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RESEARCH ARTICLE

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Double hairpin-assembled probe-mediating catalytic hairpin assembly and primer exchange reaction for sensitive and label-free miRNA analysis in gastric carcinoma

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Abstract

The aberrant expression of microRNA (miRNA) is closely associated with various pathological processes, such as the development of gastric cancer. High-efficiency quantification of miRNAs is significant for the diagnosis, prognosis, and treatment of cancers. However, the sensitive and reliable detection of miRNA remains a huge challenge. We depict here a novel fluorescent approach for sensitive and label-free miRNA detection by exploiting a designed detection scaffold to integrate the catalytic hairpin assembly and primer exchange reaction (PER). In this method, the detection scaffold that is constructed based on the hybridization between two hairpin structure probes (H1 probe and H2 probe), is capable of specifically recognizing target miRNA and activating signal amplification, and the PER process transcribes numerous G-rich sequences to induce ThT-based label-free signal generation. Based on the efficient signal amplification strategy and label-free signal generation mode, the method exhibits a wide detection range of 7 orders of magnitude and a high repeatability (coefficient of variation, 2.76%), implying that the proposed approach will be a robust tool in quantification of miRNA and early diagnosis of disease.

Keywords microRNA, Catalytic hairpin assembly (CHA), Primer exchange reaction (PER), Thioflavin T (ThT)

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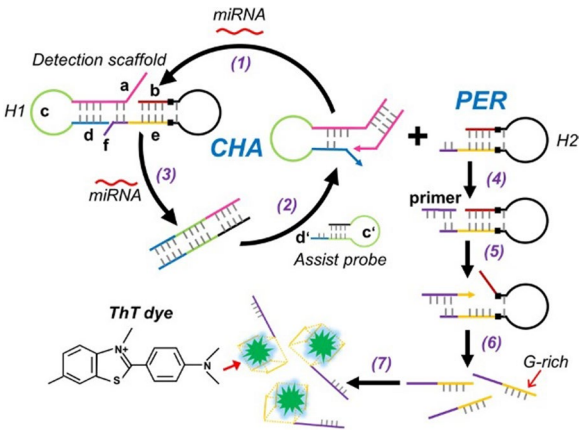
Graphical abstract



Introduction

Gastric carcinoma is the fourth most frequent cancer worldwide and accounts the second major cause of mortality from malignancies, that has a devastating effect on health globally (Schinzari et al. 2014; Smyth et al. 2020; Song et al. 2017). The mortality of gastric carcinoma will be significantly reduced by early diagnosis (Gillen 2021; Hu et al. 2018). microRNAs (miRNAs) are short, noncoding RNAs of 18–25 nucleotides that play a significant role in posttranscriptional gene suppression (Alessandrini et al. 2018; Shin and Chu 2014). Previous research has shown that miRNAs are closely associated with cell proliferation, invasion, metastasis, and angiogenesis in cancer development (Hill and Tran 2021; He et al. 2020). miRNAs have been recognized as cellular modulators that play a significant part in the diagnosis, prognosis, and treatment of gastric carcinoma based on their functions and mechanistic insights (Cuellar-Gomez et al. 2021; Hu et al. 2019). Therefore, development of a reliable approach for sensitive quantitative determination of miRNA is in urgent demand.

Currently, qRT-PCR, which is based on the thermal-cycle based chain reaction, remains the one of the most classical methods for miRNA detection and is the gold standard in clinical practice (Abel and Rederstorff 2021; Ban and Song 2022). The qRT-PCR is sensitive, but the procedure is time-consuming and involves complex primer design, heavy equipment, and knowledgeable trainers. On the basis of multiple signal transduce modes, including fluorescence (Wang et al. 2020; Guo et al. 2020; Zhao et al. 2020), colorimetry (Hosseinzadeh et al. 2020; Shahsavar et al. 2022), Raman scattering spectroscopy (Dong et al. 2022; Kim et al. 2022), and electrochemistry (Wang et al. 2021; Bai et al. 2020), numerous techniques have recently been developed for miRNA detection with higher sensitivity. These methods have made considerable progress to the qRT-PCR method to some extent by exploiting different signal amplification strategies. Among them, the fluorescent assay integrating with different signal amplification strategies has gained abundant attention due to its features of easy-to-operate and stable signal output (Fang et al. 2023). For example, Xuguo Luo et al. proposed a method for sensitive miRNA detection by integrating target recognition assisted activation of split-DNAzyme and primer exchange reaction (PER). Xianxian Zhao et al. developed a novel strategy in which rolling circle amplification (RCA) was utilized for target recognition and CRISPR-Cas12a system was used for signal generation (Guo et al. 2020). Although the above techniques executed favorable miRNA analysis, they lack enough sensitivity to detect minute amounts of miRNA and require fluorescent moiety labeled probes that are easily influenced by reaction conditions in order to generate signals, and thus adding complexity, costs, and the possibility of false positive results to these techniques. Therefore, development of a novel approach that is capable of detecting miRNA in a label-free and sensitive manner will greatly facilitate the development of diagnostic tools.

As a promising technique for isothermal signal amplification, PER has been widely applied in constructing miRNA detection approaches in recent years due to its unique capability to product single-strand DNA (ssDNA) sequences (Li et al. 2020; Huang et al. 2023). However, the signal amplification efficiency of PER cannot meet the high requirements of low abundance target

amplification that greatly limited the wide application of PER based approaches. Integration with signal amplification strategy is possibly to improve the efficiency of PER. Herein, we proposed a novel fluorescent sensing platform by integrating catalytic hairpin assembly (CHA), an enzyme-free, high-efficiency, and isothermal amplification method, with PER for signal amplification, and using a commercial fluorescent dye to generate fluorescence signal in a label-free mode.

Experimental section

Materials and reagents

All the oligonucleotide sequences used in this research are listed in Additional file 1: Table S1. The sequences were synthesized and purified by Sangon Biotech. Co. Ltd. (Shanghai, China). Thioflavin T (3,6-dimethyl-2-(4-dimethylaminophenyl)benzo-thiazolium cation, ThT) and DNA polymerase (phi29 enzyme) were obtained from Sigma-Aldrich (Shanghai, China). PBS buffer solution, commercial serum solution, BSA and diethylpyrocarbonate (DEPC)-treated water (DNase, RNase free) were obtained from New England Biolabs (NEB, Beijing, China). Deoxyribonucleoside 5′-triphosphate mixture (dNTPs) was bought from Beyotime Institute of Biotechnology (Shanghai, China).

Assembly of detection scaffold

Assembly of detection scaffold: 10 μL H1 probe (25 μM) was mixed in the tube containing 40 μL PBS buffer. The mixture was then heated to 90 °C for 10 min and was annealed to room temperature (25 °C). In the same method, 10 μL H2 probe (25 μM) was also pre-treated by heating to 90 °C for 10 min and was cooled to room temperature (25 °C). 10 μL pre-treated H1 probe (25 μM) and 10 μL pre-treated H2 probe (25 μM) were the mixed (concentration ratio of H1 and H2 probe was 1:1), and the mixture was incubated at room temperature for 30 min.

Feasibility of detection scaffold: 10 μL FAM labeled detection scaffold was added to the tube containing 10 μL miRNA-21 (25 μM). The mixture was incubated at room temperature for 30 min, and the fluorescence signal was recorded. Afterward, 10 μL assist probe was added to the mixture and was incubated at room temperature for 30 min. The fluorescence signals were detected by Shimadzu RF-5301PC fluorescence spectrometer.

Analytical performance of the approach

Ten microliters of detection scaffold, 10 μL assist probe (5 μM), and 10 μL different concentrations of miRNA-21 were mixed in the tube containing 20 μL PBS buffer. The mixture was incubated at room temperature for 20 min. Afterward, 10 μL primer sequences, 2 μL DNA polymerase, 2 μL of ThT (5 μM), and 4 μL dNTPs (0.25 mM) solution were mixed, and the mixture was incubated at room temperature for 30 min. The fluorescence signals were detected by Shimadzu RF-5301PC fluorescence spectrometer.

Results and discussion

The working mechanism of the established approach for miRNA detection

The working mechanism of the established approach is illustrated in Fig. 1. In this method, a detection scaffold is constructed by the hybridization between H1 probe and H2 probe. Particularly, the H1 probe contains three functional sections, including the **a** section for target recognition, **c** section and **d** section to induce CHA process; the three functional sections of H2 probe include the **b** section for chain replacement, **e** section to transcribe G-rich sequences, and **f** section to provide binding site for primer. In the existence of target miRNA (miRNA-21), it can bind with the **a** section in the detection scaffold and gradually dissociates H1 probe from the H2 probe, exposing the **d** section and **c** section. The **d** section and **c** section in the H1 probe bind with the **c'** section and **d'** section in the assistance probe to release the miRNA-21. The liberated miRNA identifies the **a** section in a next detection scaffold and disassociates H1 probe from H2 probe to form a signal recycle (CHA). Meanwhile, the H2 probe induces PER to transcribe G-rich sequences. In PER process, a primer sequence binds with the **f** section in the H2 probe and a ssDNA chain is attached to the 3' terminal of the primer under the assistance of the DNA polymerase. The chain extension process is stopped by the halt site, and the extended primer containing transcribed G-rich sequence is liberated from the H2 probe due to the random walk process of three-way branch migration. The G-quadruplex, a unique secondary structure that is fold by G-rich sequence, may be recognized by the fluorescent dye Thioflavin T (ThT). Following G-quadruplex recognition, ThT generates fluorescence signals with much reduced background and an enhanced signal-to-noise ratio.

Fluorescent assays to test the feasibility of the CHA and PER process

The CHA process is crucial in recognizing target miRNA and inducing PER for signal amplification. Therefore, we performed a fluorescent assay to test the feasibility of CHA and its capability in recognizing target miRNA. The mechanism of the fluorescent assay is illustrated in Fig. 2A. In the fluorescent assay, the two terminals of the H1 probe were labeled with fluorescent moiety (FAM) and corresponding quenching moiety (BHQ). After the linear H1 probe was annealed to hairpin structure, the FAM signal was quenched by BHQ. in the present of

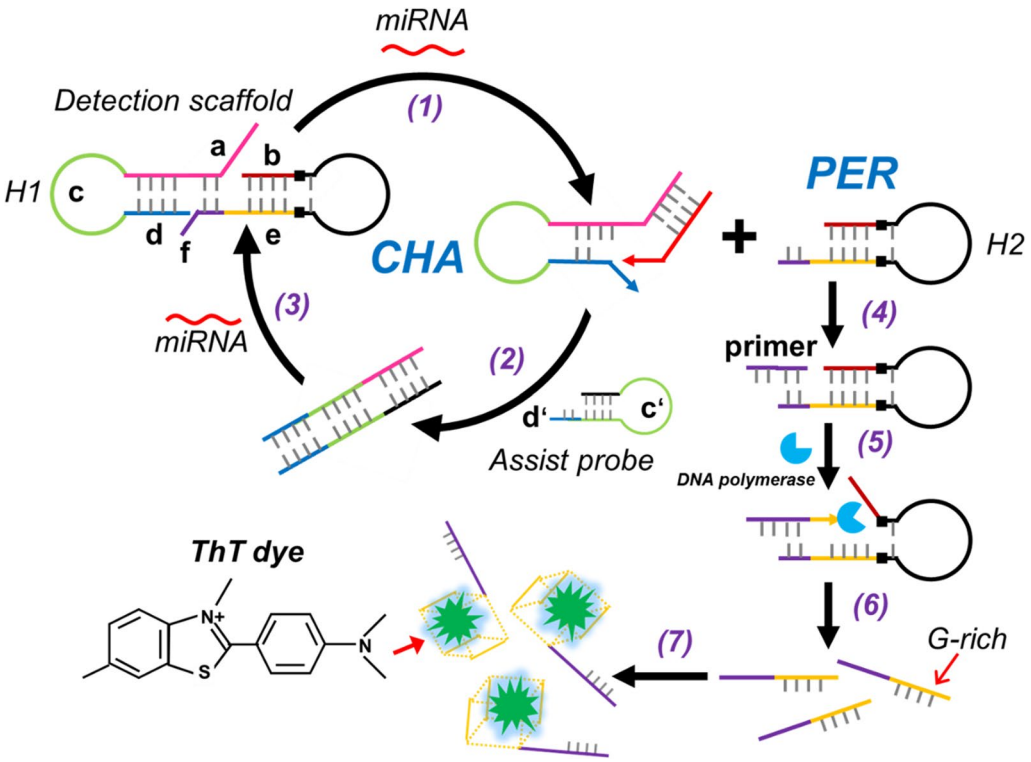


Fig. 1 The working mechanism of the approach that is established by integrating CHA with PER

target miRNA, it identified the **a** section in H1 probe and gradually unfolded H1 probe. As shown in Fig. 2B, the recorded FAM signal when target miRNA existed in the sensing system (green line) was almost 4.53 times higher than that when target absent (blue line), indicating a large amount of FAM labeled H1 probe was unfolded by miRNA. Upon the addition of assist probe, the recorded signal elevated significantly, implying that the construction of CHA process endows the method a higher signal amplification efficiency. The feasibility of PER process could be demonstrated by the recording of the ThT signal. In the PER process, primer sequences hybridized with the **f** section in the H2 probe to induce chain extension under the assistance of DNA polymerase. The result in Fig. 2C showed a significantly enhanced ThT signal when primer and DNA polymerase existed simultaneously in the sensing system, while the recorded ThT signals when primer or DNA polymerase absent were in the same level with blank group.

Optimization of experimental conditions

Above, we have demonstrated the feasibility of the established approach in analyzing miRNA. We have then optimized several experimental conditions, including the concentration of DNA polymerase, and incubation time, for a better detection performance. The DNA concentration is crucial in the chain extension process. Therefore, we investigated the detection performance when the approach was performed with different concentrations of DNA polymerase. The result in Fig. 3A showed that the recorded ThT signal elevated with the DNA polymerase concentration increased from 0.1 U/L to 0.5 U/L, and no more enhancements could be observed when the sensing system was incubated with more DNA polymerase. Therefore, 0.5 U/L DNA polymerase was selected to be applied in the following experiments. In addition, the incubation time was determined 60 min from the result in Fig. 3B.

Analytical performance of the established approach

Under the optimized experimental parameters, the proposed approach was applied to detect different concentrations of miRNA-21 and the sensitivity of the approach was evaluated. As expected, the recorded ThT signal elevated with the concentration of miRNA-21 increased from 100 aM to 100 pM (Fig. 4A). A good linear correlation between the ThT signal and the logarithmic concentrations of miRNA-21 was observed as shown in Fig. 4B. The correlation equation between the ThT signal and the logarithmic concentrations of miRNA-21 was

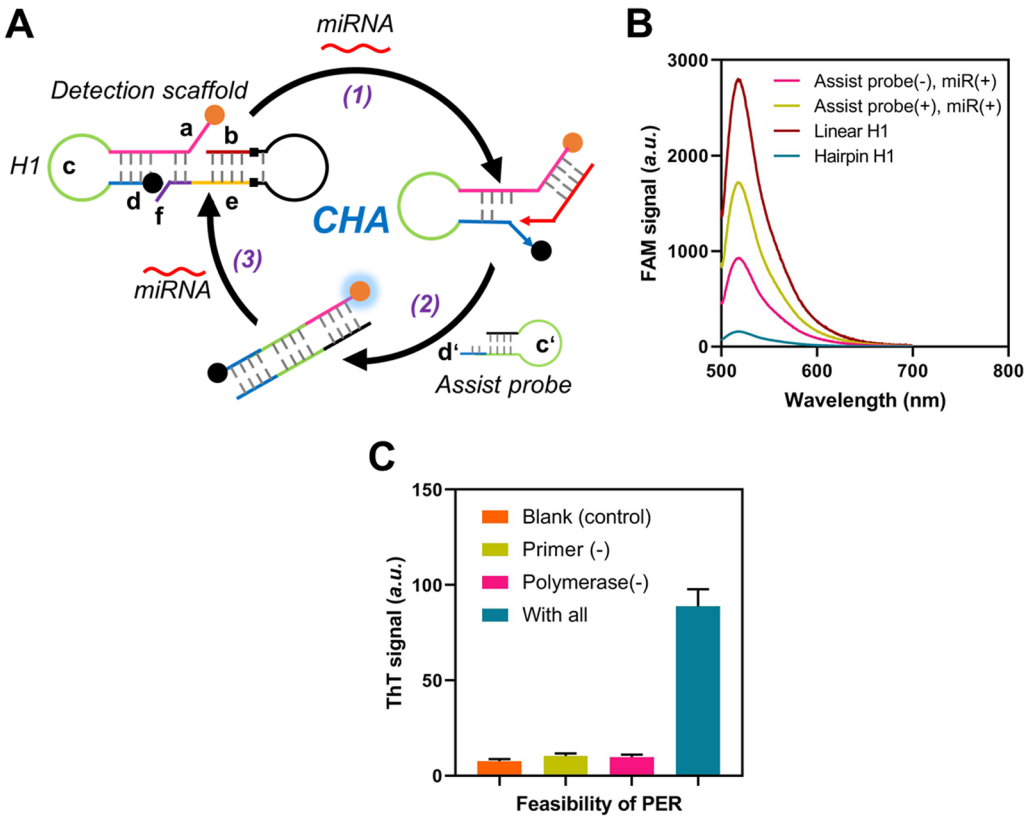


Fig. 2 Feasibility of CHA and PER process. **A** Schematic illustration of the fluorescent assay to test the feasibility of CHA. Linear H1 was the H1 probe before being annealed to hairpin structure, Hairpin H1 probe was the H1 probe that was assembled to hairpin structure. **B** Fluorescent spectrum of the FAM labeled H1 probe during the CHA process. **C** ThT signal after PER when primer and DNA polymerase existed or not

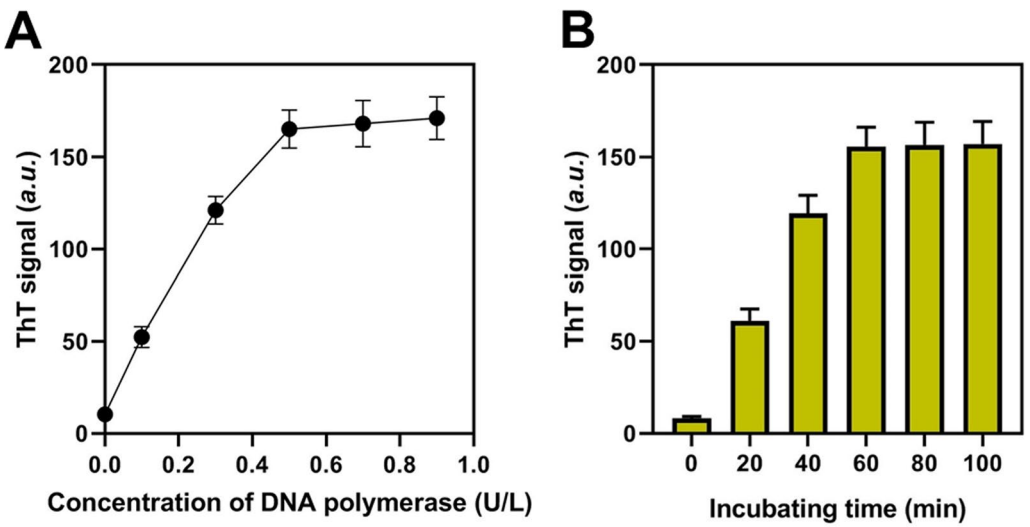


Fig. 3 Optimization of experimental parameters. ThT signals of the approach when detecting 10 Nm miRNA-21 with different concentration of DNA polymerase (**A**) and different incubation time (**B**)

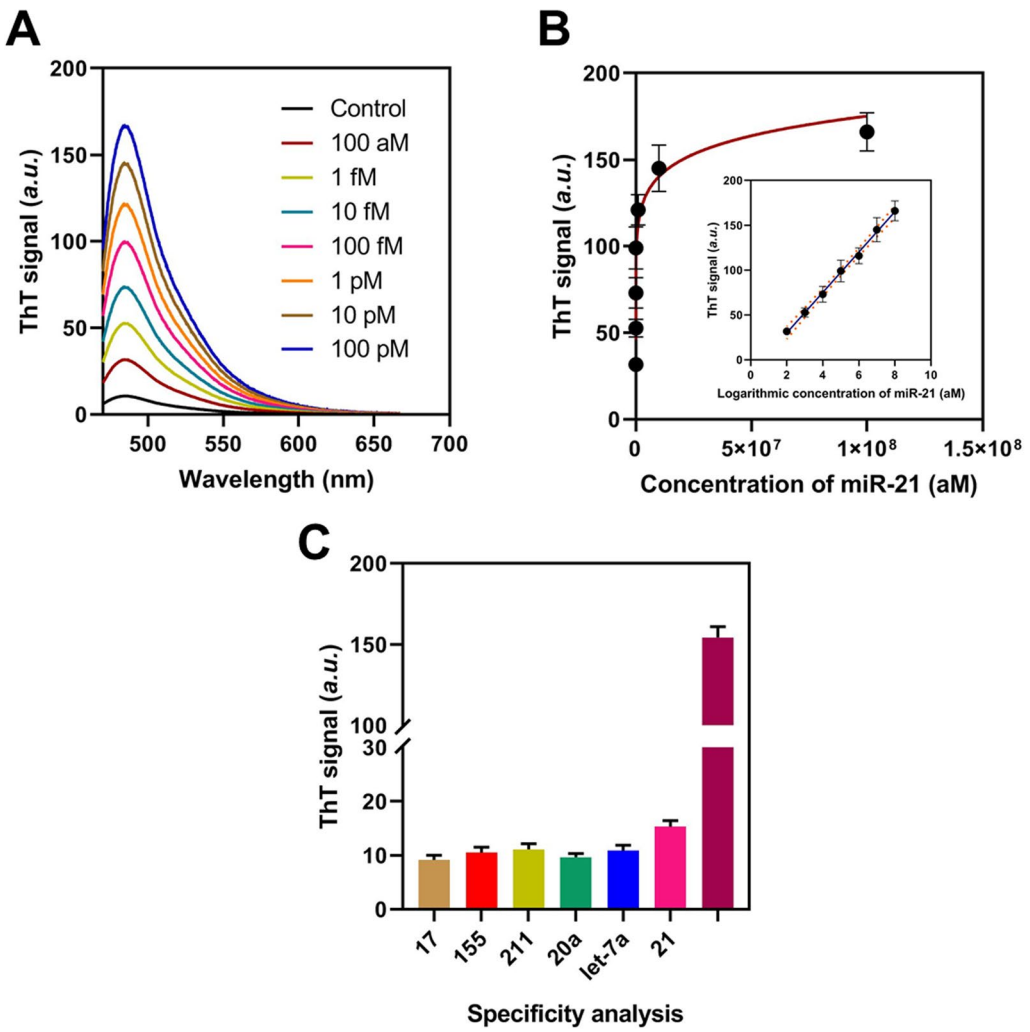


Fig. 4 Analytical performance of the established approach. **A** Fluorescent spectrum of the approach when detecting different concentrations of miRNA-21. **B** correlation between the ThT signals and concentrations of miRNA-21. **C** ThT signals of the approach when detecting different miRNAs

Y = 22.52*lgC-14.99, with the correlation coefficient of 0.9912. The limit of detection was as low as 51 aM, which is comparable or superior to most of the established label-free miRNA detection approaches.

Specificity is a crucial metric for assessing the performance of biosensors. The high sequence homology between miRNAs made it difficult to be identified. To test the specificity of the established approach, several distinct miRNAs, including miRNA-17, miRNA-155, miRNA-211, miRNA-20a, and let-7a, were examined as controls under the same experimental circumstances. The main difficulty in detecting miRNAs is their sequence homology, even down to single nucleotide differences. Many distinct miRNAs, including miRNA-17, miRNA-155, miRNA-211, miRNA-20a, and let-7a, were examined as controls under the identical experimental circumstances in order to assess the specificity of our approach. According to the experimental result in Fig. 4C, the proposed method effectively distinguishes target miRNA from the interfering ones, indicating a high selectivity of the established approach.

Stability and repeatability of the approach

Apart from the sensitivity and selectivity, many other indicators are crucial in evaluating the detection performance of an approach. To investigate the stability of the approach, miRNA-21 was diluted to 10 pM by different solutions, including the commercial serum, PBS buffer, DEPC water ((diethylpyrocarbonate-treated water) and BSA solution. As shown in Fig. 5A, no significant differences could be observed among the recorded ThT signals in the four samples, indicating a high stability of the approach. We then evaluated the applicable potential of the approach in analyzing miRNA-21 from clinical

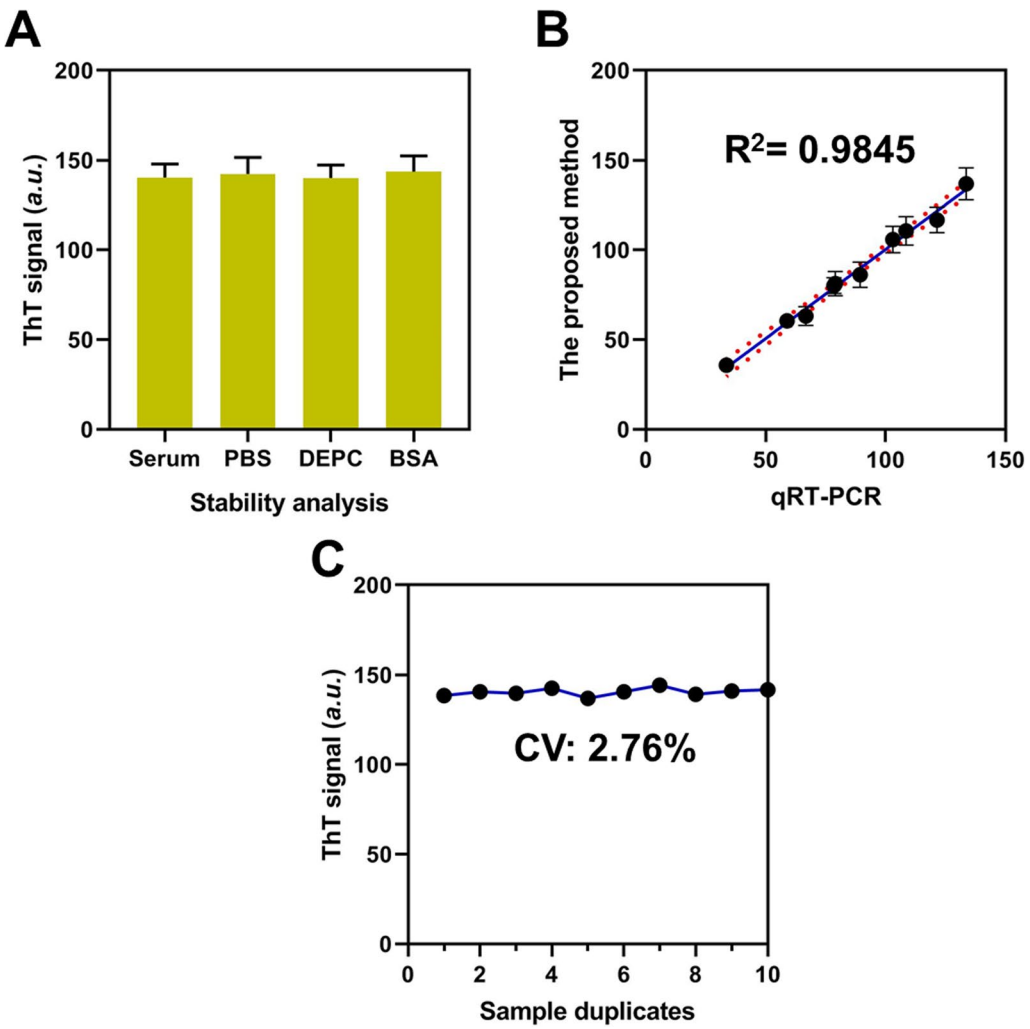


Fig. 5 Stability and repeatability of the approach. **A** ThT signals of the approach when detecting miRNA-21 from different buffer solutions. **B** correlation between the calculated miRNA-21 concentration by the method and by qRT-PCR. **C** ThT signals of the approach when detecting miRNA-21 from 10 sample duplicates

samples. The samples were prepared by diluting miRNA to different concentrations by commercial serum solution. Both the proposed approach and qRT-PCR method were utilized in quantifying miRNA-21 in these samples. The result in Fig. 5B showed the calculated miRNA-21 concentration by the proposed approach was highly consistent with the concentrations calculated by qRT-PCR. To test the repeatability of the approach, 10 sample duplicates were prepared, and the approach was utilized to detect miRNA-21 in the sample duplicates. The calculated CV (coefficient of variation) value from the result in Fig. 5C was 2.76%, implying a high repeatability of the approach.

Conclusion

In summary, we proposed a novel method for the ultrasensitive detection of miRNA by integrating CHA and PER. In this method, a detection scaffold is constructed by hybridization of two hairpin structure probes (H1 and H2). The detection scaffold is capable of specifically identifying target miRNA and inducing CHA for the first signal recycle. The liberated H2 probe works as a template to initiate the PER process under the cooperation of primer sequence and DNA polymerase. The PER process transcribes numerous G-rich sequences that can fold into G-duplex to induce ThT based signal generation. Based on the elegant design of signal amplification processes and label-free signal generation based on ThT, the approach exhibited a wide detection range of 7

orders of magnitudes and a low limit of detection in aM level. In addition, it was also proved that the proposed approach possesses a high stability and repeatability (CV, 2.76%), demonstrating considerable promise for both clinical diagnostics and the development of anti-cancer drugs.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-023-00387-0.

Additional file 1. Table S1: details of the sequences used inthis research.

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Author contributions
XL and YC are the supervisors of the team in all research steps including designing, data analysis and manuscript writing. HZ and TL, as the co-first authors, have the main role for experimental data collection, data gathering, preparation of results, and data analysis. JW, ML, YL, XT, and RW assist the data analysis.

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Availability of data and materials
Almost all details of experimental data are presented in the article or additional file.

Declarations

Ethics approval and consent to participate
The manuscript does not contain clinical or trial studies on patients, humans, or animals.

Competing interests
The authors declare that they have no competing interests.

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