Sequence-Specific Biosensing of DNA Target through Relay PCR with Small-Molecule Fluorophore

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Supporting Information

ABSTRACT: Polymerase chain reaction coupled with signal generation offers sensitive recognition of target DNA sequence; however, these procedures require fluorophore-labeled oligonucleotide probes and high-tech equipment to achieve high specificity. Therefore, intensive research has been conducted to develop reliable, convenient, and economical DNA detection methods. The relay PCR described here is the first sequence-specific detection method using a small-molecule fluorophore as a sensor and combines the classic 5′→3′ exonuclease activity of Taq polymerase with an RNA mimic of GFP to build a label-free DNA detection platform. Primarily, Taq polymerase cleaves the 5′ noncomplementary overhang of the target specific probe during extension of the leading primer to release a relay oligo to initiate tandem PCR of the reporting template, which encodes the sequence of RNA aptamer. Alternatively, dual-labeled oligonucleotide probes have been used for sequence specific detections. The TaqMan approach where PCR-induced probe cleavage coupled with fluorescent signal generation was one of the first sequence specific methods to allow real-time PCR monitoring. It utilizes the 5′→3′ nuclease activity of *Thermus aquaticus*(Taq) polymerase to achieve high specificity and sensitivity (achieving detection of 10→100 DNA copies), but the TaqMan method is expensive and requires specially modified fluorogenic oligonucleotide probes and sophisticated real-time PCR machines. To decrease the high cost of labeled oligonucleotides in sequence dependent detection techniques, several new strategies have been developed such as the mediator probe method. Nonetheless, fluorophore-modified oligonucleotide probes are still required for this methodology, though the cost is lower than that of a TaqMan probe. This study is the first report of a sequence-specific PCR system based on an unlabeled oligonucleotide, using an RNA mimic of GFP and small-molecule fluorophores as sensors for signal generation. Recently, the Jaffrey group has described an RNA aptamer, denoted “Spinach,” that mimics the function of Green Fluorescent Protein (GFP): when bound with small-molecule fluorophore 3,5-di-fluoro-4-hydroxybenzylidene imidazolinone (DFHBI), the Spinach/DFHBI complex becomes brightly fluorescent. This RNA–fluorophore complex displays fluorescence properties similar to GFP, but unlike most fluorescent proteins it is resistant to photobleaching. Further, DFHBI is fluorescent only upon binding to RNA, and it is not activated by the other components in the reaction. This unique ability of RNA aptamers to act like GFP could be exploited as a reporting fluorescent signal for target specific detection. Here, we report a relay PCR, which is based on RNA aptamer mediated DNA detection. It is comprised of three steps including (i) cleavage based target recognition PCR, (ii) a subsequent tandem PCR to accumulate multiple copies of a DNA template encoding an RNA aptamer, and (iii) finally the transcription of reporting PCR product to transcriptional reporter mRNA.
obtain RNA aptamers that emit fluorescent signals upon binding to a small-molecule fluorophore.

**RESULTS AND DISCUSSION**

The principle of relay PCR detection is illustrated in Figure 1. Unlike the high-cost TaqMan probe that is a fluorophore and quencher modified oligonucleotide, a simple unlabeled probe Pr is introduced. The Pr is composed of an 18-nt 5′ noncomplementary sequence Tr (blue) and a target complementary recognition fragment Pr (gray) that is modified with an NH2 group at the 3′ end blocking its extension by DNA polymerase. The target is primed by specific primers p-1 and p-2. The melting temperature (Tm) of the recognition fragment of Pr was kept higher than the primer p-1 to make sure that the probe Pr could form a stable duplex with single-stranded target before hybridization of p-1 at the annealing step. Taq polymerase has an inherent 5′ to 3′ exonuclease activity that could cleave the probe Pr at the point of obstruction to release the 5′ noncomplementary fragment Tr during the first PCR amplification (Figure 1a). Another template TT and primer p-3 have been included to the PCR reaction in advance. The TT consisted of three parts from the 3′-5′ end: (i) antisense sequence of the cleaved fragment Tr (blue), (ii) DNA sequence encoding the RNA aptamer (gray), and (iii) the promoter sequence of T7 RNA polymerase (pink). The p-3 primer is 24nt in length and identical to the 5′ end of TT template. Therefore, cleavage of the probe Pr to release the fragment Tr in the recognition PCR amplification of target DNA starts the tandem PCR to amplify the sequence TT by using Tr and p-3 as primers (Figure 1b). The final result of this relay PCR amplification is the accumulation of two double stranded PCR products, one is a targeted sequence and the other is a TT template. The final stage is the transcription in which the PCR product of the TT template is used. Transcription is initiated through adding T7 RNA polymerase, NTP, and the corresponding fluorophore into the PCR mixture directly. Finally, on completion of transcription, complexes of aptamer–fluorophore are formed to elicit a fluorescence signal by exposing it to ultraviolet light. Thus, a detection result could be read through the direct measurement of fluorescence change in the reaction mixture (Figure 1c). In the current study, three different RNA aptamers, namely 24–2, 17–3, and 17–2, are synthesized (Supporting Information Table S1 and Figure S1). 24–2 produces very intense emission of sea-green fluorescence with fluorophore DFHBI. 13–2min and 17–3 generate green and yellow fluorescence, respectively, in the presence of fluorophore DMHBI. The fluorescence signals could easily be observed within the visual spectrum under ultraviolet light (Figure 1d).

Here, a composite strategy based on recognition PCR, relay PCR, and transcription in the presence of a small-molecule fluorophore is employed to target sensing. The target recognition PCR that could amplify DNA targets triggers the following PCR amplification of template TT sequence through the cleavage of sequence-specific probe Pr to release a relay oligo Tr. Sequential PCRs are interlinked because the released Tr is applied as the primer of the second PCR. Therefore, the efficiency of the probe cleavage is a key factor in this system. Dual PCRs and transcription of double stranded TT in the presence of fluorophores (DFHBI or DMHBI) are integrated.
to generate a large amount of single stranded RNA aptamer that could report the presence of a DNA target through fluorescence tracking with high specificity and sensitivity.

In this study, a 105nt conserved region from 16S rDNA of *Clostridium perfringens*, a clinically significant bacterium, was selected as a template to detect and quantify through relay PCR (Supporting Information Figure S2). The PCR primers, p−1 and p−2, were designed to amplify the targeted sequence. A GC-rich region with a high melting temperature was selected to design the probe Pr from the target fragment. This allows the probe to bind to the target prior to the elongation of leading primer p−1 during the annealing step. According to our strategy, 18nt sequence Tr was added to the 5′ end of probe Pr as a noncomplementary overhang with the target. An amino modifier was also tethered to the 3′ end of probe Pr to prohibit its undesired extension on the target sequence by Taq DNA polymerase (Supporting Information Table S1). The probe attaches to the target in a manner that the ‘G’ base is cleaved by Taq polymerase at the site of obstruction in the probe−target complex during the first PCR for target recognition. Moreover, the cleaved fragment Tr having ‘G’ at the 3′ end acts as an efficient primer in the tandem PCR to amplify the TT template that encodes the aptamer reporter.

The cleavage of probe Pr by Taq DNA polymerase was verified through analysis of the PCR reaction containing the isotope-labeled Pr (Figure 2a). In the presence of target DNA, the cleaved fragment Tr with an expected length of 19nt was observed after the PCR amplification based on polyacrylamide gel (PAGE) analysis, which was released from 5′ 32P-labeled probe Pr (lane 2, Figure 2a). No cleavage of the probe Pr was found in the same PCR reaction without the target sequences as a comparison (lane 3, Figure 2a). A temperature gradient PCR was conducted to identify a suitable annealing temperature for maximum probe cleavage, affording an efficiency of 84% cleavage at an annealing temperature of 57 °C (Supporting Information Figure S3). Once the cleaved fragment Tr is released in the first PCR amplification, DNA polymerase could extend the cleaved sequence Tr on the TT template. This step was also validated with the isotope-labeled experiment (lane 3, Figure 2b). In the presence of the target sequence, two products, target DNA and the TT DNA, should be obtained after relay PCR amplification. Two clear bands with expected lengths of 105bp and 128bp were observed based on agarose gel electrophoresis analysis (lane 2, Figure 2c). Finally, the relay PCR product was successfully transcribed by T7 RNA polymerase to obtain RNA aptamer, generating a large amount of single stranded RNA aptamer that could report the presence of a DNA target through fluorescence tracking with high specificity and sensitivity.

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The reporting system in this technique is very flexible, and it is straightforward to see fluorescence as reporting signals by

![Figure 2](Image 114x461 to 511x749)
replacing the aptamer sequence in the TT template and its corresponding fluorophore. As illustrated in Supporting Information Figure S4, TT templates containing different aptamers were used in relay PCR. PCR products were then transcribed and incubated with their respective fluorophores. A qualitative difference among different samples could be visualized even with the naked eye by illuminating the reaction tubes with ultraviolet light (Supporting Information Figure S5). A real time increase of fluorescent signals could be recorded with the addition of fluorophore in the beginning of the transcription reaction (Supporting Information Figure S6). The combinations of different aptamers with corresponding fluorophores resulted in seven different fluorescent signals.\textsuperscript{24} Though all fluorophores are not present commercially available, most of those fluorescent dyes could be obtained through three steps of organic synthesis, which is not difficult for most research groups of organic chemistry to do. A simultaneous multidetection of pathogens is possible by choosing different aptamers corresponding to the same fluorophore and with nonoverlapping spectral properties.

The technique reported in this paper is sensitive enough to detect $10^2$ to $10^{10}$ copies of target DNA by visual appreciation of the reaction tubes (Figure 3a). Targets are not only realized by a qualitative fluorometric method but accurate quantitation could be measured by the progress of the transcription reaction. Relay PCRs including $10^2$ to $10^7$ copies of DNA targets were performed in three replicates to determine the linearity of the assay. The same amount of DFHBI fluorophore and transcription reagents were added into each relay PCR mixture. A time-dependent fluorometric graph was obtained, and the relationship between the concentration of the DNA template that ranged over several orders of magnitude. In Figure 3c, the solid line indicates a good linear relationship between fluorescence intensity of the reaction end point with the concentration of target molecules. An $R^2$ value of 0.9747 demonstrated a suitable linearity in the range from $10^4$ to $10^7$ copies of DNA targets. The error bars were determined by the standard deviation (SD) of the triplicate experiments. Furthermore, precision of the assay was determined by running four replicates of $10^7$ copies of target DNA along with positive control containing $10^{10}$ molecules of TT and a negative control without a target (Supporting Information Figure S7).

E. coli and Clostridium perfringens bacteria were selected as models to demonstrate the application of assay in pathogenic detection. On the basis of our verified strategy, the primers and probe for the E. coli bacterium were designed according to the malB promoter gene fragment\textsuperscript{26} (Supporting Information Table S1), and the cleavage of the probe at the right position (19nt) was confirmed with a $5'$ $^{32}$P-labeled probe (Supporting Information Figure S8). Two amplicons, a recognition PCR (89bp) and TT product (102bp) encoding aptamer 13−2min, should be obtained if the relay PCR amplification works well in the presence of target bacteria. As illustrated in Figure 4a, both products with expected lengths were obtained in the relay PCR amplification reaction containing $10^{10}$ cells of E. coli. In contrast, no PCR product was observed in the same reaction concentration of the DNA template.

![Figure 3. Concentration gradients relay PCR and fluorometric signal generation. (a) Illumination of transcribed relay PCR products under ultraviolet light with the addition of DFHBI fluorophore. Tube 1: Relay PCR without target as negative control. Tubes 2−10: Relay PCR amplifications containing concentration gradients from $10^2$ to $10^{10}$ copies of DNA targets. (b) Fluorescence measurement during transcription of relay PCR products with $10^2$ to $10^7$ copies of DNA targets. Inset: close view of the first three concentrations. PC: containing $10^{10}$ copies of DNA targets as a positive control. NC: Relay PCR reaction without target as a negative control. (c) Intensity of fluorescent signals of the reaction end point is plotted against the concentration of target DNA. The solid line indicates linear least-squares fitting between $10^4$ and $10^7$ molecules of the DNA target. The error bar graph represents the variation between triplicate experiments.](https://doi.org/10.1021/acs.chembio.5b01081)
without targeted bacteria. PCR products were transcribed in the presence of DMHBI fluorophore, and the resultant qualitative pathogenic detection could be visualized by naked eyes through UV irradiation. Relay PCR with 24−2 and 17−3 aptamers were also confirmed with E. coli (Supporting Information Figure S9), and real time increases of fluorescent signals were measured with relay PCRs of E. coli (Supporting Information Figure S10). Further, the specificity of our method was investigated by using the 10^{10} cells of Staphylococcus aureus, Clostridium perfringens, and Aeromonas hydrophila as negative controls with all primers and a probe of E. coli detection. On the basis of the agarose gel analysis, only the relay PCR amplification of the E. coli bacterium showed positive results, revealing high specificity achieved by our method (Figure 4b). Specificity was further confirmed by the detection of 10^{10} cells of Clostridium perfringens with the help of already designed primers and probes for feasibility analysis of the strategy; a yellow fluorescence was obtained with the TT encoding 17−3 aptamer (Figure 4c).

The relay PCR also allowed multiplex assays because of the separation of target amplifications from reporter accumulation. A multiplex relay PCR reaction was performed with E. coli and Clostridium perfringens. Primers and sequence-specific probes for both bacteria were used with the same sensing template TT (24−2) to report the existence of target bacteria. Three expected bands, two from recognition PCR of the target bacteria and one from amplification of the reporter TT DNA, were obtained in the multirelay-PCR reaction containing two bacteria together. There was no PCR product found in the negative control of multiplex relay PCR containing Staphylococcus aureus and Aeromonas hydrophila as negative controls, which further confirmed the specificity of the method in multiplexing (Supporting Information Figure S11). A real demonstration of signal generation with different reporting aptamers was not possible at this stage because of overlapping spectral properties of selected aptamers. The aptamers were selected on the basis of their brightness with respect to GFP to ensure the visual detection of the target.24 Successful multiplexing of target detection proved the potential of relay PCR to generate multiple signals with aptamers having distant emission spectra (Supporting Information Table S2).

In conclusion, the relay PCR based amplification scheme described here appears to have several promising features for research and diagnostic applications as follows: (i) Target recognition and reporter accumulation for DNA detection are accomplished by two different PCR reactions in the same tube. The tandem PCRs are linked together through a target-recognition probe, while the PCR amplifications occur consequentially in the presence of target DNA. (ii) Sequence-specific detection was achieved with precisely designed primers and sequence-recognition probe, which could be cleaved by Taq DNA polymerase in the presence of target DNA during PCR amplification. The cleaved fragment triggers the tandem PCR amplification of reporting template that encodes the RNA aptamer sequence. (iii) The detection result was reported through recording the fluorescence during transcription of PCR products by adding small fluorophore molecules. Though the detection limitation and dynamic range and time-consuming nature of our method is not as good as that of the TagMan strategy, no fluorophore modified probes and sophisticated machines are required to fulfill either qualitative or quantitative assays, thus making this method ready-to-use and cost-effective. (iv) The flexibility of choosing different fluorophores made the multiplex PCR assay feasible for future application. In the present study, we have combined exonuclease activity of Taq polymerase with an RNA mimic of GFP to build a label-free DNA detection platform. However, this detection also has its down side—the amplification vessels need to be opened to add other reagents following the PCR. So there is a necessity to carefully avoid contamination during the whole operation. Moreover, as the fluorescence microplate reader is indispensable to fulfilling the quantitative detection, the method now is best suited for a visual analysis after the transcription reaction, providing qualitative (a “yes or no” answer) detection.

The relay PCR is the first sequence-specific PCR detection method using a small fluorophore molecule as a sensor. The high specificity and sensitivity was achieved by retaining the key advantage of the PCR induced probe cleavage approach, namely, the elegant use of 5'-exonuclease activity of Taq polymerase. Meanwhile, the application of RNA aptamers that could produce various kinds of fluorescence with corresponding fluorophores makes this method versatile in detecting single or multiple targets. This technique paves the way to connect the PCR amplification with functional RNA molecules, such as a ribozyme or riboswitch. We are currently working to optimize their use in different assays.

## METHODS

### Material and Apparatus.

The oligonucleotides (Supporting Information Table S1) were PAGE-purified except the phosphorylated probes that were HPLC-purified from Sangon Biotech Co., Ltd. (Shanghai, China). Taq DNA polymerase, Taq buffer, dNTPs, and NTP were purchased from TransGen Biotech (Beijing, China). T4 polynucleotide kinase (PNK) was purchased from Alfa Aesar, and [γ-32P] ATP was purchased from Furuil Biological Engineering (Beijing, China). Fluorophores (DFHBI and DMHBI) were synthesized in our lab as described by Paige et al.24 The relay PCR reaction was manipulated in a C-1000 thermal cycler (Bio-Rad). 32P-labeled PCR products were analyzed with a cyclone plus phosphor imager from PerkinElmer Co. The fluorometric detection was carried out in a Varioscan Flash (Thermo Scientific) machine.

### Probe Labeling.

Probe Pr was labeled with [γ-32P] ATP at the 5′ end to confirm the cleavage of noncomplementary relay oligo Tr through S′−3′ exonuclease activity of the Taq polymerase during recognition PCR. A reaction containing 4 μM probe DNA, 1× PNK buffer-A, 10 units of PNK enzyme, and [γ-32P] ATP was incubated at 37 °C for 2 h, and a labeled probe was purified through 10% denaturing PAGE.

### Cleavage PCR with Labeled Probe.

PCR was performed to confirm the cleavage of S′ non-complementary relay oligo Tr through S′−3′ exonuclease activity of Taq polymerase. A PCR reaction of 50 μL containing 10^{10} copies of the target template, 1× taq buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM (NH_{4})SO_{4} and 2 mM MgSO_{4}), 0.2 mM dNTPs, 0.4 μM of each primer, 2.5 units of EasyTaq polymerase, and a 5′-32P-labeled probe was run. The PCR cycle consisted of initial template denaturation at 95 °C for 2 min, followed by 25 cycles of 94 °C for 10 s, 54 °C for 20 s, and 65 °C for 30 s with a final extension for 2 min at 72 °C. The PCR product was purified and run through 10% denaturing PAGE followed by cross-linking of the gel with a phosphor screen for 15 min in the dark. The image was analyzed through a cyclone plus phosphor imager.

### Synthesis and Dilution of Fluorophores (DFHBI and DMHBI).

Fluorophores DFHBI and DMHBI were synthesized as described by Paige et al.24 The fluorophore was first dissolved in DMSO at a concentration of 40 mM and then diluted into 100 mM HEPEs buffer (pH 7.5) with a final concentration of 5% DMSO and 2 mM fluorophore.

### Amplification and Transcription of RNA Aptamers.

The DNA sequences of three RNA aptamers (TT), namely 24−2, 17−3, and 13−2min (Supporting Information Table S1), were synthesized and
amplified separately through PCR in a 25 μL reaction mixture containing 10^6 copies of TT (24–2, 17–3, and 13–2min), 1X taq buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM (NH₄)₂SO₄, and 2 mM MgSO₄), 0.2 mM dNTPs, 0.4 μM of each primer (p-3 and Tr), and 2.5 units of EasyTaq polymerase. The PCR cycle consisted of initial denaturation at 95 °C for 2 min, followed by 25 cycles of 94 °C for 10 s, 52 °C for 20 s, and 72 °C for 30 s with a final extension for 2 min at 72 °C. PCR products were run in 3.5% agarose gel. PCR products were transcribed in a reaction containing 25 μL of PCR product, 1.5 mM of NTP, 1X T7 RNA polymerase buffer (40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 10 mM DTT, 6 mM MgCl₂, 2 mM spermidine), and 50 units of T7 RNA polymerase at 37 °C for 4 h. Afterward, 0.2 mM DFHBI (24–4) and DMHBI (13–2min, 17–3) were added, and fluorescence was measured at respective wavelengths (Supporting Information Table S2) and photographed under 365 nm UV light.

**Relay PCR.** Relay PCR was performed in a 50 μL PCR reaction having 10^10 copies of the target template, 1X taq buffer (20 mM Tris-HCl (pH 8.4), 20 mM KCl, 10 mM (NH₄)₂SO₄, and 2 mM MgSO₄), 0.2 mM dNTPs, 2.5 units of EasyTaq polymerase, 0.4 μM of each primer, 0.4 μM of probe, and 10^5 copies of TT (24–2, 17–3, 13–2min). The PCR cycle consists of initial template denaturation at 95 °C for 2 min, followed by 35 cycles of 94 °C for 10 s, 57 °C for 20 s, and 65 °C for 30 s with a final extension for 2 min at 72 °C. **Fluorescence Assay.** Double stranded PCR product was transcribed by using T7 RNA polymerase. A transcription reaction containing 25 μL of relay PCR product, 1.5 mM of NTP, 1X T7 RNA polymerase buffer (40 mM Tris-HCl (pH 7.9), 10 mM NaCl, 10 mM DTT, 6 mM MgCl₂, 2 mM spermidine), and 50 units of RNA polymerase were incubated at 37 °C for 4 h. Fluorophore DFHBI/DMHBI was added to the transcribed product at a final concentration of 0.2 mM at RT, and fluorescence was measured at respective excitation and emission wavelengths (Supporting Information Table S2). The fluorometric detection, in which about 450 readings with a 30 s interval were recorded, was carried out in a Varioskan Flash (Thermo Scientific) machine. Fluorescence was also imaged directly under the illumination of 365 nm of UV light.

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