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Catalytic Assembly of DNAzyme Integrates with Primer Exchange Reaction (CDiPER) for Highly Sensitive Detection of MicroRNA

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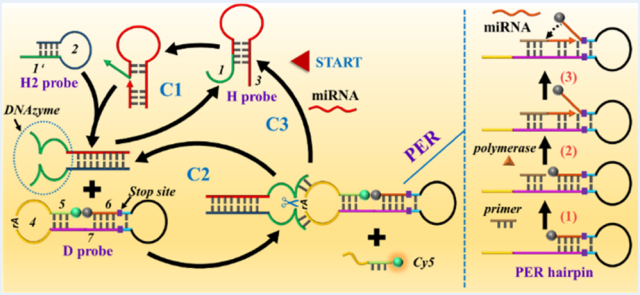
ABSTRACT: MicroRNAs (miRNAs) have significant regulatory functions in the modulation of gene expression, making them essential biomarkers for the diagnosis and prognosis of diseases. Nevertheless, the identification of miRNA poses significant difficulty in terms of its low abundance, necessitating sensitive and reliable approaches. Herein, we develop a simple approach, termed **C**atalytic assembly of **D**NAzyme **i**ntegrates with **P**rimer **E**xchange **R**eaction (CDiPER), for reliable and sensitive miRNA detection through the target recognition-triggered DNAzyme assembly and primer exchange reaction (PER) strategy. In this method, target miRNA can precisely bind with a specifically designed hairpin probe (H probe) to induce the conformation changes of the H probe, releasing DNAzyme sections to activate the PER process for signal amplification and fluorescence signal production. The established method displays a high dynamic range of over 6 orders of magnitude and a low detection limit of 312 aM. The created method has a number of unique advantages, such as (i) a better sensitivity than existing systems using PER for signal amplification as a result of its integration with the target recognition-triggered DNAzyme assembly and (ii) streamlined operating procedures. Further, the technology was used to detect the expression of miRNA in collected clinical samples from diabetes mellitus patients, revealing that miRNA was decreased in patients and demonstrating the significant clinical promise of the method.



1. INTRODUCTION

MicroRNAs (miRNAs) are known to exert a substantial influence on a wide range of biological activities.[1,2] The dysregulation of microRNAs (miRNAs) has been implicated in the pathogenesis of cancer and a range of other disorders. Diabetic foot ulcers (DFU) are recognized as significant consequences of diabetes, particularly prevalent among the elderly population, and have a substantial impact on their daily functioning.[3] In the context of DFU, miRNA-21 emerges as a promising biomarker that exhibits the potential to enhance the reparative processes of ischemic tissue and stimulate angiogenesis in individuals with diabetic foot conditions.[4] Hence, the thorough and exact investigation of miRNA-21 assumes great significance in the realm of prognosticating the prognosis of diabetic foot and other ailments.

Northern blotting, microarray technology, and real-time polymerase chain reaction (RT-PCR) are common analytical techniques utilized for the purpose of identifying and quantifying miRNAs.[5−8] Nevertheless, the intrinsic properties of miRNAs, such as their limited abundance, significant sequence similarity, and susceptibility to degradation, pose challenges to the sensitivity and specificity of the current detection approaches for miRNAs. In order to surpass these limitations, various methods for amplifying molecular signals have been devised with a particular focus on nuclease-assisted signal amplification techniques. Notably, techniques such as rolling circle amplification (RCA),[9,10] polymerization/nicking reaction (PNR),[11,12] and loop-mediated isothermal amplification (LAMP)[13,14] have been developed to enable simple, rapid, and effective amplification of target miRNAs.[15] Nevertheless, the utilization of nuclease-driven amplification methods invariably necessitates intricate primer design and involves the involvement of several nucleases. Furthermore, the efficacy and stability of the amplification are constrained by the presence of nuclease activity. The stability of target amplification is significantly influenced by these parameters. In recent years, the primer exchange reaction (PER) cascade technique has emerged as a potential technology for the amplification of the target.[16,17] The PER cascade employs a catalytic DNA hairpin, referred to as the PER hairpin, as a template. This hairpin facilitates the continuous growth of a short single-stranded DNA (ssDNA) primer following specific reaction pathways with the assistance of a strand-displacing polymerase. This process enables the stable amplification of

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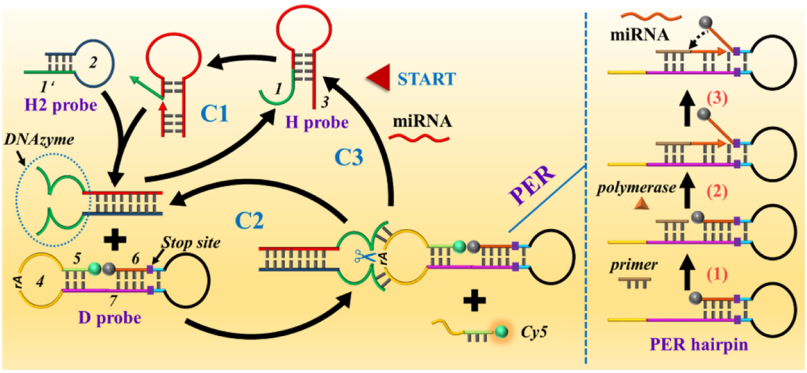

Figure 1. Working mechanism of the established approach for miRNA detection.

target sequences.[18,19] Significantly, the production of a new strand in situ occurs only when the appropriate set of PER hairpins and its associated PER primer are present simultaneously. This autonomous process minimizes signal leakage and efficiently mitigates the background response.[20] The utilization of the PER cascade has been widely employed in the development of very sensitive detection methods for miRNAs using colorimetric, fluorescent, and electrochemical technologies owing to the influence of this particular reaction.[18,21,22] However, in these PER-based methods, commonly integrated several nuclease-assisted target amplification approaches which require full consideration of enzyme properties for the amplification greatly limited their further application.

DNAzymes are nucleic acids that replicate the biofunction of protein enzymes.[23,24] They have found extensive use in the development of adaptable amplification sensing platforms. This is mostly owing to their intrinsic advantages, such as excellent stability in complex experimental circumstances and decreased nonspecific absorption.[25] Nevertheless, the inherent limitations of DNAzyme-based sensing systems, such as their relatively low catalytic activity, lack of flexibility, and insufficient sensitivity, have significantly hindered their progress, in terms of further development.

Herein, we developed a method, termed **C**atalytic assembly of **D**NAzyme **i**ntegrates with **P**rimer **E**xchange **R**eaction (CDiPER), for the sensitive and selective fluorescence detection of target miRNA by integrating target miRNA identification-induced DNAzyme cleavage feedback with a PER approach. Furthermore, by analyzing miRNA-21 in human serum and its expression level, this biosensor practicability in real sample analysis and accurate disease diagnosis has been verified.

2. EXPERIMENTAL SECTION

2.1. Materials and Apparatus. All oligonucleotides used in this experiment (Table S1) were purchased from Sangon Biotech., Co., Ltd. (Shanghai, China). DNA polymerase was obtained from New England Biolabs (NEB, Beijing, China). A deoxyribonucleoside 5′-triphosphate mixture (dNTPs) was provided by Tiangen Biotech., Co., Ltd. (Beijing, China). Diethypyrocarbonate (DEPC)-treated water (DNase, RNase free) obtained from the Beyotime Institute of Biotechnology (Shanghai, China) was used in all experiments.

2.2. Feasibility of the Approach. The synthesized fluorescence moiety and quenching moiety labeled double-loop probe (D probe) were diluted to 20 μM with DEPC water. Twenty microliters of the diluted D probe were heated to 90 °C and kept for 10 min. Afterward, the mixture was gradually cooled to room temperature to form the double loop in the D probe. The fluorescence signals of the D probe before and after assembly were detected by the Fluorolog 3-211 fluorescence spectrophotometer (Horiba Jobin Yvon, France). The same procedures were used to prepare the H probe. 10 μL of the assembled D probe and H probe were then mixed with 10 μL of miRNA (1 μM) and incubated for 20 min. The fluorescence signal of the D probe was recorded and compared with that before mixing with target miRNA.

2.3. miRNA Detection. First, 1 μL of target miRNA, 1 μL of H probe (100 nM), 1 μL of H2 probe (100 nM), and 1 μL of primer sequences (1 μM) were mixed with 1 μL of D probe (500 nM) and incubated for 10 min. DNA polymerase was added to the mixture, and the mixture was incubated for 30 min at 60 °C. The fluorescence spectrum was recorded.

2.4. Statistics Analysis. The data in this research were listed as mean ± standard deviations and statistical significances were calculated by GraphPad Prism 8.0. The differences between the two groups were obtained by two-tailed Student's t test, and $P < 0.01$ was a threshold for significance. Three technical replicates were used for statistical analysis.

3. RESULTS AND DISCUSSION

3.1. Principle of the Target Recognition-Triggered DNAzyme-Driven PER Strategy. The proposed fluorescent biosensor consists primarily of two amplification processes: (i) DNAzyme-triggered signal cycle and (ii) DNAzyme-driven PER strategy (Figure 1). The details of the target sequence in this design are shown in Table S1. Through prehybridization between the "1" and "3" sequences, the hairpin probe (H probe) comprising the target recognition region and DNAzyme section is created. The "1" strand can be released in the presence of target miRNA by toehold-mediated strand displacement reactions accompanied by the formation of a miRNA/"3" hybrid, thereby exposing DNAzyme-1 ("1" section). H2 probes bind with "3" section (loop section in H probe) in the miRNA/"3" hybrid and form a H/H2 hybrid. The released miRNA recognizes the next H probe and forms the first signal cycle (C1). The "1" and "1" sections in the H/H2 hybrid form an intact DNAzyme. The DNAzyme recognizes the "4" segment in the D probe and creates an active secondary conformation. DNAzyme is activated by the addition of Mg^{2+} to specifically cut the "4" segment, unleashing the Cy5-labeled "5" sequence (C2). Consequently, Cy5 is

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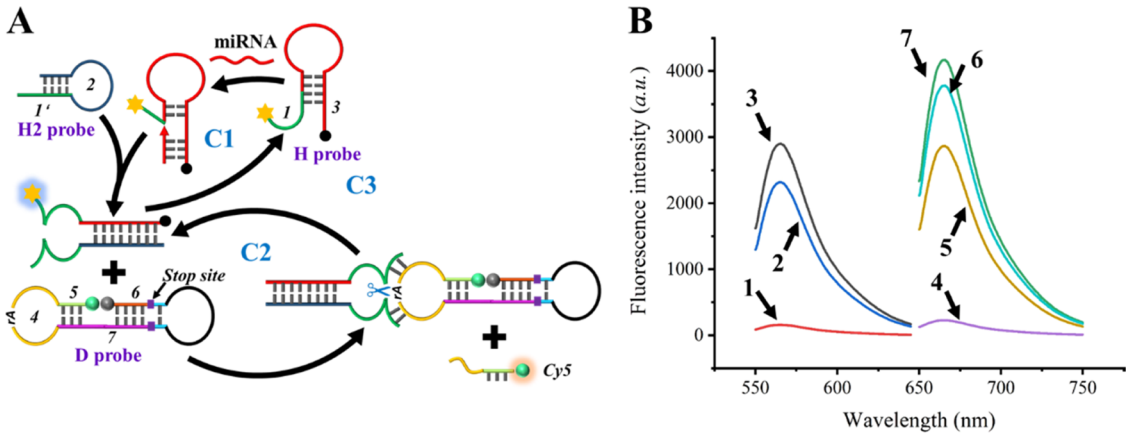

Figure 2. Assembly of the probes and feasibility of the method. (A) Schematic illustration of the fluorescent assay to test the feasibility of DNAzyme-based D probe cleavage. (B) Fluorescence spectrum of the probes when detecting miRNA by the approach. (1) assembled H probe (H probe in hairpin structure); (2) H probe + target miRNA; (3) H probe (L, H probe in linear state before being assembled to hairpin structure); (4) assembled D probe (D probe in dumbbell structure); (5) H probe + target miRNA + H2 probe + D probe; (6) H probe + target miRNA + H2 probe + D probe + primer + DNA polymerase; (7) D probe (L, D probe in linear state before being assembled to dumbbell structure).

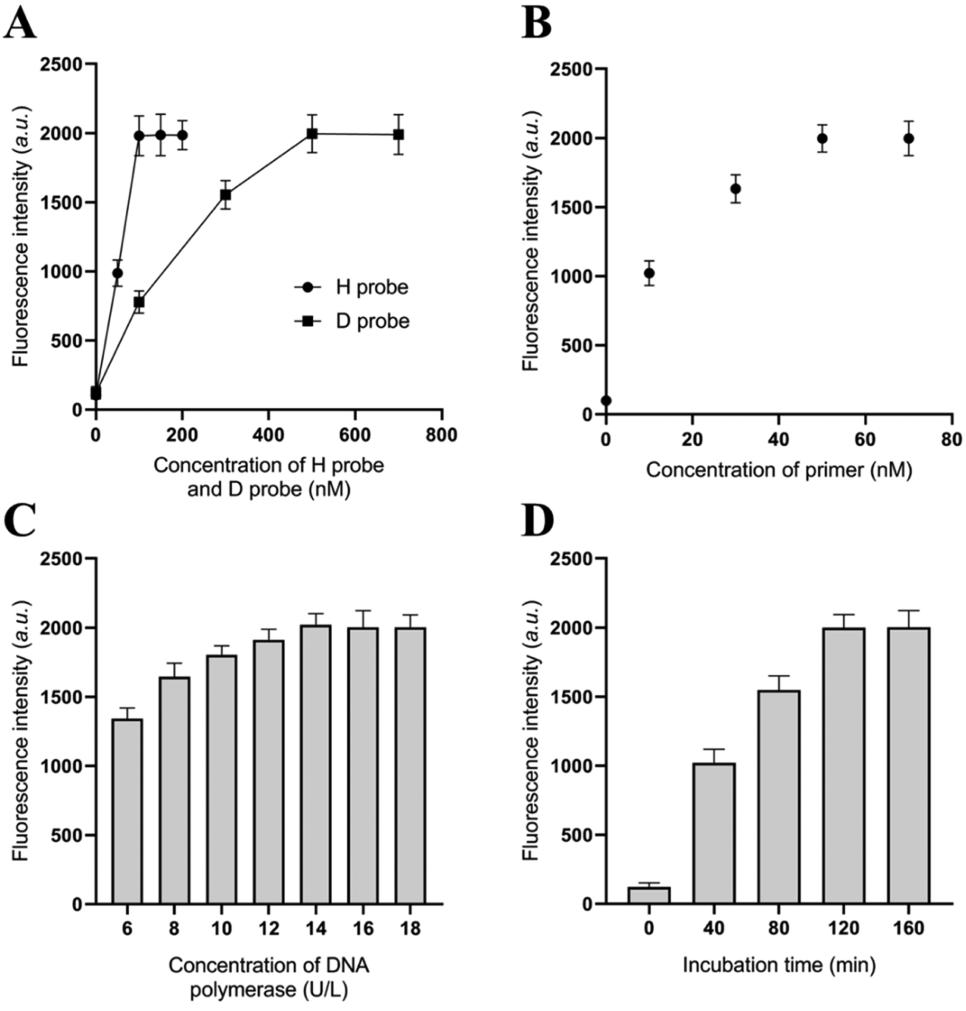

Figure 3. Optimization of the experimental conditions. Fluorescence intensities of the approach with different concentrations of H probe, D probe (A), primer (B), DNA polymerase (C), and incubation time (D). Three technical replicates were used for statistical analysis.

dissociated from the BHQ moiety labeled at the end of the "6" segment, resulting in the recovery of Cy5 signals. In addition, the primer binding site in the PER probe is exposed due to the release of the Cy5-labeled "5" sequence, allowing it to bind with the primer and initiate the PER process. With the assistance of Bst-DNA polymerase and dNTP, the sequence identical with the target miRNA is copied, and the primer is extended to the C-G termination site (stop site). Then, the

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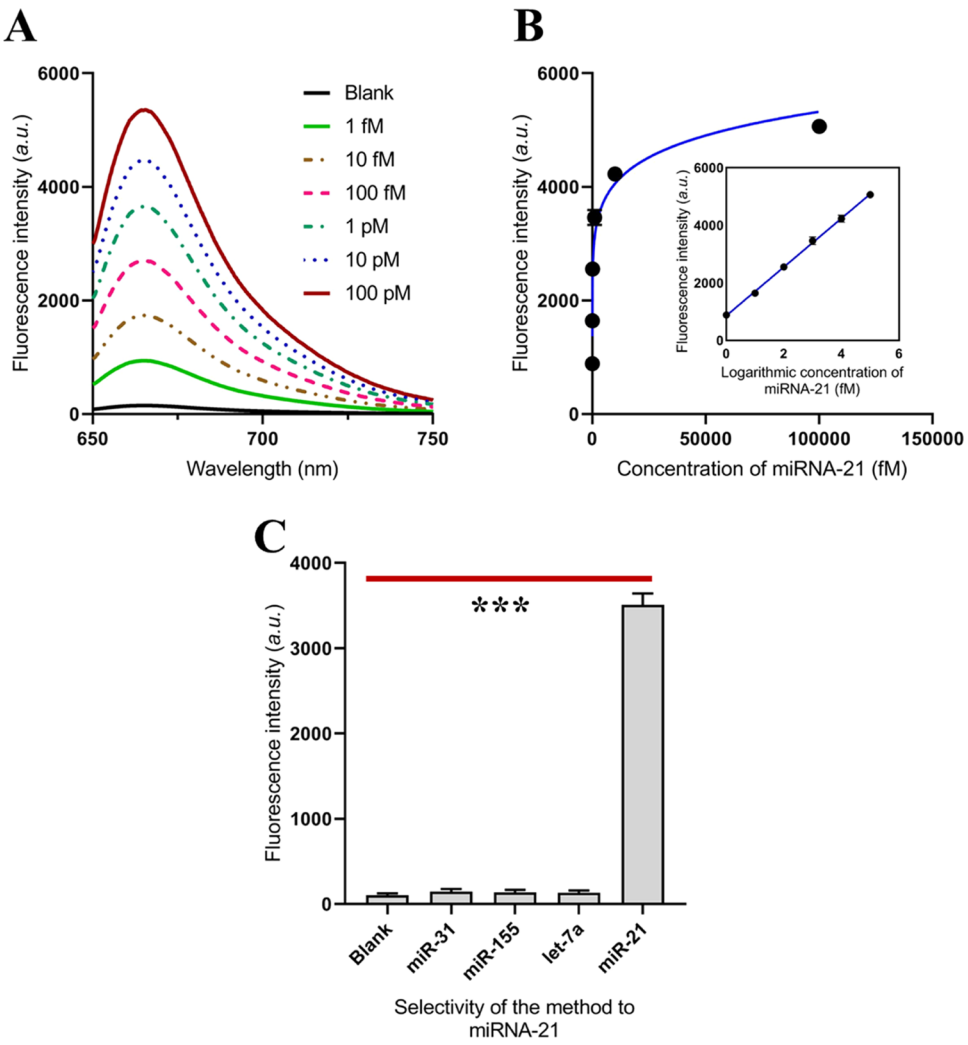

Figure 4. Analytical performance of the approach for miRNA. (A) Fluorescent spectrum of the approach when detecting different concentrations of miRNA. (B) Correlation between the obtained peak fluorescence intensities (570 nm) and the concentrations of miRNA. (C) Fluorescence intensities of the approach when detecting target miRNA and interfering miRNAs. ***, $P < 0.01$, $n = 3$ technical replicates.

sequence domain in the hairpin competes with the extended sequence to combine with the hairpin stem, which, in turn, releases the extended sequence from the hairpin spontaneously. The released extended sequence bonds with a net H probe and initiates signal recycling, resulting in the production of copious quantities of Cy5 signals (C3).

3.2. Feasibility Analysis. As shown in Figure 2A, a fluorescent assay was conducted to confirm the construction of the H probe and the D probe. Before the assembly of the H probe, the Cy3 signal was strong; however, after assembly of the H probe into a hairpin structure, the Cy3 signal was substantially attenuated by the BHQ. Figure 2B demonstrates that the Cy5 signal of the D probe decreased significantly after assembly, indicating formation of the dumbbell structure. The target recognition-triggered DNAzyme cleavage of the "4" segment in the D probe was then evaluated. The Cy3 signal increased substantially in the presence of target miRNA, indicating the successful unfolding of the H probe. With the addition of the D probe and Mg^{2+}, the Cy5 signal was significantly amplified, indicating that the "4" segment was cleaved by the DNAzyme section in the H probe. The Cy5 signal was recorded to investigate the PER process enhanced signal amplification. Only when primer and polymerase were present in the sensing system did the Cy5 signal significantly increase. If neither the primer sequence nor the polymerase were present, there was no significant increase in fluorescence. The PAGE result also demonstrated the feasibility of the target recycling process and the PER process (Figure S1).

3.3. Influence of Experimental Conditions. In order to optimize the efficacy of miRNA detection, an investigation was conducted to assess the impact of varying concentrations of the H probe, D probe, primer, DNA polymerase, and incubation period. As the concentration of the H probe gradually increased from 0 to 100 nM, there was an initial rise in the value of the Cy5 signal, as depicted in Figure 3A. When the concentration of the H probe was increased in the sensing system, no further increases in observations were recorded. Subsequently, the optimal concentration of the D probe was determined to be 500 nM. As illustrated in Figure 3B, the fluorescence response exhibited a steady increase as the concentration of the primer increased, eventually reaching a plateau at 50 nM. Hence, it was established that a concentration of 50 nM for the primer yielded excellent results. Following the optimization of primer concentration, the dosage of DNA polymerase was seen within the range of 6 to 18 U, with increments of 2 U. The corresponding data may

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be found in Figure 3C. The fluorescence intensity exhibited a positive correlation with the quantity of DNA polymerase, with a maximum value observed at 14 U. Consequently, the quantity of DNA polymerase utilized in following tests was determined to be 14 U. Figure 3D illustrates the relationship between the reaction time of the enzyme and the corresponding fluorescence response. The fluorescence response increases fast within the time frame of 30 to 120 min when the reaction time is extended. However, there is no significant change observed in the fluorescence response after 120 min.

3.4. Analytical Performance of the Established Approach. The technique was implemented under ideal experimental circumstances to detect varying quantities of miRNA, ranging from 1 fM to 100 pM. The findings depicted in Figure 4A demonstrate a progressive augmentation in the peak fluorescence signals of the spectra as the concentration of miRNA, spanning from 1 fM to 100 pM, increases. The observed fluorescence intensities exhibited a direct proportional relationship with the logarithmic concentration of miRNA. By analyzing the data, a correlation equation was established to quantify the association between the fluorescence intensity and the concentration of miRNA:

$$Y = 844.2 \times \lg C + 862.4$$

with a correlation coefficient of 0.9956 (Figure 4B). The limit of detection was determined to be 312 aM according to the triple standard deviation method. Compared with former methods, the proposed approach exhibited a lower LOD (Table S2). In order to assess the selectivity of the developed approach for detecting homologous miRNAs, miRNA was employed as the target for detection. Additionally, three additional miRNAs (miRNA-31, miRNA-155, and let-7a) were chosen as potential sources of interference, all of which were present at equivalent concentrations. The fluorescence signal acquired from the technique, as depicted in Figure 4C, did not exhibit any statistically significant increases in fluorescence compared to the control group, indicating the absence of interfering miRNAs. The fluorescence signal exhibited a significant increase to 3456 a.u. only in the presence of miRNA. This value was found to be 3.73 times greater than that of the control group, suggesting a remarkable level of selectivity in the employed approach ($P < 0.01$). The result in Figure S2 demonstrated that the method could accurately distinguish target miRNA from one or two mismatched sequences.

3.5. Method Comparison. In the process of technique comparison, the initial step involved dilution of synthesized miRNA into various concentrations. The artificial serum samples were prepared by diluting the target miRNA by the commercial serum solution. The established approach was employed to identify miRNA in the fabricated samples, and the amounts of miRNA in each sample were determined by fitting the recorded fluorescence signals to the correlation equation derived in Figure 4B. RT-PCR is a common analytical technique utilized for the purpose of identifying and quantifying miRNAs and has been widely used in clinical practice for amplifying and quantifying nucleic acids. Therefore, RT-PCR was used as the gold standard method to validate DCiPER. The findings shown in Figure 5 demonstrate a strong correlation coefficient between the computed quantities of miRNA.

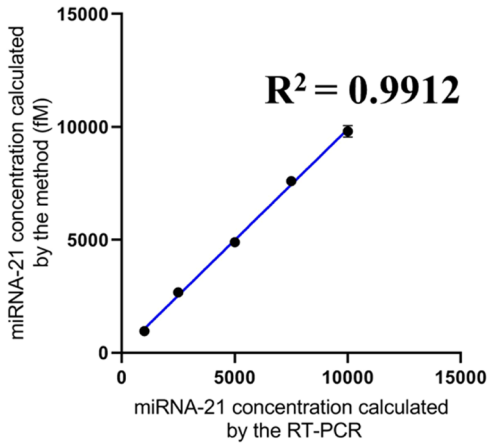

Figure 5. Calculated miRNA concentrations by the proposed method and by the traditional RT-PCR method.

3.6. Clinical Application of the Approach for miRNA Analysis. The above findings provide evidence for the viability of the established approach in detecting miRNA-21 with high sensitivity and reliability. In order to assess the clinical applicability potential, the established approach was employed to examine the concentration of miRNA-21 in serum samples obtained from clinical subjects. Peripheral blood samples were obtained from a cohort of 10 individuals diagnosed with DUF and 10 healthy volunteers at the hospital throughout the period of July 2022 to August 2023. The purpose of this study was to investigate and compare the levels of miRNA in the two groups. Prior to conducting this study, informed consent was obtained from all participants. The study protocols were approved by the Ethic Committee of the Hospital. Based on the findings presented in Figure 6, it was seen that the levels of miRNA-21 exhibited a reduction in individuals diagnosed with diabetes, in comparison to the levels observed in the healthy control group.

4. CONCLUSIONS

In this study, we present a novel method for the detection of miRNA that is both reliable and highly sensitive. Our approach

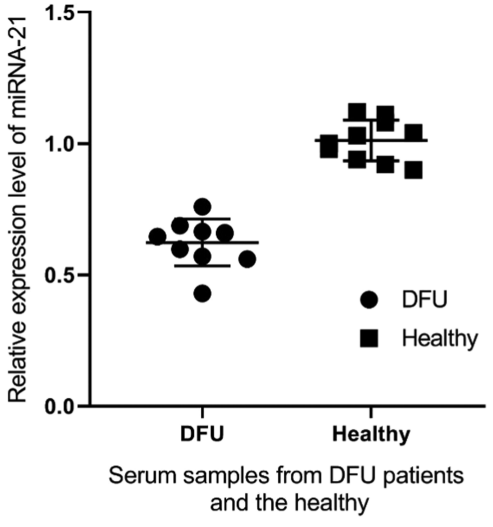

Figure 6. Relative miRNA-21 concentrations in the serum samples from patients and the healthy. $P < 0.01$.

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utilizes a primer exchange reaction strategy driven by target recognition-triggered DNAzyme. The method demonstrated a high level of detection sensitivity, with a limit of detection as low as 312 aM, due to the strong ability of PER to generate significant quantities of ssDNA sequences. Furthermore, the proposed technique was also utilized to detect miRNA in clinical specimens, thereby highlighting the involvement of miRNA-21 in the pathogenesis of diabetes mellitus. Considering the relatively high catalytic activity, flexibility, and high sensitivity, the CDiPER method has significant promise for its application in biosensing and clinical assays. However, the CDiPER method can be applied only for target miRNA detection. Redesign of probes is essential for expanding CDiPER for other biomarkers' detection. In the future, we will focus on expanding the method into a universal platform that can be applied to the analysis of multiple biomarkers without the need for redesign of probes.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c10003.

Sequences of oligonucleotides used in this work (Table S1); brief comparison of the CDiPER with former methods (Table S2); experimental section; PAGE result of the target recycling and the PER process (Figure S1), and selectivity of the approach for target detection (Figure S2) (PDF)

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Author Contributions

K.Z. and S.J.: conceptualization; K.Z.: investigation and validation; S.J.: resources; S.J.: project administration; and Y.L. and S.J.: data curation. All authors have read and agreed to the published version of the manuscript.

Notes

The authors declare no competing financial interest.

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