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Ultrasensitive, rapid, and highly specific detection of microRNAs based on PER-CRISPR/CAS

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ABSTRACT

Abnormal microRNA (miRNA) expression levels are confirmed as diagnostic biomarkers of the emergence and development of diseases. In this study, we developed a fluorescence biosensor for detecting miRNAs based on double amplification reactions with the primer exchange reaction (PER) and CRISPR/Cas12a. In the absence of target miRNA-21, PER hairpins remained locked by the protector strands and the primers did not extend. In the presence of target miRNA-21, the miRNA-21 bound to the guard sequence and exposed primer binding sites. Also, the closed PER hairpin was unlocked to specifically extend primers into single-stranded DNA (ssDNA) of unequal lengths. These ssDNAs of unequal lengths could activate the cleavage of a reporter by Cas12a, leading to an increase in detectable fluorescence signals. A large number of short nucleic acid fragments were amplified by PER-CRISPR multiple cycle cleavage fluorescent probes. Based on PER-combined CRISPR/Cas12a established dual signal amplification method was characterized by a low limit of detection of 10fM. The fluorescent biosensor for miRNA detection had the advantages of low detection cost, simple operation, and mobility, providing a very promising platform for the point-of-care testing of miRNA-21.

Introduction

MicroRNAs (miRNAs), a class of short noncoding RNAs (18–25 nt),[1,2] play an important role in a variety of biological processes.[3] Abnormal miRNA expression were usually related to cancer development, maintenance, and progression levels are confirmed as diagnostic biomarkers of the emergence and development of diseases.[4] Therefore, miRNAs need to be quantified precisely enough to be identified as the ideal biomarkers. Monitoring miRNA expression level is extremely crucial for observing the role of miRNA in biological processes. However, the detection of miRNAs is challenging because of short sequences and low abundance.[5]

A variety of amplification techniques have been used for analyzing the low concentrations of miRNAs in biological samples.[6] Real-time quantitative polymerase chain reaction (RT-qPCR) is now considered a gold standard for all miRNA detection techniques.[7] However, complex and time-consuming RT-qPCR technology requires expensive reagents, specialized equipment, and skilled personnel.[8] Isothermal amplification techniques, including hybridization chain reaction, loop-mediated isothermal amplification, rolling circle amplification, primer exchange reaction (PER), and catalyzed hairpin assembly, have been gradually developed to overcome these limitations.[9,10]

PER was a novel and powerful isothermal amplification technique, and the synthesis of PER has attracted much attention.[11] A single PER hairpin serves as a catalytic template, with the help of DNA polymerase, to repeatedly add newly formed single-stranded sequences to specified short primers.[12] Some high-performance biosensors have been developed based on PER and applied to the analysis of pathogens, proteins, and nucleic acids. For example, wang et al. constructed an electrochemical biosensor based on primer exchange amplification reaction and target-triggered circular chain displacement reaction to detect miRNAs in exosomes with high sensitivity.[13] Bu et al. developed a rapid electrochemical biosensor for the point-of-care testing of *Escherichia coli O157:H7* based on the cascade signal amplification of CRISPR/Cas12a and PER without specific nucleic acid extraction.[7,14]

Recently, a CRISPR/Cas system[15] has been developed as a nucleic

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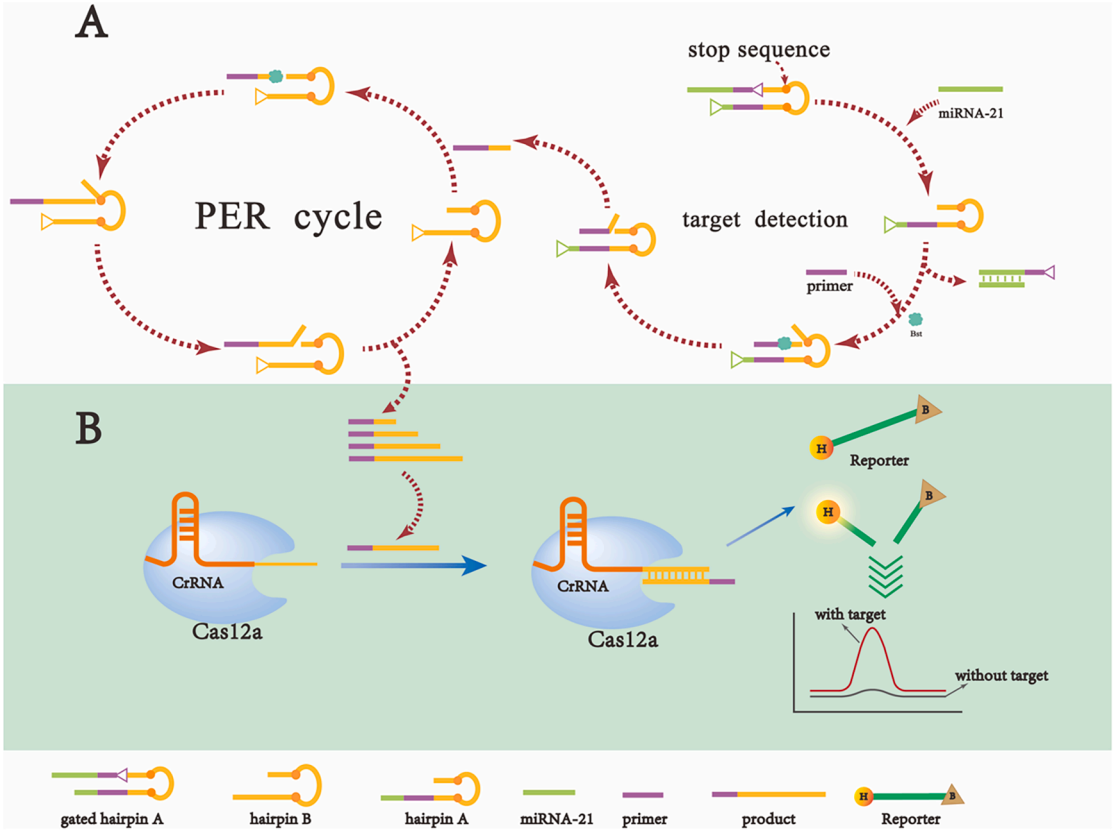
acid detection strategy due to the specific cleavage capability of the collateral DNA/RNA.[15] Of these, the CRISPR-associated protein Cas12a (previously known as Cpf1) has been also demonstrated to possess "collateral cleavage" activity.[16] After the gRNA binds with the complementary target DNA, the *trans*-cleavage activity of this Cas12a enzyme can be triggered to cleave collateral reporter.[17,18] The reporter is a labeled fluorophore quencher (FQ), and it generates a sensitive visualized readout for target amplicons. CRISPR-based diagnostic[19] platforms have exhibited the great potential of CRISPR systems and are poised to improve some long-standing problems of biosensing such as poor sensitivity and difficult operations.[20,21] A DETECTR nucleic acid detection strategy based on DNA endonuclease-targeted CRISPR *trans*-reporter, CRISPR-Cas12a, was developed and gained attention in molecular primary diagnostics. Zhang et al. have proposed a strategy about CRISPR-Cas12a enhanced rolling circle amplification method for ultrasensitive miRNA detection.[22] Gong et al. put forward a duplex-specific nuclease-assisted CRISPR-Cas12a strategy for miRNA detection using a personal glucose meter.[23] Recently, CRISPR/Cas12a has been used to detect miRNAs as a sensitive tool. Although it requires simple instruments to obtain a stable signal, such as an electrochemical workstation, and a UV − vis spectrophotometer with high detection efficiency, it demands complex operation procedures, which restrains the point-of-care applications of CRISPR-Cas12a for miRNA detection. Therefore, it is necessary to further develop a portable strategy to detect miRNA.

In this study, we designed PER-combined CRISPR/Cas12a, as a fluorescence biosensor, to establish a method with simple operation, short detection time, and high sensitivity (Scheme 1). In the absence of target miRNA-21,[24] PER hairpins were locked by molecules, and primers were prevented from extending. And the presence of target miRNA-21 could expose the binding domain of the primer, and the primer bound to the hairpin A. The PER reaction would, in turn, be the trigger, and the primer was extended into strands of ssDNA (PER product) of unequal lengths, which produced Cas12a-gRNA–DNA (ternary complex with Cas12a-gRNA). After the ssDNase cleavage activity of Cas12a was activated, the triggered Cas12a system could cleave the reporter labeled FQ, generating a sensitive fluorescence visualized readout[25] Based on PER-combined Cas12a enzyme, the biosensor displayed a change in the fluorescent signaling probe (reporter labeled FQ) depending on the target,[26,27] thus causing an obvious change in the fluorescent signa.[28,29]. The whole detection time needed only 1 h, and no complex operation process existed. Only Cas12a and nucleic acid amplification were required, saving costs. Besides detecting miRNA21, this method can also detect other types of miRNAs, showing strong generality, high sensitivity detection can be achieved by one-step method.

All DNA oligonucleotides (Table 1) used in this study were synthesized by Sangon Biotech Co., ltd. (Shanghai, China). All of the DNA oligonucleotides were stored using 1 × TE buffer (1 mM EDTA, 10 M Tris, pH 6.5) and RNase-free water. An HiScribe T7 Quick High-Yield RNA synthesis kit, EnGen Lba Cas12a, and 10 × NEBuffer 2.1 (0.01 M BSA, 0.5 M NaCl, 0.1 M $MgCl_2$, and 0.1 M Tris-HCl, pH 7.9) were obtained from New England Biolabs (MA, USA). Bst DNA polymerase (Bst), 10 ×

Table 1
Comparisons among methods developed for miRNA determination.

Type of biosensor	Detection time	Detection range	Detection limit	Ref.
Fluorescent detection	60 min	500pM to 10nM	500pM	[30]
Electrochemiluminescence	2 h	1fM to 100pM	2.67fM	[31]
Colorimetry	2 h 15 min	5pM to 50nM	0.28pM	[32]
Fluorescent detection	50 min	10fM to 1μM	10fM	This work



Scheme 1. Schematic illustration of the PER-Cas12a strategies for miRNA-21 detection.

Bst reaction buffer (0.02 M $MgSO_4$, 0.2 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, 0.1 M KCl, 0.1 % Triton X-100, pH 8.8), and dNTP set solution (100 mM each) were obtained from Sangon Biotech Co., ltd (Shanghai, China). DNase/RNase-free deionized water was obtained from Tiangen Biotech Co., ltd. (Beijing, China). RNasin ribonuclease inhibitor was acquired from Promega Biotech Co., ltd (Beijing, China). All solutions were prepared with ultrapure water.

Fluorescence signals were measured using a NanoDrop ND-3300 fluorescence spectrometer (Gene Company Limited). The fluorescence emission was recorded at a wavelength of 553 nm, and the excitation wavelength was set to 535 nm. Ultrapure water was acquired from Nanjing Xinfeida Optoelectronic Science and Technology Co. The pH meter (PHS-25) used in this study was obtained from Shanghai INESA Scientific Instrument Co.

First, 2 μL of CRISPR RNA (CrRNA) template and 2 μL of a T7 promoter (100 μM) were added to 14 μL of RNase-free water, and the mixture was heated to 95 °C for 5 min. Then, the mixture was allowed to cool gradually to room temperature for at least 20 min. The mixture was reheated to 37 °C and held for 15 min. DNase I was used to degrade single and heterozygous DNA. Then, 45 μL of RNase-free water was added to the mixture and kept at 37 °C for 16 h. Subsequently, the mass of CrRNA transcription products was depurated with an miRcute miRNA isolation kit and centrifuged at 13,000 rpm for 1 min. Then, the byproducts were removed by washing buffer (washing three times). Finally, the CrRNA concentration was measured using NanoDrop 1000 and it was stored at −80 °C in an enzyme-free tube.

First, 10 μL of different samples were taken and mixed with 2 μL of 6 × loading buffer. Then, these different samples were injected into the polyacrylamide 15 % gel electrophoresis (PAGE) system. The PAGE buffer was 1 × TBE (89 mmol Tris, 89 mmol Boric acid, 10 mmol EDTA) and a constant voltage of 120 V was used for 70 min. The electrophoresis column was stained with SYBR Green nucleic acid for 20 min and observed on a Gel Doc EZ imager (Bio-Rad, USA).

PER cascade is a programmable, autonomous, stepwise fashion, which with the help of a strand-displacing polymerase isothermally. PER can produce lots of single-stranded DNA with arbitrary user-prescribed sequences. The PER cascade starts with a specific primer. Using a DNA hairpin mediator, PER appends to the existing primer a new user-specified independent sequence. The new sequence can trigger the next step extension, thus forming a programmable PER cascade to autonomously grow a nascent DNA strand along a prescribed pathway to produce an arbitrary user-prescribed sequence.

The three components required to operate the miRNA-21 detector PER system were gated hairpin A, telomerase hairpin B, and primer. The chain replacement reaction was needed to design primer binding sites of PER and complete target detection. First, hairpin A (100 nM) and protector strand (150 nM) were maintained at 95 °C for 5 min to a higher binding efficiency. When the miRNA-21 signal was present, the target miRNA-21 could attach to a domain on a protector strand bound to the hairpin A, which was only exposed in the presence of the cognate miRNA-21 signal. The gated hairpin A domain was fully changed. The protector strand could spontaneously dissociate from the hairpin A and exposed primer binding base sequences (b). When exposed, the primer joined at the PER hairpin facilitated b*. In the presence of the Bst polymerase, short pieces (b* + c*) were produced. These pieces continued to bind to hairpin B, producing a large number of fragments (c*) of varying lengths.

Before the experiments, the protector strand (150 nM) and hairpin A (100 nM) were heated at 95 °C for 5 min, and slowly cooled down to 25 °C to gain a stable hairpin (gated hairpin A). Then, hairpin B was heated at 95 °C for 5 min and cooled to room temperature. The PER reaction cycle was performed by adding 4 μL of the gated hairpin A, 2 μL of hairpin B (10 μM), 2 μL of 10 × Bst buffer, 2 μL of dNTP (1 mM), 1 μL of Bst poly enzyme (8U/μL), and 5 μL of 1 × TE buffer (pH 6.5). Different concentrations of targets (2 μL) were maintained at 37℃ for 30 min. Then, add 2 μL Primer (2 μM) attain at a volume of 20 μL mixed liquor. Finally, the PER reaction was performed at 37℃ for 10 min, and Bst poly was thermally inactivated by incubating at 80 ℃ for 20 min. A total of 4 μL of the PER product were taken from the final product as an ssDNA activator to activate the Cas12a-mediated cleavage reaction. Subsequently, 14 μL of Cas12a cleavage reaction system contained 4 μL of LbaCas12a (0.3 μM), 5 μL of crRNA (0.25 μM), and 4 μL of the PER product; 1 μL of RNase inhibitor was added. Finally, the mixture was incubated at 37 °C for 40 min, and the results were obtained.

A total of 1 μL of the reporter was added to the reaction solution. Then, the results were obtained using an ND-3300 fluorescence spectrophotometer (Gene Company Limited). The slit widths of the emission and excitation were both kept at 10.0 nm and the excitation wavelength at 535 nm, whereas the fluorescence of hexachlorofluorescein was measured with an emission of 553 nm. All experiments were repeated three times.

Various concentrations of target miRNA-21 were added to the PER system reaction solution to demonstrate the analytical performance of the proposed fluorescence sensor.

The same concentrations (1μM) of miRNA21-1, miRNA21-2, miRNA21-3, miRNA21-4, and miRNA-144 were added to the reaction solution to further evaluate the selectivity of the designed sensor.

The designed biosensor was analyzed for miRNA-21 in real serum samples. Briefly, 100 μL of the serum was equally mixed with 800 μL of 1 × TE buffer. Next, 100 μL of miRNA-21 at different concentrations was added to the mixture to prepare artificial pollution samples. Thus, spiked samples containing different concentrations (10^8fM, 10^7fM, and 10^6fM) of miRNA-21 were obtained. Finally, the target detection process was the same as that mentioned earlier. The recovery rate was calculated by measuring the ratio of the miRNA-21 signal to the amount of miRNA-21 added to the system.

Under optimal detection conditions, the change in the fluorescence signal with the concentration of target miRNA-21 was studied to evaluate the analytical performance of PER-combined CRISPR/Cas12a strategy for miRNA-21 detection. As shown in Fig. 1A, with different concentrations of miRNA-21 increasing from 10 to 1 μM, the light intensity at 555 nm was also continuously increased. We chose 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, 10 pM, 1 pM, 100fM, and 10fM miRNA-21 to measure separately and found that the lowest detection limit was 10fM. Fig. 1B shows a good linear correlation between F/F0 and the logarithm of the target miRNA-21 concentration (fM) with the range from 100fM to 100 nM. The regression equation was $Y = 1.414*X - 1.105$ with R^2 (a correlation coefficient) of 0.9831. The measurement results of each experiment were repeated three times. The linear range of the PER assisted Cas/Cas12a, and high sensitivity was attributed to the multi-turnover *trans*-cleavage activity of Cas12a and nucleic acid amplification. When compared with a series of previously reported miRNA detection methods (Table 1), the results showed that the developed sensor had high sensitivity for detecting miRNAs (where F and F0 represent the fluorescence strengths in the experimental and control groups, respectively).

The miRNA-21 was substituted with miRNA-141 to validate the specificity of the proposed approach; mutation bases were 1–4 (21–1, 21–2, 21–3, and 21–4), and the control was the blank. Fig. 2 shows that the fluorescence value was high only when the target was added. The fluorescence values were similar to those of blank controls when non-targets were added. The result suggested that the constructed method had high specificity.

The study explored whether the proposed approach could be applied for the recovery detection of the target in a complex biological material with an accurate recovery rate. For comparison, we used the recovery of a series of miRNA-21 standards at different concentrations. As shown in Table 2, the concentrations of miRNA-21 measured using the proposed approach were in good agreement with those in serum, with recoveries of 103 %, 97 %, and 95 % for samples spiked with 10^8, 10^7, and 10^6fM miRNA-21, respectively. This proved that the proposed strategy achieved a good detection effect in biological samples.

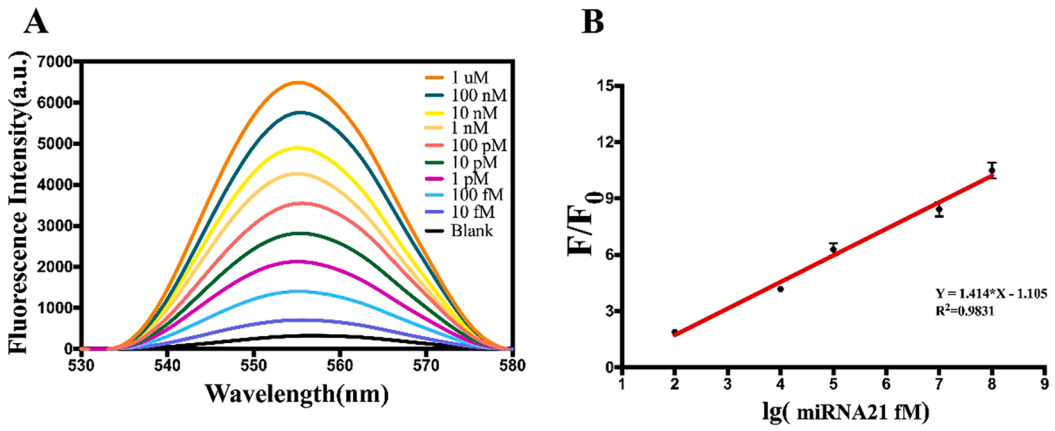


Fig. 1. Quantification of target miRNA21 using the proposed method. (A) Fluorescence spectra of the PER-Cas12a system in the presence of different concentrations of miRNA-21. (B) Linear analysis results for different concentrations of miRNA-21.

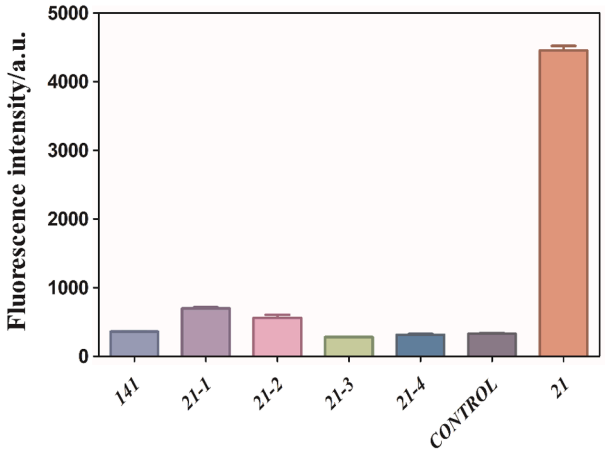


Fig. 2. Evaluation of the selectivity of the fluorescence biosensor. Error bars represent standard deviations of three parallel experiments.

Table 2
Detection of miRNA in the sample serum.

Original value (fM)	Added (fM)	Detected (fM)	Recovery ± RSD (%)
0	1×10^8	1.03×10^8	103 ± 3.9
0	1×10^7	0.97×10^7	97 ± 2.4
0	1×10^6	0.95×10^6	95 ± 2.3

This study proposed a PER-combined CRISPR cleavage system by optimizing the key conditions of PER. As an ascending CRISPR cleavage system, PER can be used in more comprehensive scenarios of nucleic acid detection. In summary, a biosensor for miRNA-21 based on a dual signal amplification strategy was successfully developed. Using a PER-CRISPR system for the detection signal amplification, we realized a PER-combined CRISPR/Cas12a dual signal amplification method miRNA-21 concentrations as low as 10fM could be detected with good specificity, high sensitivity, and a wide linear range from 10fM to 1 μM. In PER, a large number of fragments were amplified in just 10 min to activate CRISPR, greatly shortening the reaction time and achieving the purpose of efficient and rapid detection. The results showed that using multiple DNA probes could improve the detection sensitivity and accuracy. The constructed biosensor was also used to detect miRNA-21 in real serum samples and showed great potential in detecting miRNAs in complex samples. The successful application of the combination of PER with CRISPR/Cas12a provided a new thought to improve the use of CRISPR/Cas12a for common applications. This strategy significantly improved the convenience of the fabricated sensors using PER strategies and offered a new perspective for the development of extensive and sensitive analysis tools for nucleic acid detection in the future.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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