Talanta 116 (2013) 330–334

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Long-tail probe-mediated cycled strand displacement amplification: Label-free, isothermal and sensitive detection of nucleic acids

Hongyan Su [a], Jiabao Long [a], Qiuping Guo [a], Xiaochun Meng [b], Yongjun Tan [a], Qingyun Cai [a], Zhuo Chen [a], Xiangxian Meng [a,*]

[a] College of Biology, State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Chemistry and Chemical Engineering, Hunan University, Changsha 410082, China
[b] Department of Traditional Chinese Medicine, The Fourth Hospital of Changsha, Changsha 410006, China

ARTICLE INFO

Article history:
Received 7 February 2013
Received in revised form
6 May 2013
Accepted 11 May 2013
Available online 30 May 2013

Keywords:
Long-tail probe
Isothermal
Cycled strand displacement amplification
Label-free

ABSTRACT

We design a long-tail shaped DNA probe for the label-free, isothermal and sensitive detection of nucleic acids based on cycled strand displacement amplification (Ltail-CSDA). The long-tail probe, a stem-loop structure with a long poly(T) tail at 5′-termini, integrates target-binding and amplification and signaling within one multifunctional design. The specific binding between the long-tail probe and the target triggers a polymerization reaction, during which a long dsDNA product is synthesized and the hybridized target is displaced by the strand displacement activity of polymerase. The displaced target forms another specific probe-target binding and prompts cycled polymerization reactions. The proposed Ltail-CSDA has the distinct advantages of its isothermal nature, free-label, simplicity and attomolar sensitivity compared with other existing technologies. More significantly, the dynamic range of the method is extremely large, covering nine orders of magnitude. Using total RNA samples extracted from hepatitis C virus (HCV) as targets, we further demonstrate the detection capability of the method for complex nucleic acid samples, indicating its potential applicability for clinic molecular diagnostic assays.

1. Introduction

The invention of polymerase chain reaction (PCR) revolutionizes medical molecular diagnostics that rely on detecting and quantifying nucleic acid targets of interest [1]. Its applications to genetic analysis, DNA cloning, in vitro diagnosis, rapid screening of infectious diseases and others have been already well documented [2–4]. However, the requirement of precise control of temperature cycling and the resultant instrumental restraint has been hampering its wider and more versatile applications. There is a continuous demand in developing isothermal approaches for sensitive and convenient DNA detection.

Accordingly, various strategies for the nucleic acid detection were proposed, such as pyrene-excimer probes [5], enzyme conjugates [6,7], rolling circle amplification [8,9], locked nucleic acid (LNA)-based northern blot [10] and microarray-based flow cytometry [11]. These strategies involved methodological improvement in designing and labeling of primers or probes, chemical modification of nucleotides and incorporation of signal amplification. Notably, polymerase-based strand displacement amplification (SDA) has become increasingly popular in the detection of nucleic acids as a result of its robustness, specificity and isothermality [12–17]. Park et al. reported a SDA-based colorimetric method for DNA detection combined with RNase H [13,14]. However, rigorous optimizations are necessary for the activities of the polymerization of polymerase and the cleavage of RNase H in the same solution. Ward et al. developed a RNase H-free SDA-based method for detection of plant pathogens by introducing four to six primers' reactions [15]. Guo et al. proposed the cycled SDA method using molecular beacon, reaching a DNA target detection limit of 6.4 fM [16]. However, the multi-primers make their experimental design very sophisticated, and the double-labeled probes would make DNA detection quite expensive.

In this work, we designed a novel long-tail probe to perform label-free, simple, low-cost and isothermal detection of nucleic acids with high sensitivity and wide dynamic range based on target-triggered cycled SDA. We named the method as long-tail probe-mediated cycled SDA (Ltail-CSDA). The long-tail probe is a stem-loop structure DNA with a long poly(T) tail at 5′-termini and integrates target-binding, amplification and signaling within one multifunctional design. We reached the aims of the detection limit of 50 aM and the dynamic range of nine orders of magnitude relying on cycled SDA and the extensive polymerization products.

* Corresponding author. Tel./fax: +86 731 88887861.
E-mail address: xxmeng@hnu.edu.cn (X. Meng).

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http://dx.doi.org/10.1016/j.talanta.2013.05.021

2. Experimental section

2.1. Materials

Long-tail probes and other oligos were commercially synthesized by TaKaRa Bio Inc. (Dalian, China). Sequences of these oligos were listed in Tables S1 and S2 in the Supporting information. Polymerase klenow fragment exo$^-$ (KF$^-$) was purchased from New England Biolabs, Inc. The 10,000 × SYBR Green I (SG) was purchased from Invitrogen (CA, USA). The deoxynucleotide solution mixture (dNTPs) was purchased from TaKaRa Bio Inc. All other reagents were of analytical grade. Deionized water was obtained from the Nanopure Infinity™ ultrapure water system (Barnstead/Thermolyne Corp., Dubuque, IA, USA). 0.1% DEPC water was prepared for RNA experiments. The total RNA samples extracted from HCV were obtained from Ambion (Austin, USA).

2.2. Polymerization reaction catalyzed by polymerase KF$^-$

Unless specified, the experiments were performed in 100 μl solution consisting of 5×10^{-10} M target, 5×10^{-8} M long-tail probe2 and a basal solution, which contained 20 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 10 mM NaCl, 1 mM DTT, 5 U KF$^-$, 100 μM dNTPs and 50 nM primer. These samples were incubated at 37 °C for 2 h, and then heated at 85 °C for 5 min to inactivate KF$^-$. According to variant experiments, the concentration of the target and the type of the probe were adjusted. Additionally to investigate the influence on polymerization, we adjusted the concentrations of dNTPs, KF$^-$, $MgCl_2$, NaCl and DTT, and the polymerization time interval.

2.3. Fluorescence measurement

Polymerization product was mixed with 10 μl 10 × SG dye. The fluorescence intensities were recorded using F-2500 fluorescence spectrophotometer (Hitachi, Japan) with an aqueous thermostat (Amersham) accurate to 0.1 °C. The fluorescent spectra were measured using the spectrofluorophotometer. The excitation wavelength was 497 nm, and the spectra were recorded between 507 and 650 nm. The fluorescence emission intensity was measured at 530 nm.

2.4. Gel electrophoresis

Polymerization products were analyzed by a 20% non-denaturing PAGE, which was carried out in 1 × TBE (pH 8.3) at 80 V constant voltage for about 3 h. Bands were analyzed under the silver-straining method using an Image Master VDS-CL (Amersham Biosciences).

3. Results and discussion

3.1. Principle of Ltail-CSDA

The design of long-tail-mediated, label-free and isothermal detection for nucleic acids is illustrated (Fig. 1). The detection system consists of a long-tail probe, a short primer and polymerase. The long-tail probe contains three domains, a stem domain (SD), a long-tail domain (LTD) and a target-binding domain (TBD). The SD is 10-nt-long two complementary sequences, of which one strand at the 3′-end is matched to a 9-nt-long primer. The LTD, a long poly(T) sequence at the 5′-end, extends the length of dsDNA products when polymerization is triggered. The 4-nt poly(T) at the 3′-end prevents probe's own polymerization reaction. The TBD is where the specific binding occurs between the probe and the target. In the presence of the target Fig. 1b), the TBD-target binding undergoes a conformational change, leading to the stem separation of the SD (Step 1). Then, the primer anneals with the open stem and triggers a polymerization reaction in the presence of dNTP/polymerase (Step 2). Notably, during the primer extension, a long dsDNA product is synthesized and the hybridized target is displaced by the strand displacement activity of polymerase (Step 3). The displaced target hybridizes with another long-tail probe, triggering another polymerization reaction (Step 4) to generate a large number of long dsDNA polymerization products. Thus, fluorescent dye (SYBR green I, SG) preferably binds to the dsDNA products via intercalation [18], and leads to strong fluorescence signals, which is directly proportional to the concentrations of the target. In the absence of the target, there is no polymerization reaction and the long-tail probe keeps the hairpin structure and leads to weak fluorescence background (Fig. 1a).

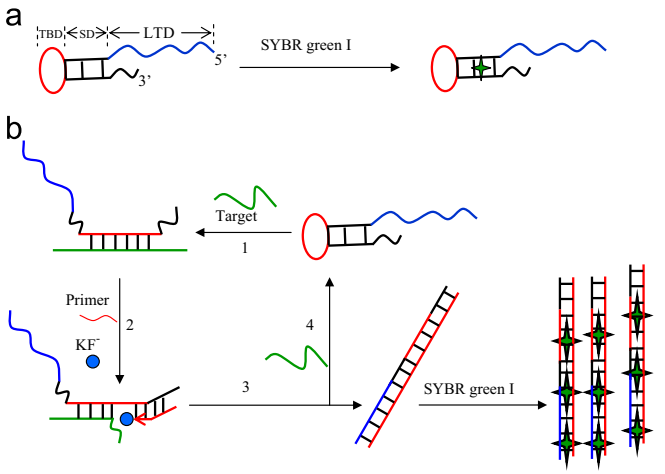

Fig. 1. Reaction mechanism of Ltail-CSDA. (a) In the absence of the target, the long-tail probe keeps hairpin structure and leads to weak fluorescence background. (b) In the presence of the target, the binding between the probe with the target undergoes a conformational change, leading to the stem separation of the probe (Step 1). A primer anneals with the open stem and triggers a polymerization reaction in the presence of dNTP/polymerase (Step 2). A long polymerization product is synthesized and the hybridized target is displaced by polymerase in the process of primer extension (Step 3). The displaced target hybridizes with another long-tail probe, and triggers another polymerization reaction (Step 4). Cycled polymerization amplification produces a large number of long dsDNA products, leading to strong fluorescence along with the intercalation of SG molecules.

The dsDNA polymerization products were quantified via the fluorescence intensity of SG. We reason that the long-tail probe could improve both the sensitivity and the specificity of nucleic acid detection. The fact that long polymerization products resulting from the long-tail probe can bind more molecules of SG and offer additional signal amplification for nucleic acid detection. Moreover, the presence of the hairpin structures in the long-tail probe brings about high conformational constraint, and the competition between the stem region and the loop/target binding could significantly improve the specificity [8,19–23].

3.2. Design of the long-tail probe

To achieve the desired degree of amplification, our design relies upon both the annealing of the primer with the long-tail probe and the length of dsDNA polymerization products. From the principle of Ltail-CSDA (Fig. 1), the long-tail probe in the study must have a stem long enough to ensure that stem hybridization affinity will be stronger than hybridization affinity with the primer, in order to prevent polymerization reaction in the absence of the target. On the other hand, the stem cannot be too long as it may restrain the conformational change of the probe when