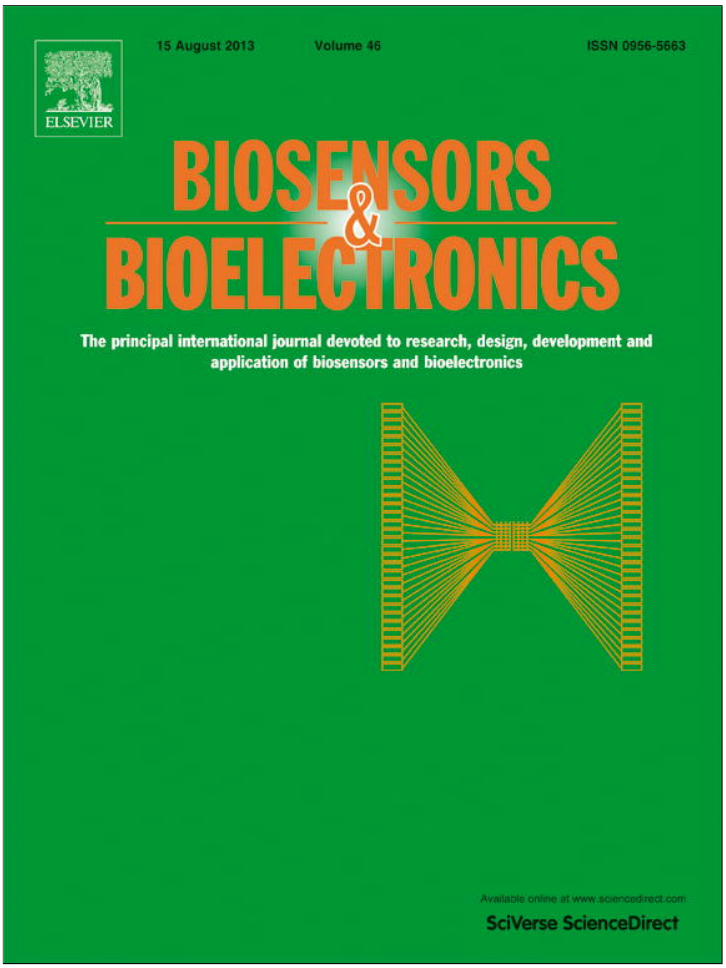

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An isothermal and sensitive nucleic acids assay by target sequence recycled rolling circle amplification

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ABSTRACT

Sequence-specific nucleic acid detection is playing a more and more important role in modern life sciences. Traditional rolling circle amplification (RCA) involves multiple distinct reaction steps and the experiment result is influenced by multiple factors. What's more, a main limitation of traditional RCA is that each target strand hybridizes with only one padlock probe, and this 1:1 hybridization ratio limits the sensitivity. Here we have proposed target sequence recycled rolling circle amplification (TR-RCA) to increase sensitivity by one step. We demonstrated that our method can not only make RCA occur, but also one target DNA can be reused and thus achieving self-recycle. In TR-RCA, the dumbbell probe recognizes the target DNA and hybridizes with it, and then the stem of the dumbbell probe is opened, after that the opened area anneals with the primer and triggers RCA. At the same time, after a target is displaced, it recognizes and hybridizes with another dumbbell probe, triggering the next cycle of RCA. This amplification method is achievable at a constant temperature simply by mixing dumbbell probes, target DNA, primers, and other chemical complexes together in one tube. Our method has significant advantages in ease of operation. And the results indicate that the target DNA can be detected at fM level with high specificity.

1. Introduction

Sequence-specific DNA detection is of central importance in modern life sciences owing to its applicability ranging from diagnostic tests for mutations to the assessment of medical treatment (Wang, 2000; Grossmann et al., 2007; Wang et al., 2003). Amplification is one of the best ways to detect and analyze nucleic acids since it permit the highest analytical sensitivity (Weizmann et al., 2006a, b). With the advent of polymerase chain reaction (PCR), the field of biological research and diagnostics has been revolutionized since specific DNA sequence in genome can be amplified and thus lead to enormous sensitivity (Saiki et al., 1992; Klein, 2002). However, the PCR method is not free of limitations, it is considered to be complicated for requiring complicated thermal cycling steps and the resultant instrumental restraint has been hampering its wider and more versatile applications. What's more, nonspecific amplification generated by thermal cycling also limited its routine use in many laboratories (Nallur et al., 2001). As a result, several isothermal amplification methods have been developed, among which, rolling circle amplification (RCA) could be the unique one and would be one of the best choices for DNA target amplification among other various fields (Schweitzer and Kingsmore, 2001). Hyperbranching rolling circle amplification (HRCA) has become a significant amplification tool for its isothermal and exponential amplification capacity (Lizardi et al., 1998). It can detect as few as 10 copies (Zhang et al., 2006). However, excess probe molecules carried over from the ligation step could lead to ligation-independent amplification, and then result in high background. Therefore, without purification step or change the reaction into a solid phase, the process may increase background signals (Hafner et al., 2001; Wang et al., 2005). Zhou et al. designed dumbbell probe-mediated rolling circle amplification to detect micro RNA with high sensitivity (Zhou et al., 2010). Although effective, RCA based technology should be at first obtain circular probes and then conduct amplification, these two reactions are in separate and it is difficult to conduct both reactions simultaneously (Nilsson et al., 1994). The requirement for a separate enzyme not only prevents application in intact cells and tissues but also adds to the complexity and cost of such detection method (Harcourt and Kool, 2012). In order to overcome these drawbacks, Murakami et al. developed a novel mode of RCA which called primer generation-rolling circle amplification (PG-RCA), the method can detect the sample DNA isothermally in a single reaction and reach a high sensitivity comparable to HRCA in 2 h

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(Murakami et al., 2009). Unfortunately, when they applied the method to detect the genome of *Listeria monocytogenes*, target sequence was required to contain nicking endonucleases site, which can be limited in the number of target sequences against which they can be deployed (Zuo et al., 2010). Guo et al. presented a sensitive DNA amplified detection method based on isothermal strand-displacement polymerization reaction (Guo et al., 2009). Among the numerous advantage of the method, the most excellent one was the target can activate a circular polymerization reaction in the method, and thus allows it to self-detect. The method also involved only one reaction. What's more, the react system only needs one enzyme and thus the technology is being free from troublesome design. But when the method applied to detect a sample in a complicated environment, since the reaction was conducted under 37 °C, the short, nonspecific DNA may bind on the loop of the beacon and then nonspecific amplification may occur.

Here we proposed a target sequence recycled rolling circle amplification (TR-RCA) based on dumbbell probe-aided strategy for isothermal, high sensitive and high specific nucleic acids sequence detection. On the one hand, the method takes the advantage of 5′-3′ polymerase activity and strand displacement activity but lacking 3′-5′ proofreading property of the Bst DNA polymerase large fragment. On the other hand, it makes use of the mechanism of the dumbbell probe can change its conformation while the target binds on it. Also, the primer can combine to the dumbbell probe and the amplification can occur. After amplification, the SYBR Green I, which is an intercalative fluorescence dye, was added into the react product and then the signal can be analyzed via fluorescence instrument.

2. Experimental section

2.1. Reagents

The Bst DNA polymerase large fragment and their corresponding buffer are purchased from New England Biolabs (NEB), the DNAmate kit, Exonuclease I, Exonuclease III, T4 DNA ligase and their corresponding buffer are from Takara Bio (Shiga, Japan), Water (≥ 18.2 MΩ) used throughout the experiments was generated by a Milli-Q water purification system (Millipore, Bedford, MA, USA). SYBR Green I (10,000 ×) was purchased from Invitrogen and diluted with dimethyl sulfoxide (DMSO) to a concentration of 20 × before use. The deoxynucleotide solution mixture dNTPs is ordered from MBI (Fermentas, USA). All oligonucleotides used in our research are synthesized and purified by HPLC at Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (SSBE) (Table 1). The padlock probes are chemically 5′-phosphorylated.

Table 1
Oligonucleotide sequences used in target sequence recycled rolling circle amplification. The italicized region of the linear probe identifies the part which would form stem structure. The underlined italics region identifies the complementary sequence to the primer.

Name	Sequence (5′-3′)
Linear probe	Phosphate-*TCGGACA*TCACAGTAAAGAGAGGTGCGCC CAT*TGTCCGACCATGACA*TCACAGTAAAGAGAGGTG CGCCCAT*TGTCATGG*
Target	ATGGGCGCACCTCTCTTTACTGTGATGTTTTGTTT
Target for linear RCA	ATGGGCGCACCTCTCTTTACTGTGATGT
Random DNA	TGCAAGGTGTCAGTATAATCCGACGACATTTCTTC
Primer	TGTCATGGTCG

2.2. Preparation of dumbbell probes

The 2 μM padlock in the ligation buffer (66 mM Tris–HCl, pH 7.6, 6.6 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.1 mM ATP) was conducted at 95 °C for 5 min and then 37 °C for 1 h in a 30 μl reaction system. Self-ligation reaction was occurred by adding 35 U of T4 DNA ligase. After that, the ligation products were added to 60 μl exonuclease mixture containing 67 mM Glycine-KOH (pH 9.5), 1 mM DTT, 6.7 mM $MgCl_2$, 10 U Exonuclease I and 20 U Exonuclease III. The mixture was incubated at 37 °C for 2 h, followed by inactivation at 95 °C for 10 min. The exonucleasis products were purified by DNAmate kit.

2.3. Amplification reaction

Unless specified, TR-RCA was conducted at 55 °C for 3 h in 60 μl reaction containing amplification buffer (20 mM Tris–HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), 4U Bst DNA polymerase large fragment, 50 μM dNTP, 400 nM primer and 40 nM dumbbell probe.

In the experiment of evaluating feasibility and specificity of current assay, traditional linear rolling circle amplification as control was conducted at 55 °C for 3 h in 60 μl reaction containing amplification buffer (20 mM Tris–HCl pH 8.8, 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100), 4 U Bst DNA polymerase large fragment, 50 μM dNTP, 40 nM dumbbell probe and 10 nM target.

2.4. Fluorescence measurement

All fluorescence measurements were carried out on a Perkin-Elmer LS-55 fluorometer equipped with a xenon lamp excitation source. The excitation wavelength was 488 nm, and the spectra are recorded between 500 and 600 nm. The fluorescence emission intensity was measured at 520 nm. Excitation and emission slits were set for 10.0 nm and 5.0 nm band-pass, respectively.

Aliquots of 10 μl amplified product and 1 × SG were diluted to 50 μl with 10 mM phosphate buffer (pH 7.5). After incubation for 10 min at room temperature, the fluorescence spectra were measured in a quartz cuvette. The relative fluorescent intensity was the net intensities values which derived from the sample of target DNA, where the background signal recorded in the absence of target DNA had been subtracted for each value.

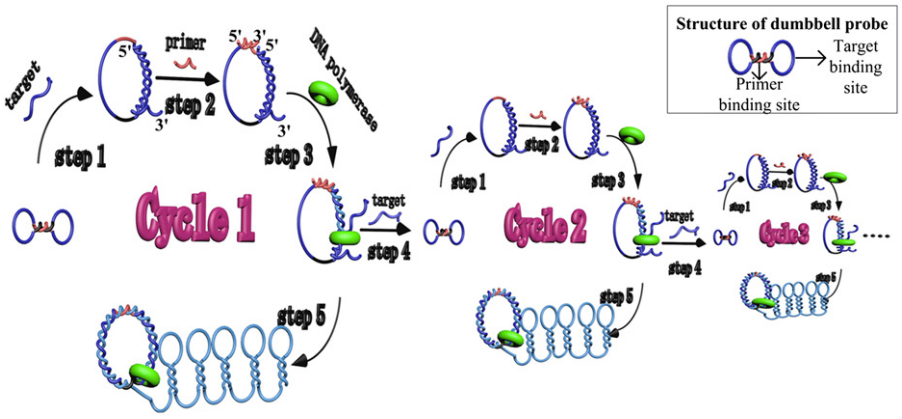
The threshold values to define a specific sample for detecting is calculated based on formula (1), the Vnegative and Vstdev (neg) represent the averages and standard deviations of the relative fluorescence intensity from the analysis of negative control samples. A sample with a relative fluorescence value higher than the threshold values (V) is considered to be positive.

$$V_{\text{threshold}} = V_{\text{negative}} + 3V_{\text{stdev(neg)}} \quad (1)$$

3. Results and discussion

3.1. The principle of TR-RCA

The principle of our designed TR-RCA method is illustrated in Scheme 1. In this method, the dumbbell probe contains three domains. The two target-binding domains, which are exactly the same loop sequence, are located in the two ends of the dumbbell. Besides, the middle stem of the dumbbell is SG (SYBR Green I)-binding domain which contains a region that is complementary to primer. In the absence of a target, the conformation of dumbbell probe can not change, the stem would still in complementary conformation, thus the primer is unable to anneal with the probe



Scheme 1. The principle of isothermal and sensitive nucleic acids assay by target sequence recycled rolling circle amplification. (step 1) Target binding on dumbbell probe and the conformation of dumbbell probe change, (step 2) primer binding on dumbbell probe, (step 3) amplification triggered by Bst DNA polymerase large fragment and displaced the target, (step 4) target open next dumbbell probe and be recycled, and (step 5) dumbbell rolling circle amplification occur.

to induce amplification. In contrast, when the target present in the system, the loop of dumbbell probe can recognizes the target and hybridizes with it. Thus, the dumbbell probe undergoes a conformational change, which can lead to stem separation and the binding site for elongated primer would expose (step 1). In this situation, the primer can recognizes it and binds on it (step 2). Therefore, under the strand-displacement property of Bst DNA polymerase large fragment, the primer which hybridized with the changed dumbbell probe can be extended isothermally and the target can be displaced (step 3). In this process, the displaced target then would recognize another dumbbell probe and trigger the second cycle of amplification (step 4) Simultaneously, the primer will continue extension, and the elongated products contain hundreds of copies of the sequence complementary with the dumbbell probe, which also form numerous SG-binding regions (step 5). Consequently, the target can be reused and lead to self-recycle, which can assure high sensitivity. In this process, the dumbbell probes act not only as a template for amplification but also can be regarded as a cause for self cycling of target for its special conformation. The resulted products contain multiple binding regions for SG, so we could detect the target with high sensitivity under isothermal condition by monitoring the increasement of fluorescence intensities.

3.2. Feasibility and specificity of current assay

At first, the self-ligation of dumbbell probe was confirmed. (See Supplemental information). After obtained the dumbbell probe, we conducted the experiment to verify the method is target-recycled RCA but not traditional RCA whose target would hybridizes with only one padlock probe. We designed a target whose 3′ exactly complementary to the dumbbell probe and at the same time the target can act as primer of traditional RCA to contrast TR-RCA. Besides, two other controls were designed to find out whether the primer can initiate the amplification and whether the target can cause the amplification. Fig. 1A displays the electrophoresis results obtained from traditional linear rolling circle amplification mode (1), dumbbell probe with primer (2), target recycled rolling circle amplification (3), and dumbbell probe with target (4). There are obvious amplification products in lane 1 and lane 3, what's more important, the product in lane 3 is more than that in lane 1. It proved that in the TR-RCA amplification process, the target achieve self-recycle and thus caused more product than traditional RCA whose target can not be recycled. In contrary, no amplification products were found in

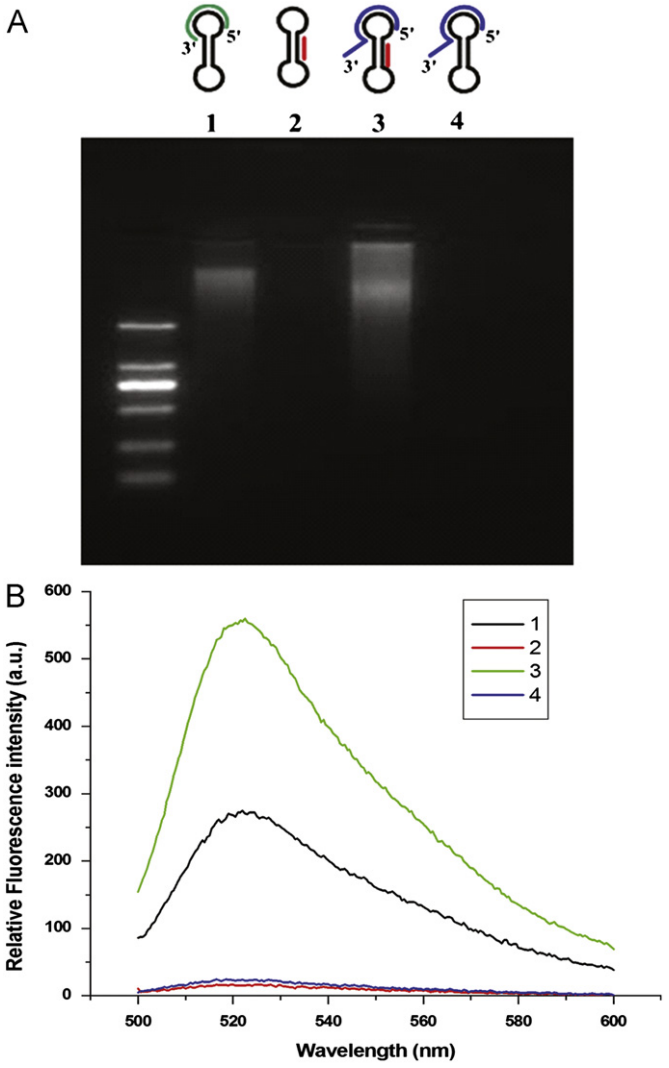


Fig. 1. The specificity assessment of current method. **(A)** Green I was added into each sample and the products are electrophoresed in a 1% agarose gel. Lanes 1, 2, 3, 4 represent: 1, linear dumbbell probe rolling circle amplification; 2, dumbbell probe and primer; 3, dumbbell probe-aided rolling circle amplification; and 4, dumbbell probe and target. (B) Fluorescence intensity obtained from the signal labeled with 1, 2, 3, 4 represent the same as the A.

lane 2 and 4. It indicated that the amplification product was not caused by primer or by target alone, it was caused by target open the dumbbell probe firstly and then the primer bind on it to initiate the amplification. The success of recycle depends on two main characteristic of Bst DNA polymerase large fragment; the strand displacement property and the lacking of 3′-5′ proofreading exonuclease activity (Aliotta et al., 1996). The lacking of 3′-5′ proofreading exonuclease activity makes the target avoid proofreading into a sequence whose 3′ exactly complementary to the dumbbell probe. Because in case of nonspecific binding at 3′ state, the target would not be elongated and then can trigger the recycle, so the target can achieve self-detect. What's more, the strand-displacement property of Bst DNA polymerase large fragment make the RCA occur at the same time, the products contain numerous dumbbell probes, which can be intercalated by SYBR Green I (SG). After that, the resulting products mentioned above were detected on a fluorescence instrument. As shown in Fig. 1B, the signal labeled with 1, 2, 3, 4 represent the sample which is already explained above. It was observed that the fluorescence signal obtained from linear rolling circle amplification was much lower than that of TR-RCA. And there are no fluorescence signal observed in sample 2 and 4. Besides, the amplified product includes multiple units of sequences complementary to the dumbbell probe, and forms duplex stem structure which is SG-binding domain. The fact that one SG-binding domain can bind to several molecules of SG offers additional signal amplification for target detection. The results of fluorescence detection were consistent with the results of gel electrophoresis. It is demonstrated that the proposed method involved target recycling and the efficiency is higher than linear rolling circle amplification.

A time profile of target DNA and random DNA experiment was conducted in order to verify the detection method. As can be seen in Fig. 2, in sample DNA, the fluorescence intensity maintained its increase with time, indicating that the continuous formation of dumbbell probe-target complex and TR-RCA happened. Furthermore, the relative fluorescence intensity increased rapidly with the reaction time up to 180 min. However, the signal exhibited no further significant increase when the reaction duration went beyond 240 min. It may be attributed to the fact that the activity of the Bst DNA polymerase had decreased and the dNTPs was used up. In the absence of a target, no fluorescence intensity change was observed, indicating that no amplification was triggered. Besides, the control experiment contains random single-stranded DNA revealed that amplification was not triggered even in the presence of dNTP/polymerase. The result proved that dumbbell probe can not recognize the random DNA and thus the amplification could not happen, which assure the detection be specific.

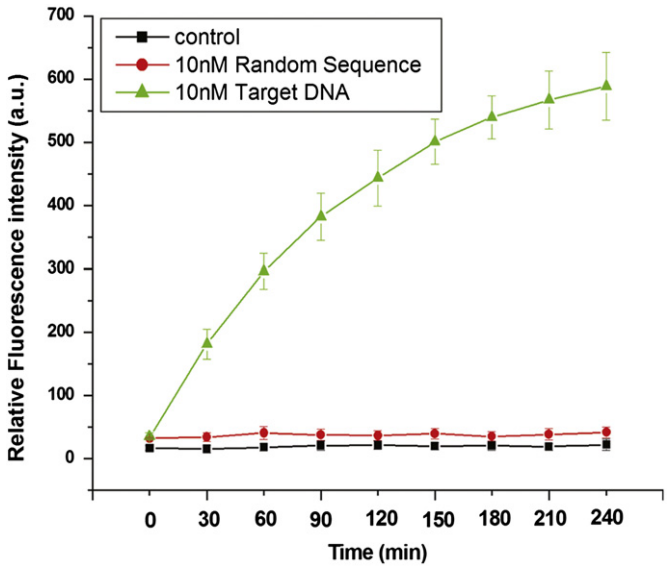


Fig. 2. Time profile of 10 nM target, 10 nM random DNA and control.

3.3. Optimization of TR-RCA experiment condition

There are several factors which would affect the detection limit, it is necessary to find the optimum condition for the reaction. The concentration of target was set at 1 nM for all optimized experiments. Amplification temperature is one of the core factors in our method. So the effect of amplification temperature was evaluated carefully. The temperatures was increased from 45 °C to 70 °C with an interval of 5 °C were evaluated. As presented in Fig. 3A, the fluorescence intensity peak was observed at around 55 °C, while the fluorescence intensity of control was always low and changed scarcely. It may be attributed to lower activity of the Bst DNA polymerase large fragment when the temperature was below 55 °C. While the temperature was higher than 55 °C, the *Tm* of primer is not high enough and hence can not anneal to the dumbbell probe to initiate amplification. Thus, 55 °C was chosen for hybridization of the dumbbell probe and target in all following experiments.

In order to improve the limit of detection further, it is important to optimize the dNTPs concentration to improve the signal/background ratio of TR-RCA. Several concentrations of dNTPs substrates were compared (Fig. 3B). We found that when the concentration of dNTPs reached 500 μM, negative control containing only circular dumbbell DNA probe, enzymes, primers and dNTPs substrates provided high background. On the other hand, when the concentration of dNTPs at 50 μM, the ratio of the positive sample to negative controls is higher than other concentrations. Suggesting that too much dNTPs would influence the activity of Bst DNA polymerase large fragment and initiating the nonspecific amplification.

The effect of the concentration of the primers on the ratio of the positive sample to negative controls was also investigated. As shown in Fig. 3C, the fluorescence intensity response increased rapidly with the increasing of the concentration of primers. However, when the concentration of primers reached 800 nM, the fluorescence intensity of negative control went up too, whose ratio of sample to control is lower than the condition of when the primer concentration is 400 nM. That phenomenon is mostly probably because the primer can compete with the stem of dumbbell probe and triggers the false amplification. Therefore, the primer concentration was fixed at 400 nM throughout the experiment.

In the proposed method, the concentration of dumbbell probe is a vital factor to influence the fluorescence intensity. Since the appropriate amount of concentration can assure enough amplification product, thus improving the sensitivity. Nevertheless, we found that excess dumbbell probe could cause background. Fig. 3D demonstrates the effect of the different concentrations of dumbbell probe from 20 nM to 160 nM on the fluorescence intensity. It was observed that when the concentration of dumbbell probe is 20 nM the signal was low, indicating that there were not enough amplification products be produced. But when the concentration beyond 40 nM, the signal of sample did not increase but the control is even higher. It was due to the fact that too many dumbbell probes whose stem containing the same sequence the same as primer would compete with the primer and thus false amplification occurred. The results showed that the 40 nM dumbbell probe was set as an optimized system.

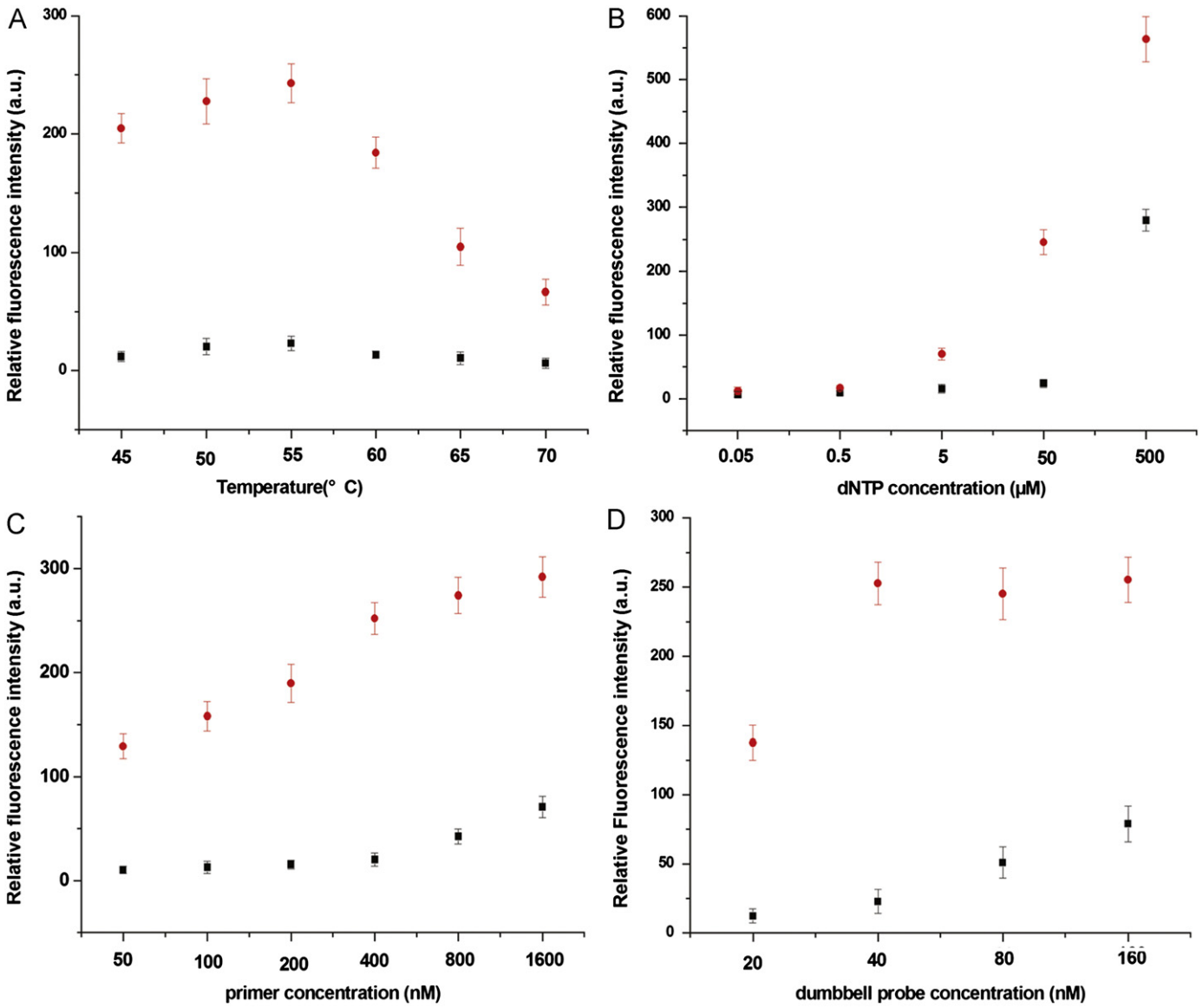


Fig. 3. Optimization of experiment conditions: (A) amplification under different temperatures, (B) fluorescence intensity of different concentration of dNTP, (C) the effect of the quantity of primer on the fluorescence intensity, and (D) the influence of different concentrations of dumbbell probe on signal. All reactions are carried out as described in the experimental section. Error bars show standard deviation which was determined by at least three replicates.

3.4. The sensitivity of TR-RCA

We subsequently examined the detection limit of the TR-RCA under optimized conditions. In Fig. 4, we recorded the relative fluorescence intensities for the amplification product at a series of dilutions of the target. The experiments were repeated three times. The results showed that the relative fluorescence intensities increase as the concentration of the targets increased, indicating that hybridization and polymerization were going on. The response of fluorescence intensity showed that the detection of as little as 100 fM can be achieved. At this concentration, the relative fluorescence value of target and control was 46.3 ± 7.6 and 16.4 ± 6.9, according to the formula (1), the threshold value was set as 37.1, indicating the special target could be discriminated apparently. When the target concentration dropped to 10 fM, the relative fluorescence value obtained was 28.4, which was not exceeding the threshold line, there are no apparent changes in fluorescent signal. The insert shows fluorescence spectra of increase in emission intensity with increasing target concentration obtain from a single experiment. With the TR-RCA method, the sensitivity exceed the target-primed rolling circle amplification (Li et al., 2008a), RCA with direct hybridization by beacon (Li et al., 2008b), and basic nicking enzyme signal amplification (NESA) (Li et al., 2008b). The sensitivity is comparable with extended NESA method, which also takes advantage of target recycle and rolling circle amplification to further boosts the sensitivity (Li et al., 2008b).

The success of the current assay depends on two important factors. The first is the design of the dumbbell probe. On the one hand, the stem should be long enough to ensure that stem hybridization affinity will be stronger than hybridization affinity with the primer. Besides, it should be stable and not open under 55 °C. One the other hand, stem that is too long would not only restrain hairpin probe conformational change upon hybridization with target but also would cause a large background signal (Guo et al., 2009; Spits et al., 2006; Coskun and Alsmadi, 2007). The other factor is that the lacking 3′-5′ exonuclease activity of Bst DNA polymerase large fragment, this feature avoid the target be consume to a blunt end with dumbbell probe and as a primer to initiate amplification. Only with the intact target which possess a

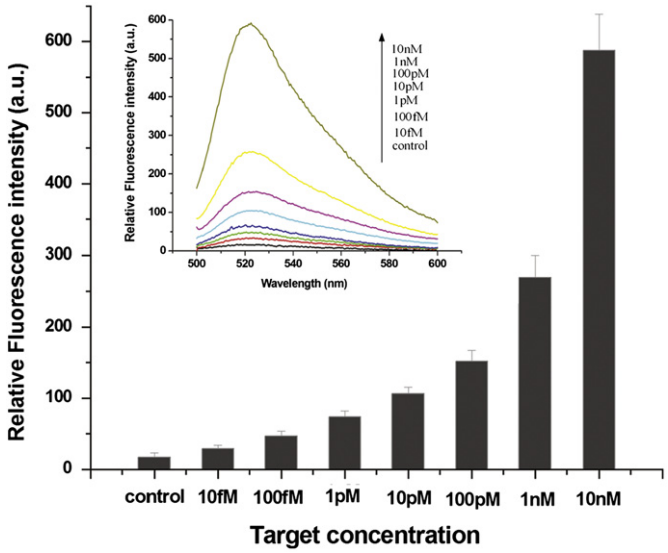


Fig. 4. The sensitivity assessment of current method.

sticky end can it be reused and assure high sensitivity. To further confirm the feasibility of the proposed method, TR-RCA has been used for analyzing mRNA of *Listeria monocytogenes*. (See Supplementary Information).

Additionally, Spits et al. found that Phi29 DNA polymerase is preferred over Bst DNA polymerase because it showed higher efficiency and lower error rate (Spits et al., 2006; Coskun and Alsmadi, 2007). However, the strong exonuclease activity of Phi29 DNA polymerase can cleave ssDNA as well as RNA (Ibarra et al., 2009; de Vega et al., 1998; Lagunavicius et al., 2008). This property confined its use in TR-RCA since it will cleave the nonspecific binding at 3′ part of the target, leading to the target recycle can not be occur. But by using of Phi29 mutants or of other nuclease-deficient and thermostable polymerases may improve signal in future experiments.

4. Conclusions

In summary, a TR-RCA method was presented in this paper. Our experiment results showed that a specific DNA sequence can be successfully detected in a single-tube experiment under isothermal conditions. Compared with PCR, avoiding sophisticated thermal cyclers should be a great advantage. Compared with other isothermal amplification reactions such as RCA, HRCA, extended NESA, etc. the RT-RCA only need one step and thereby can react in one tube at one time. Compared with other one step-isothermal amplification reactions such as PG-RCA and basic NESA, our method only need one kind of enzyme in the reaction system. Last but not least, the react temperature of TR-RCA is under 55 °C, in which the high specificity can be guaranteed.

TR-RCA can be used to detect biological samples with single-strand genomic DNA, cDNA and RNA. Before applying to actual biological detection, a pretreatment, such as enrichment, may be necessary for trace amount of samples. Additionally, its simplicity can make it be useful in direct in situ detection and can be easily extended to a high-throughput, multipurpose and automatic screening format.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bios.2013.02.003.

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