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Simple, sensitive, and label-free miRNA analysis through strand displacement reaction integrating with G-quadruplex-based signal generation

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Abstract

MicroRNAs (miRNAs) play a significant role in the pathogenesis of various diseases throughout biological processes, and the accurate detection of miRNA biomarkers holds great potential for early stage disease diagnosis and treatment. In this study, a novel method is developed to detect miRNA-21, a biomarker for drug-induced liver injury, by combining target sequence recycling with G-quadruplex-based signal production. This approach is highly sensitive and does not require the use of labels. The target sequence facilitates the cyclic exposure of G-rich regions in the detection probe by toehold-mediated strand displacement processes, with the aid of the catalytic chain. The G-quadruplex sequences that have been produced subsequently interact with thioflavin T (ThT), resulting in a significant increase in its fluorescence intensity. This enhanced fluorescence is utilized for the purpose of detecting miRNA-21, with a remarkably low detection limit of 4.4 fM. The suggested technique also allows for the very specific identification of the target miRNA-21. Due to its non-label format, excellent selectivity, and sensitivity, this technology presents a straightforward and versatile approach for detecting a wide range of biomarkers in the early phases of illness detection.

Keywords MicroRNAs (miRNAs), G-quadruplex, Thioflavin T (ThT), Strand displacement reaction, Nonalcoholic fatty liver disease (NAFLD)

Introduction

Nonalcoholic fatty liver disease (NAFLD) affects a significant proportion, specifically more than 33%, of the population and exhibits associations with several metabolic disorders (Pafili and Roden 2021; Polyzos et al. 2019; Younossi et al. 2019). The word comprises a broad range of illnesses, starting from mild steatosis to nonalcoholic steatohepatitis, fibrosis, and, eventually, cirrhosis, which may lead to the formation of hepatocellular cancer. At present, the existing diagnostic approaches for nonalcoholic fatty liver disease (NAFLD) either involve invasive procedures or suffer from limited accuracy (Papatheodoridi and Cholongitas 2018). Additionally, assessing the effectiveness of therapy interventions for NAFLD poses significant challenges in terms of monitoring.

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MicroRNAs (miRNAs) are a class of short non-coding RNA molecules ranging from 18 to 24 nucleotides in length. MicroRNAs (miRNAs) have the ability to attach to target messenger RNAs (mRNAs) and play a significant role in a wide array of biological activities, with a special emphasis on intercellular communication (Fang et al. 2021; Hochreuter et al. 2022). Multiple studies indicate that miRNAs play a crucial role in the transfer of miRNAs, hence presenting promising clinical applications for the diagnosis, prognosis, and treatment of liver disorders (Long et al. 2019; Mahmoudi et al. 2022). Nevertheless, the challenge of achieving high-performance monitoring of miRNA arises from several factors, including the great similarity of sequences, low concentration, and susceptibility to degradation.

Traditional methods for miRNA detection have included reverse transcription polymerase chain reaction (RT-PCR) (Takei et al. 2020; Xu et al. 2019), northern blotting (Varallyay et al. 2008; Zia and Flynt 2018), and microarrays (Jin and Xu 2021; W. Li and Ruan 2009). Despite the widespread success of these methods in areas like fundamental medical study and early clinical application, miRNA detection with high sensitivity remains a technical obstacle. Although RT-PCR has a low detection threshold and good specificity, designing PCR primers for miRNA is challenging because of their short length (usually 22–25 nt). Northern blotting is a common method for detecting microRNA, but it has drawbacks, including low sensitivity and the need for potentially dangerous radioactive isotopes. Current microarray technology can be utilized for high-throughput detection, but it is expensive and time-consuming to use (Cheng et al. 2018). Therefore, it is critical to develop a simple yet effective detection method for the speedy identification of miRNA.

Integration of isothermal amplification with other signal transducer technologies has allowed for highly accurate and precise miRNA identification in recent years (Ma et al. 2017; Mader et al. 2012). Instances include electrochemiluminescence (ECL) (M. Y. Wang et al. 2023a, b), surface-enhanced Raman scattering (SERS) (D. Li et al. 2020), and fluorescence (FL) (Wang et al. 2020; Zhang et al. 2020). Because of its great sensitivity, rapid detection, and excellent repeatability, fluorescent methods that incorporate with different types of isothermal signal amplification have received a lot of attention, isothermal amplification methods such as the strand displacement reaction (SDR) (Zhou et al. 2021), enzyme-assisted amplification (Qian et al. 2019), and hybridization chain reaction (Wang et al. 2022). Because of its isothermal and enzyme-free properties, SDR is a prototypical example of isothermal amplification. Toeholds are DNA strands that can be enrolled in with an invading strand to initiate branch migration, and SDR typically progresses via branch migration in threes or fours. Nonetheless, SDR's amplifying effect leaves much to be desired. The experimental complexity, cost, and risk of false positive results are all increased since the fluorescence assays were developed using expensive and meticulously engineered dual-labeled nucleic acid probes that are easily impacted by reaction conditions (such as pH). Thus, there is an immediate need for the development of a label-free approach with comparable or superior detection sensitivity and specificity. G-quadruplex, a specific type of four-stranded DNA, can interact with the tiny chemical molecule Thioflavin T (ThT) to produce extremely bright fluorescence (X. Li et al. 2019; Q. Wang et al. 2023a, b). Its low background and high fluorescence quantum yield make it ideal for use as a label-free fluorescence biosensor, which eliminates the requirement for fluorescent labeling.

This work presents a newly developed label-free fluorescent sensing platform that combines via strand displacement reaction with G-quadruplex-based signal production. The platform demonstrates high sensitivity in the detection of miRNA-21. The method involves the construction of a detection probe through the hybridization of a hairpin probe (referred to as H probe) with the "5" sequence, as depicted in Fig. 1. Target miRNA-21 binds to the toehold region at the 3′-terminus of the "1" section to generate a novel complex (miRNA-21/H probe), which can then activate the strand displacement reaction integrating with G-quadruplex-based signal production. Simultaneously, a novel region referred to as Toehold 2 becomes accessible, enabling its hybridization with a catalytic chain, thereby liberating miRNA-21 and the "4" segment. Subsequently, the miRNA-21 that has been displaced persists in initiating a further recycling process targeting a novel entity, resulting in the exposure of the "3" portion, which comprises a G-rich region. The formation of an intact endonuclease recognition site occurs through the hybridization of the catalytic chain with the H probe. Within this site, the "2′" segment can be cleaved by the endonuclease, resulting in the formation of a nicking site. With the aid of DNA polymerase, a prolonged chain is appended to the "7" segment, while the "6" sequence, which possesses identical nucleic acids to miRNA-21, is liberated. The "6" sequence that has been released forms a binding interaction with a subsequent detection scaffold, so initiating a subsequent signal cycle. This interaction results in the exposure of multiple G-rich sequences. Hence, the targeting of a single miRNA can result in the exposing of several G-rich regions. Subsequently, the G-quadruplex structures, which effectively include ThT molecules resulting

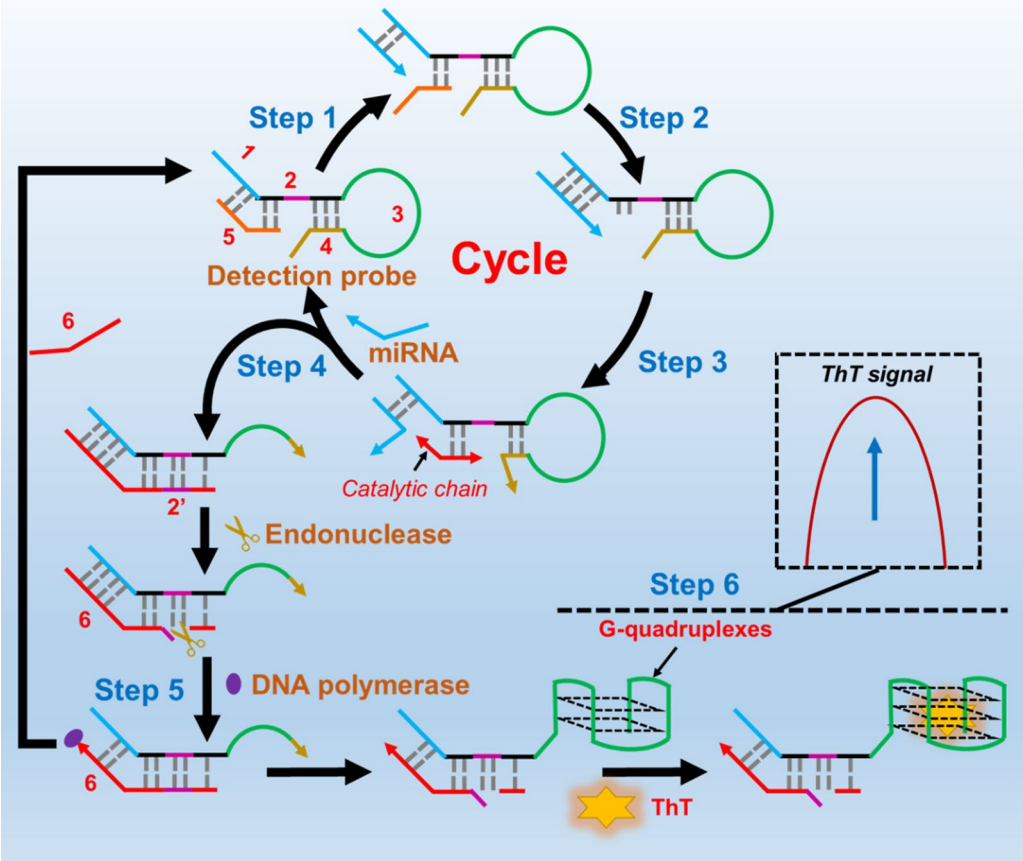

Fig. 1 The principle of strand displacement reaction method integrating with G-quadruplex-based signal generation for non-label and fluorescent detection of miRNA-21

in amplified fluorescence signals, are employed for the purpose of detecting miRNA-21 with a notable degree of sensitivity.

Materials and methods

Reagents and materials

The following chemicals were purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China): Tris–HCl, $MgCl_2$, KCl, and NaCl. ThT and normal human serum were both generously donated by Sigma-Aldrich (St. Louis, MO). New England Biolabs, Inc. (Beijing, China) supplied the Bst-DNA polymerase, Nb.BbvCI, and the Bst reaction buffer. Huzhou Hippo Biotechnology Co., Ltd. (Zhejiang, China) produced all the oligonucleotides (Additional file 1: Table S1).

Amplified sensing of miRNA-21

Detection probe for sensing was prepared by mixing H probe and "5" sequence in a 2:1 vol ratio in TE buffer, annealing for 5 min at 95 °C, and then cooling to 25 °C before the tests were performed. Then, the detection probe (50 nM), different concentrations of miRNA-21, catalytic chain (100 nM), 1 Bst reaction buffer, Bst DNA polymerase (5 U), endonuclease (10 U), and dNTP (500 mM) were mixed together and put into a 20 mM Tris–HCl reaction buffer (10 mM KCl, 12 mM $MgCl_2$,100 mM NaCl, pH 7). The resulting mixture was held at 80 °C for 20 min before being cooled to room temperature. After a further 40 min of incubation with the ThT dye (10 mM), the mixture was diluted with Tris–HCl solution and the fluorescence was recorded. The serum was diluted 10 times with buffer before miRNA-21 was introduced and analyzed for use in a recovery assay.

Results and discussion

Construction of the detection scaffold and feasibility of the method

In order to validate the fabrication of the detection probe, a fluorescence assay was conducted, as depicted in Fig. 2A. In order to evaluate the assembly between the H probe and the "5" sequence, the 3′ end of the H probe was labeled with Cy3, while the 5′ end of the "5" sequence was tagged with BHQ. Prior to assembly, the Cy3 signal of the H probe exhibited a high intensity

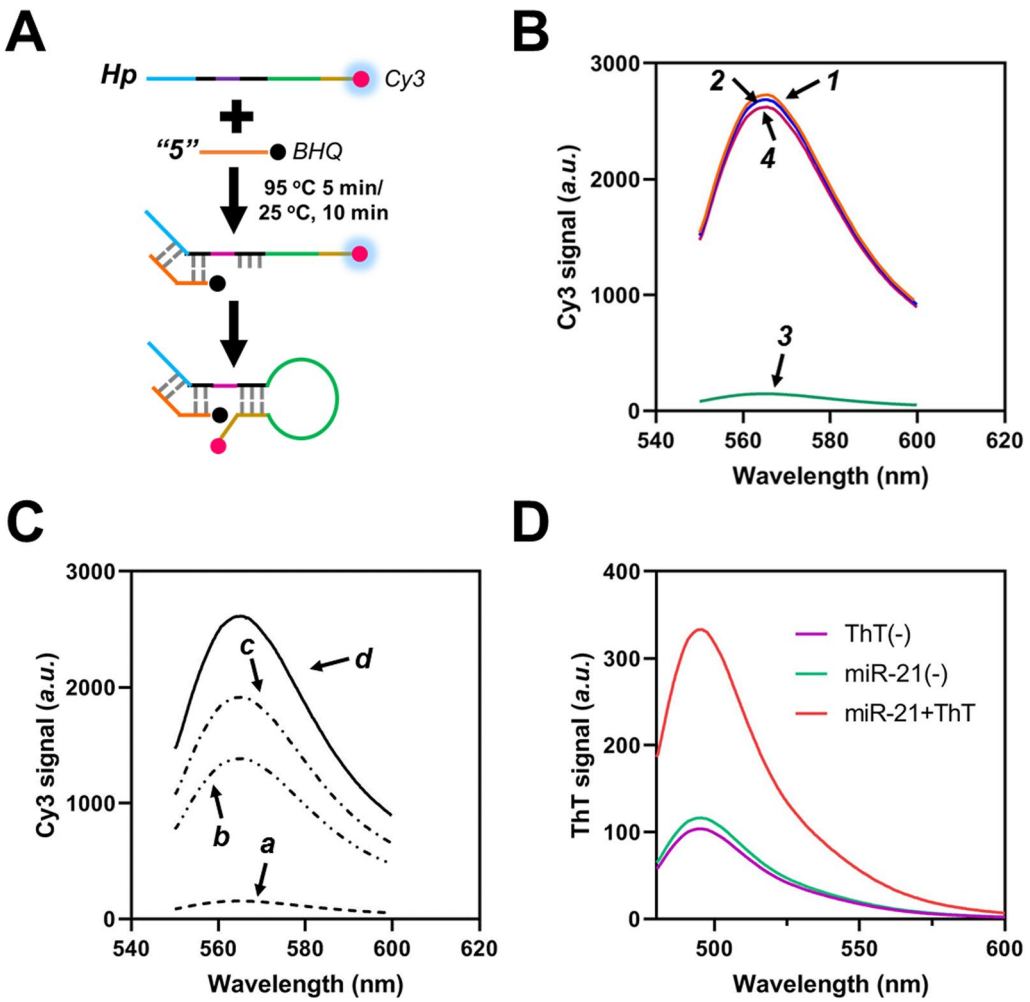

Fig. 2 Feasibility study of the established approach. **A** Schematic illustration of the fluorescence assay to test the feasibility. **B** Fluorescence spectrum of the Cy3 labeled hairpin probe (H probe, Hp) during the construction of the detection probe. curve 1: Cy3 labeled H probe (linear), curve 2: Cy3 labeled H probe (assembled to hairpin structure), curve 3: Cy3 labeled H probe (assembled to hairpin structure) + BHQ-labeled "5" sequence, curve 4: Cy3 labeled H probe (linear) + BHQ-labeled "5" sequence. **C** Fluorescence spectrum of the Cy3 labeled H probe during the target recycling process. Curve a: detection scaffold, curve b: detection scaffold + miRNA, curve c: detection scaffold + miRNA + catalytic chain, curve d: detection scaffold + miRNA + catalytic chain + DNA polymerase. **D** ThT signals of the approach when miRNA-21 and ThT existed or not

(curve 1). Subsequently, the H probe was subjected to thermal treatment at a temperature of 90 °C for a duration of 10 min, followed by a cooling process to attain room temperature. This thermal manipulation resulted in the formation of the hairpin structure, as indicated by curve 2. The Cy3 signal exhibited a significant drop after the BHQ-labeled "5" sequence was immobilized on the H probe. This decrease can be attributed to the quenching effect of BHQ on the Cy3 signal. This observation suggests that the detection scaffold was effectively assembled, as demonstrated by the recorded signal in curve 3. Furthermore, it was observed that the Cy3 signal exhibited sustained elevation in cases where the H probe was not subjected to hairpin structure assembly prior to the construction of the detection scaffold (curve 4). The aforementioned findings provide robust evidence for the effective assembly of the detecting scaffold, as illustrated in Fig. 2B. The signal cycle was then tested by using the assembled detection scaffold. As shown in curve a in Fig. 2C, the fluorescent signal of detection scaffold was low. In the presence of the target miRNA, the Cy3 signal was shown to be amplified as a result of the release of the BHQ-labeled "5" sequences from the detection scaffold (curve b). The introduction of a catalytic chain resulted in a significant increase in the observed Cy3 signal, as demonstrated by the higher curve c. The observed phenomena can be elucidated by the involvement of a catalytic

chain that facilitates the recycling of the target, hence amplifying the fluorescence signals. The coexistence of DNA polymerase and endonuclease in the sensing system resulted in a significant increase in the Cy3 signal. Specifically, the signal was found to be 1.47 times greater compared to curve c (curve d). The aforementioned findings provide evidence for the efficacy of the strand displacement response in facilitating target recycling, as depicted in Fig. 2C. The process of signal production using G-quadruplex is illustrated in Fig. 2D. In the presence of both target miRNA and ThT within the sensing system, a discernible increase in the ThT signal was seen, demonstrating a statistically significant difference compared to the absence of miRNA-21 in the system.

Optimization of experimental parameters

The aforementioned trials suggest that the ability to detect miRNA is critical to the success of our proposed technique. In order to optimize the performance of the method, we conducted an investigation into many experimental parameters. These factors encompassed the concentration of the catalytic chain, the duration of the enzyme reaction, the quantities of the enzymes (endonuclease and DNA polymerase), as well as the quantity of ThT. The optimization findings are presented in Fig. 3, where F and F0

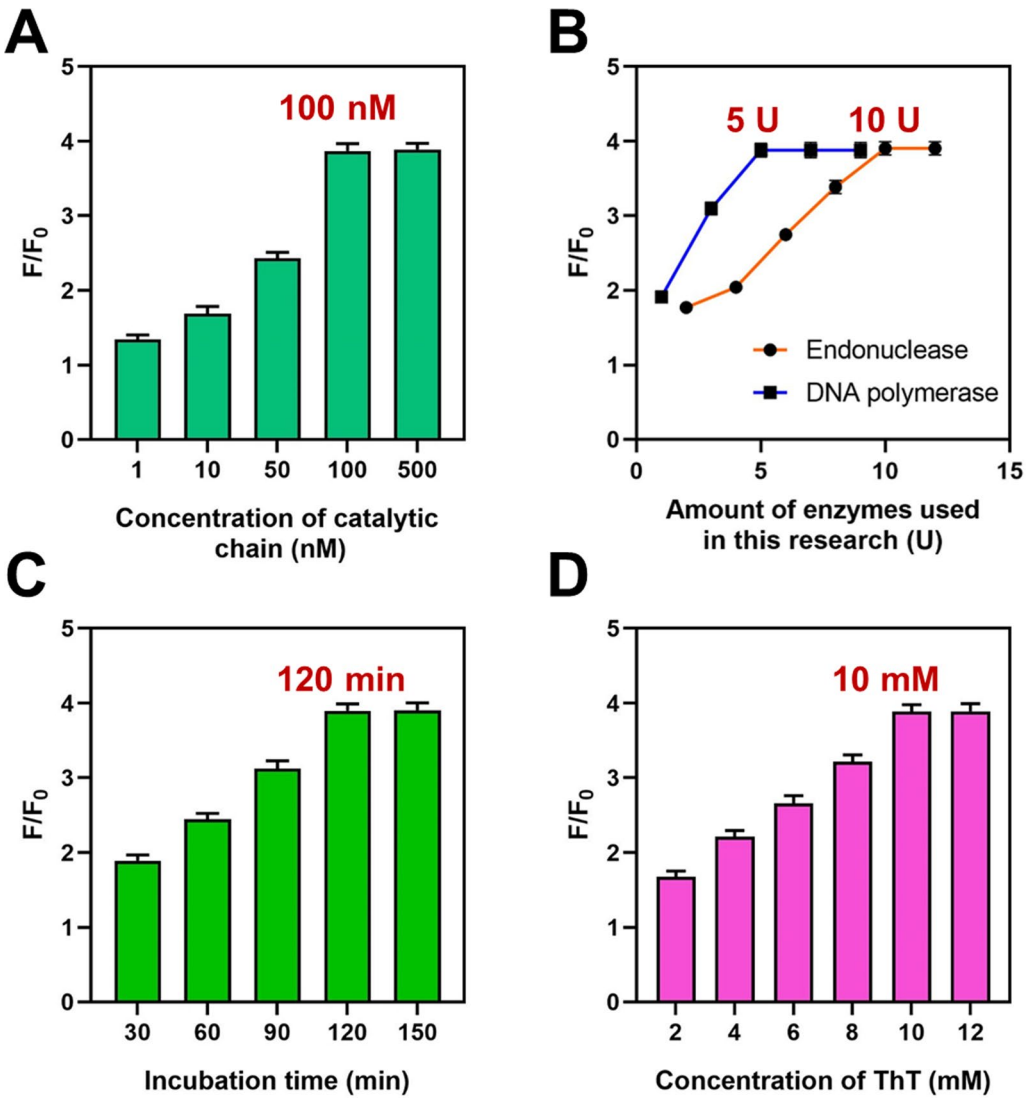

Fig. 3 Optimization of experimental parameters. F/F0 of the approach with different concentration of catalytic chain (nM) (**A**), amounts of enzymes (**B**), incubation time (**C**), and ThT concentration (**D**)

denote the fluorescence intensity of the reaction mixture at 560 nm in the presence and absence of 1 nM of miRNA-21, respectively. The analysis of the concentration of the catalytic chain was initially conducted. As illustrated in Fig. 3A, the fluorescence response exhibited a steady increase with the rise in the concentration of the catalytic chain, eventually reaching a plateau at 100 nM. Hence, it was concluded that a concentration of 100 nM of the catalytic chain yielded the most favorable results. Following the optimization of catalytic chain concentration, the dosage of endonuclease and DNA polymerase were also tuned. The outcomes of this optimization process are presented in Fig. 3B. The fluorescence intensity elevated with the quantity of endonuclease ranged from 2 to 10 U, with a maximum value observed at 10 U. Consequently, the quantity of endonuclease utilized in future trials was 10 U. In the present study, the concentration of the optimized DNA polymerase was determined to be 5 U. The impact of reaction time on the fluorescence response is illustrated in Fig. 3C. The acquired fluorescence response increases quickly as reaction time is extended in the region of 30–120 min, although the change is not immediately apparent after 120 min. The investigation also included an examination of the impact of various concentrations of ThT on the fluorescence emission intensity of the reaction solution (Fig. 3D). In a similar vein, it can be shown that the fluorescence intensity exhibits an upward trend within the examined range of concentrations. This trend culminates in a peak value at 10 mM, signifying the ideal concentration of ThT to be 10 mM.

Detection sensitivity analysis

To investigate the sensitivity of the sensing probes towards the target sequence, a series of experiments were conducted under the best reaction conditions. The concentrations of miRNA-21 ranged from 10 to 10 nM. As depicted in Fig. 4A, the fluorescence response exhibits a gradual increase with the increment in miRNA-21 concentration. Figure 3B presents the calibration graph illustrating the relationship between the fluorescence emission intensity and the logarithmic value of miRNA-21 concentration. The obtained regression equation is $F=45.96^{*}\lg C+120.2$ ($R^2=0.9936$). The calculation of the detection limit is determined to be 4.4 fM using the 3 s standard. The ability to achieve a low detection limit can be due to the autonomous exposure of the G-quadruplex sequence, which is comparable to several other approaches, as indicated in Table 1. This approach offers the advantage of simple and straightforward operation, without the need for complex procedures like sequence labeling or coupling, while still reaching low detection limits.

Selectivity study

Additionally, other miRNAs such as miRNA-211, miRNA-141, miRNA-155, and one- and two-base mismatch miRNA-21 (mis-1, mis-2) were chosen at random as control molecules for selectivity research in order to examine the sequence discrimination capacity of the proposed detection approach for miRNA-21. The measured fluorescence intensities of these reference molecules are depicted in Fig. 5A. Although the concentration of the control molecules (10 nM) is ten times higher than that of miRNA-21 (1 nM), the matching fluorescence intensities

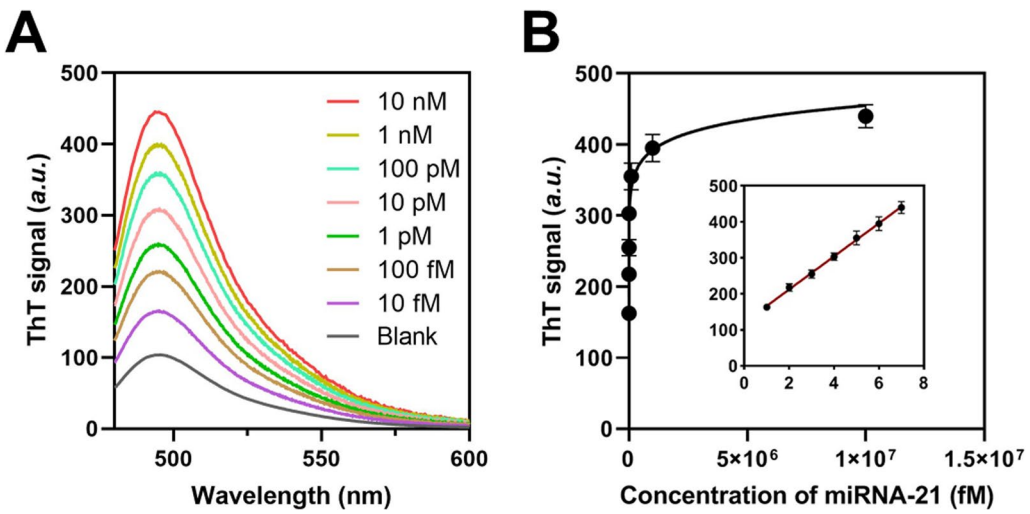

Fig. 4 Sensitivity of the proposed approach. **A** Fluorescence spectrum of the approach when detecting different concentrations of miRNA-21. **B** Correlation between the ThT signals and the concentration of miRNA-21

Table 1 A brief comparison of the approach with former methods

Title	Mechanism	LOD	Labeling	Complexity	Ref
The method	Target recycling + G-quadruplex-based signal generation	4.4 fM	ThT		
Target recycling	Target recycling + PER	49.4 fM	ThT	Two stage signal cycles	Li et al. (2022)
RACE	RCA + CRISPR-Cas9	90 fM	FAM and BHQ	Multiple steps	Wang et al. (2020)
Strand displacement	Strand displacement + Exo-III assisted signal process	25.0 pM	FAM and BHQ	Multiple steps	Nie et al. (2023)

RCA Rolling circle amplification; *PER* Primer exchange reaction; *RACE* Rolling Circular Amplification (RCA)-Assisted CRISPR/Cas9 Cleavage

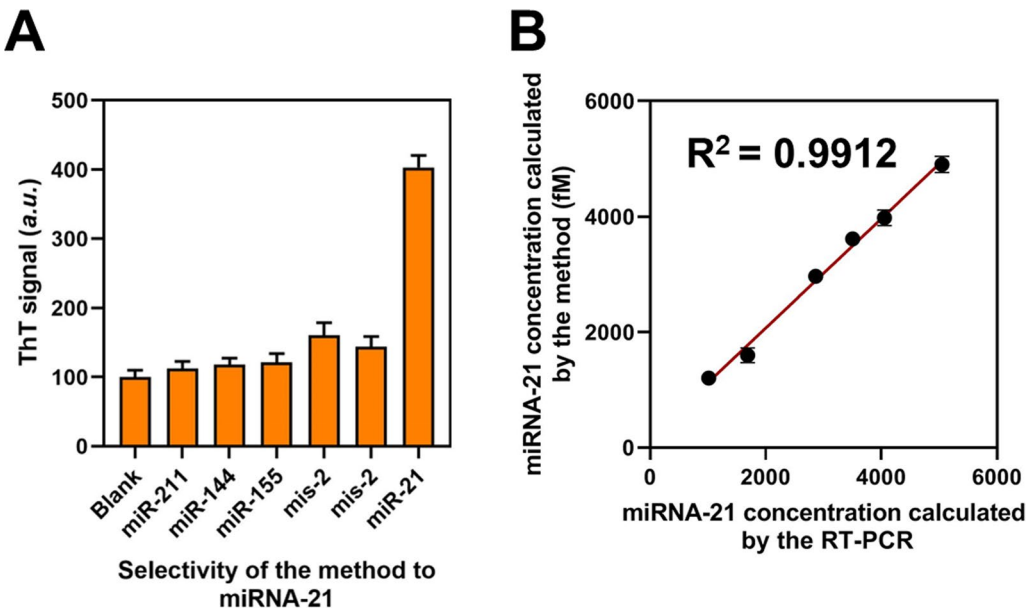

Fig. 5 Selectivity of the proposed approach. **A** ThT signals of the approach when detecting different miRNAs. **B** Correlation between the calculated miRNA-21 by the proposed method and by RT-PCR method

show only slight differences when tested against a blank. However, the fluorescence response was greatly amplified in the presence of the miRNA-21 (1 nM) target molecule in the reaction mixture. Since only miRNA-21 can selectively initiate the catalytic reaction and G-quadruplex-based signal production, our proposed technique has been shown to be highly selective for miRNA-21. The approach's applicability was evaluated by detecting miRNA-21 in 6 samples with varying quantities using both the method and the RT-PCR technique. Figure 5B demonstrates a strong correlation between the two methods' miRNA-21 concentration calculations, providing further evidence that the suggested method has therapeutic promise.

Application for diluted serum samples

Target miRNA-21 at concentrations of 100 fM, 100 pM, and 1 nM was injected into tenfold diluted healthy human serum for recovery tests to evaluate the applicability of the assay technique for human serum diluted by buffer. Table 2 shows that the miRNA-21 recovery rate was between 99.2 and 102.1%, indicating that the suggested approach is able to quantify miRNA-21 in complicated matrix samples.

Table 2 Recovery experiments for miRNA-21 in diluted samples ($n = 3$)

Sample	Added	Calculated	Recovery (%)
1	100 fM	102.1 fM	102.1
2	100 pM	101.3 pM	101.3
3	1 nM	0.9921 nM	99.2

Conclusion

In conclusion, it has been established that easy and label-free high sensitivity detection of miRNA-21 is possible using a signal amplification method based on strand displacement response combining with G-quadruplex-based signal production. Targets as tiny as 4.4 fM can be detected thanks to the synergistic combination of strand displacement reaction-induced target sequence recycling. Furthermore, this approach can finish the detection of miRNA-21 even in diluted complicated samples, demonstrating its good selectivity and highly specific recognition of miRNA-21. This signal amplification method can accomplish the detection of different cancer biomarkers through the acceptable design of sequences, and it has the advantages of simple and easy operation, label-free, and high sensitivity compared to conventional detection methods.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-023-00410-4.

Additional file 1. Table S1: Sequences of oligonucleotides used in this work

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Author contributions

GY is the supervisor of the team in all research steps including designing, data analysis and manuscript writing. HZ, as the first author, has the main role for experimental data collection, data gathering, preparation of results, and data analysis. JL, HH, HW, SQ assisted 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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