic acid	Sequence (5'–3')
16S rDNA of *E. coli*	AACAGGAAACAGCTTGCTGTTTCGCTGACG
([a]J01859.1; [b]70–158)	AGTGGCGGACGGGTGAGTAATGTCTGGGAA
	ACTGCCTGATGGAGGGGGATAACTACTG
B1	CAGTAGTTATCCC
P1	CAAACTATACAACCTACTACCTC
	AGCGTTTCCCAGACATTACTCAC
P2	GCTGACGAGTGGC
Molecular beacon	FAM-~~*AGGTAGTAG*~~CCTCTCCTCAGCACTCCGAATC
	TCCTCCTCAGCAAACTATACAAC~~*CTACTACCT*~~-DABCYL
One-base mismatch P1	CAAACTATACAACCTACTACCTCAGCGTTTCCCAGACATT
	ACTCA***T***

Note. The dotted line in specific sequence 16S rDNA of *E. coli* represents the complementary sequence of B1. The boxed portion is the complementary sequence of P1. The underlined sequence shows recognizing sequences of nicking endonuclease *Nb.BbvcI*. The strikethrough portion indicates the stem of the molecular beacon. Mismatch base of P1 is shown in bold and italic.
[a]GenBank accession number.
[b]Position of specific sequence in genomic DNA.

Nb.BbvCI nicking enzyme (10 U/μl), and a mixture of deoxynucleoside triphosphates (dNTPs) were purchased from New England Biolabs.

Amplification reaction

The target DNA was a specific sequence from *Escherichia coli (E. coli)* 16S ribosomal DNA (rDNA). The reaction in 10 μl contained 1.5×10^{-8} M B1, 1.0×10^{-7} M P1 and 2.0×10^{-7} M P2, 2.0×10^{-7} M molecular beacon, 2.5×10^{-4} M dNTPs, 1.0 U of Klenow fragment polymerase (exo^{--}), 2.0 U of *Nb.BbvCI* nicking enzyme, and 1 × NEB CutSmart buffer (50 mM potassium acetate, 20 mM Tris–acetate, 10 mM magnesium acetate, and 100 μg/ml bovine serum albumin (BSA), pH 7.9). The different concentrations of target DNA were added to initiate the amplification reactions and incubated at 37.0 °C for 75 min. The real-time fluorescence detection was performed using a CFX96 Real-Time PCR Detection System (Bio-Rad) at 1-min intervals.

Isolation of genomic DNA

The genomic DNA of *Vibrio parahaemolyticus (V. parahaemolyticus)* was extracted according to the method described in the literature [27].

E. coli genomic DNA samples were extracted from *E. coli* K-12 by the use of a genomic DNA extraction kit (Tiangen, China) following the manufacturer's instructions.

Results and discussion

Principle of the approach

This method is performed by a DNA polymerase with high strand displacement activity, a nicking enzyme, a pair of amplification primers and a bumper primer, and a double-nicked beacon, realizing multiple autocycling strand displacement DNA reactions. As shown in Fig. 1, the amplification primer P1 containing a single-stranded nicking enzyme recognition sequence first bound to target single-stranded DNA (ssDNA) and extended by Klenow fragment polymerase (exo^{--}). The bumper primer B1, which was a few bases shorter than the complementary bases of target DNA with P1 and lower in concentration than P1, slowly hybridized to target DNA and triggered strand displacement synthesis in which the extended product of P1 was displaced. Then, P2 bound to the complementary domain of the extended product of P1 and extended by a DNA polymerase, forming the recognition site of nicking enzyme. In the presence of the nicking enzyme, the nicking enzyme nicked the recognition site, and DNA polymerase extended the 3′ end at the nick while continuously displacing the downstream strand. The displaced downstream strand (product I), coupled with the double-nicked beacon, triggered the subsequent cycles of polymerization, nicking, and displacement reactions. The product I hybridized to the double-nicked beacon and extended, switching the molecular beacon from "fluorescence off" to "fluorescence on" to generate fluorescence signal. In the presence of nicking enzyme and DNA polymerase, the heterogeneous hybrid of product I and single-strand "fluorescence on" beacon that contained two nicking enzyme recognition sites was regenerated to keep on releasing products II and III. The product II as a "primer" bound to another new molecular beacon and initiated another cycle of polymerization, nicking, and displacement, also keeping on generating product III. The product III from the above-described two cycles could also open new molecular beacons and generate fluorescence signals. Products I, II, and III were continuously produced, resulting in the highly sensitive cascade signal amplification.

Sensitivity performance of ssDNA detection

To evaluate the sensitivity of the proposed method for ssDNA detection, we used a specific sequence from *E. coli* 16S rDNA as a target. The different concentrations of target DNA were used to originate the amplification reactions that were measured by real-time