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Sensitive and reliable miRNA analysis based on cyclic reverse transcription and CRISPR-Cas12a-assisted signal cycle

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Abstract

MicroRNAs (miRNAs), a category of small molecules that possess significant regulatory capabilities, have been extensively employed as biomarkers in the domain of biosensing to facilitate the early detection of diverse ailments. However, sensitive and accurate miRNA detection remains a huge challenge due to the high similarity between the homologous sequences and low abundance. Therefore, it is essential to develop methods with high sensitivity and specificity for miRNA detection. In this study, we present the development of a signal cycle-based platform that utilizes cyclic reverse transcription (CRT) and CRISPR-Cas12a to enable the precise and sensitive detection of microRNAs. The CRT mechanism facilitates precise target recognition in the presence of target miRNA, thereby converting miRNA signals to DNA signals. The *trans*-cleavage activity of the Cas12a protein is triggered by the formation of complete hairpin-shaped CRT products; this results in the cleavage of the DNA section contained in the H probe, while the RNA section ("4"@MBs) remains loaded onto the surface of magnetic beads (MB). By binding with the "reporter" sensor, the "4" sequences create an RNA/DNA duplex that the duplex-specific nuclease (DSN) can recognize. The "reporter" probe is thus metabolized, leading to the reappearance of the fluorescence signal. By capitalizing on the exceptional fidelity and selectivity of CRISPR/Cas12a, as well as the substantial impact of triggered enzymatic cycle amplification, this approach demonstrated remarkable sensitivity and specificity in miRNA detection, even in a complex environment containing 10% fetal bovine serum (FBS) and a serum sample. In contrast, a detection limit of 3.2 fM is conceivable. Furthermore, this approach maintained a notable degree of stability, which was anticipated to result in the detection of miRNAs in an effective and sensitive manner.

Keywords MicroRNAs (miRNAs), Cyclic reverse transcription (CRT), CRISPR-Cas12a, Duplex-specific nuclease (DSN)

Introduction

In recent years, microRNA (miRNAs) have emerged as potential biomarkers for various diseases (Andersen and Tost 2020; Ramanathan and Padmanabhan 2020; Roy et al. 2023), including cancer and cardiovascular disorders, due to their dysregulated expression being closely associated with the pathological process of diseases. miRNAs are endogenous and noncoding small single-strand RNAs, typically measuring approximately 18–25 nucleotides in length (Iacomino 2023; Lobera et al. 2023). Antiphospholipid syndrome (APS) is an autoimmune disorder that affects the entire body (Sammaritano 2020), and is characterized by the formation of blood clots in the veins and/or arteries, as well as repeated miscarriages, when antiphospholipid antibodies (aPLs) are present (Schreiber and Hunt 2019; Ruiz-Irastorza et al. 2010). APS, similar to other autoimmune disorders, may

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be profoundly influenced by miRNAs, which regulate the function of innate and adaptive immune responses, according to multiple lines of evidence (Kotyla and Islam 2020). Precisely identification and measurement of miRNAs is crucial for clinical diagnosis and treatment of disorders. However, the small size, low abundance, and sequence similarity among miRNA family members make this process highly complex (Lu and Rothenberg 2018).

The existing conventional techniques for miRNA detection encompass next generation sequencing (Dard-Dascot et al. 2018; Coenen-Stass et al. 2018), microarray-based hybridization (Iizuka et al. 2016), and quantitative reverse transcription polymerase chain reaction (qRT-PCR) (Ban and Song 2022). However, these methods are often constrained by the need for costly equipment, intricate operational procedures, and the necessity for skilled operators. Hence, there is an urgent need for a simple, precise, and highly sensitive analytical technique to identify miRNA. The CRISPR/Cas system is an adaptive immune system present in archaea and bacteria, functioning as a protective mechanism against the infiltration of foreign genetic material, such as viruses (Zhao et al. 2020; Knott and Doudna 2018). The CRISPR/Cas system has garnered significant interest from researchers in the gene editing area owing to its exceptional ability to modify genes (Hanna and Doench 2020). CRISPR/Cas12a, a DNA recognition and cleavage system, exhibits highly effective *trans*-cleavage activity, and high target recognizing capability under the guiding of crRNA. Briefly, the Cas12a protein forms a Cas12a/crRNA complex with crRNA in order to recognize the target DNA specifically. Subsequently, the protein initiates its *trans*-cleavage activity, which cleaves the DNA molecules with a high efficiency in a nonspecific manner (Guan et al. 2023; Zhao et al. 2022; Feng et al. 2023). The CRISPR/Cas12a system excels in the realm of molecular diagnostics due to its exceptional sensitivity and specificity. Although CRISPR-based methods demonstrate potent and resilient signal amplification characteristics, their capacity for detecting clinical samples without nucleic acid amplification remains inadequate. Numerous nucleic acid amplification strategies, including rolling circle amplification (RCA) (Wang et al. 2020; Zhang et al. 2020), recombinase polymerase amplification (RPA) (Yin et al. 2023), and catalytic hairpin assembly (CHA) (Zhao et al. 2022), have been incorporated into the CRISPR/Cas system in order to increase the sensitivity of detection. Zhao et al. introduced an approach called RACE, which combines RCA with Cas12a to achieve fast and sensitive miRNA detection (Zhang et al. 2020). Despite that the experiments showed that the inclusion of various amplification strategies does enhance the sensitivity of Cas12a-based sensing devices, the complicated primer design and laborious experimental procedures constrain their further applications.

In this study, we presented a signal cycle platform that detects miRNAs sensitively using cyclic reverse transcription (CRT) and CRISPR-Cas12a. The platform comprised two components: i) Enzymatic cyclic amplification utilizing the CRT process; ii) Signal production facilitated by the CRISPR/Cas12a system and duplex-specific nuclease (DSN). By capitalizing on the superior fidelity and cleavage efficiency of the CRISPR/Cas12a system and the DSN, this component integrated the cleavage activity of the DSN with the high-efficiency *trans*-cleavage activity of the CRISPR/Cas12a system to generate a substantial quantity of "4"@magnetic beads ("4"@MBs") products, thereby accomplishing signal amplification. Through the strategic integration of CRISPR/Cas12a and CRT, we effectively achieved the detection of complex samples with exceptional sensitivity and a limit of detection of 3.2 fM. The platform exhibited both practicality and reliability, making it an indispensable tool for clinical diagnostics and biomedical research.

Experimental section

Materials and Reagents

The nucleic acid strands used for the target recognition and signal amplification were designed according to former articles, and the details of these sequences are listed in Additional file 1: Table S1. All these oligonucleotides were synthesized and purified by Shanghai Sangon Biotech Co., Ltd. (Shanghai, China). Takara Biotech. Inc. (Dalian, China) provided us with the RNase inhibitor, RNase-free water, and deoxynucleotide triphosphates (dNTP). New England Biolabs Inc. (Ipswich, MA, U.K.) provided the Engen Lba Cas12a, and the DSN was obtained from Beyotime Biotech Co., Ltd. (Shanghai, China). The obtained streptavidin-coated MBs were 1 μm and were provided by the Beyotime Biotech Co., Ltd. (Shanghai, China).

Fluorescent assay to test the CRT reaction

A first-strand cDNA synthesis kit from Thermo Fisher Scientific was used. The reaction solution contains 0.5 μL of reverse transcriptase, 2 μL of ribozyme inhibitor (5 U), 1.5 μL of dNTPs (0.5 mM), 2 μL of the FAM labeled target miRNA, 2 μL of BHQ labeled primers, and 15 μL of PBS buffer. The mixture was incubated at 42 °C for 60 min to construct the CRT process according to former references (Long et al. 2023). The mixture was then heated to 90 °C for 10 min to degrade the CRT process.

miRNA detection by the system

A first-strand cDNA synthesis kit from Thermo Fisher Scientific was used. The reaction solution contains 0.5 μL of reverse transcriptase, 2 μL of ribozyme inhibitor (5 U), 1.5 μL of dNTPs (0.5 mM), 2 μL of the target miRNA, 2 μL of primers, and 15 μL of PBS buffer. The mixture was incubated at 42 °C for 60 min to construct the CRT process. The mixture was then heated to 90 °C for 10 min to degrade the CRT process. 1.5 μL of Cas12 protein and 4 μL of H@MB complex were added to the mixture. The mixture was incubated at room temperature for 30 min to allow the Cas12a protein to degrade the "5" fragment in the H probe. The mixture was heated to 70 °C for 10 min to degrade the Cas12a protein. 1.5 μL of the "reporter" probe, and 1.5 μL of the DSN were added to the mixture. The fluorescence signals were recorded after incubating for 30 min.

Results and discussion

The working mechanism of the approach for miRNA detection

The operational mechanism of the suggested method is depicted in Fig. 1. The linear primer sequence contains three functional sections, including the "1", "2", and "3", in accordance with the before-mentioned design principle of CRT (Lan et al. 2019). The target miRNA can be recognized by "1", which also functions as the loop of the hairpin structure that is subsequently formed. The sequence of part "2" is identical to that of the 5'-terminal regions of the target miRNA. Component "3" includes the PAM and the non-target strand (NTS) of LbCas12a. The primer undergoes elongation to form an incomplete hairpin structure (2*–1–2–3) upon hybridization with the target in the presence of reverse transcriptase. This elongation makes the hybridization between 2* and 2 sections, which results in the liberation of the miRNA encoding the CRT. By replicating itself using the incomplete hairpin (3–2–1–2*) as a template, the structure continues to develop into a complete hairpin under the assistance of DNA polymerase. The synthesized hairpin (3*-2*-1–2-3), comprising the TS/NTS duplex and the PAM, subsequently facilitated the activation of the LbCas12a/crRNA complex, resulting in the cleavage of the "5" fragment of the H probe. The "5" fragment is DNA fragment, whereas the "4" fragment in the H probe is RNA. As a consequence, the "5" fragment undergoes *trans*-cleavage

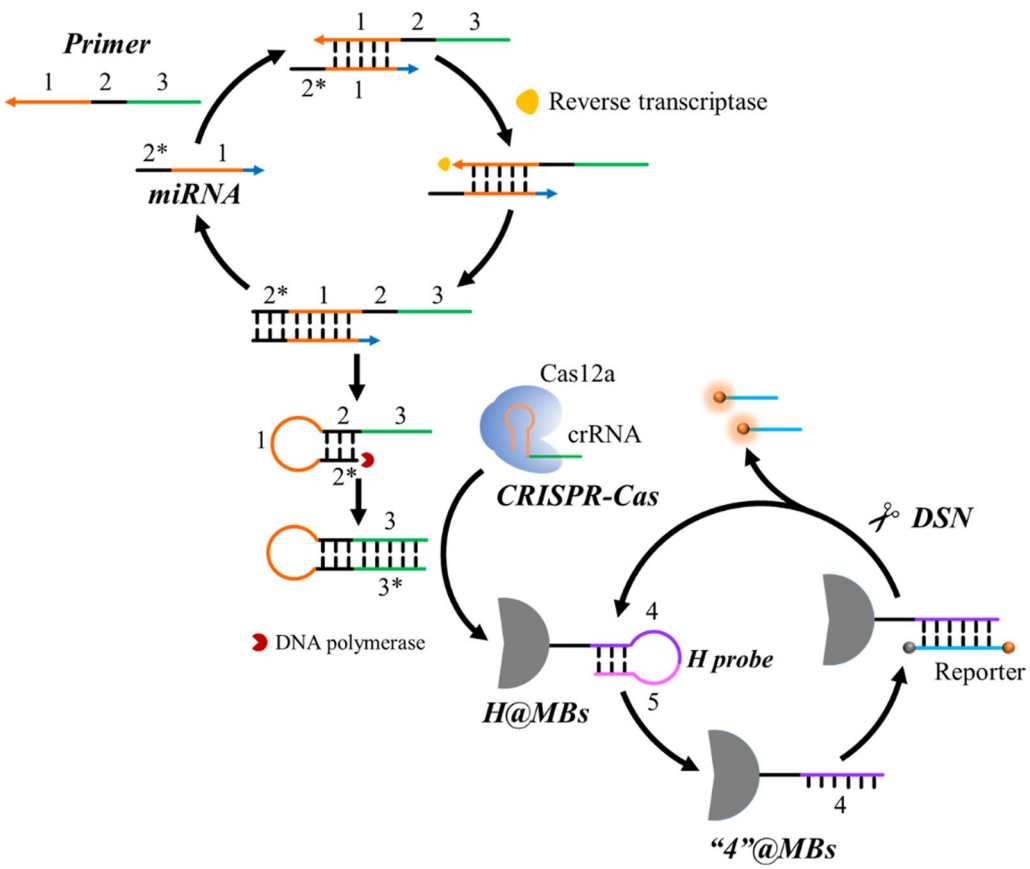

Fig. 1 The working mechanism of the approach for miRNA detection

activity facilitated by Cas12a, leading to the magnetic isolation of the "4"@MBs. A duplex of RNA and DNA is produced when the RNA "4" fragment hybridizes with the DNA "reporter" probes. With the aid of the DSN, the "reporter" probe undergoes digestion, resulting in the resurgence of the fluorescence signals.

Feasibility of the CRT process and the CRISPR-Cas12a system

Initially, we confirmed the viability of CRT by conducting fluorescence analysis. The model target chosen was miRNA-21, which has a crucial function in various biological processes by controlling gene expression after transcription. The operational mechanism of the fluorescent CRT is performed through labeling FAM and BHQ at the 3' end of miRNA and 5' end of the primer sequence. The efficacy of CRT is validated through the fluorescence changes, which can disclose the specific products generated during the course of the reaction. According to Fig. 2A, the measured fluorescence intensity dropped, suggesting that the target miRNA bonded with the primer sequence. The reverse transcriptase facilitated an increase in fluorescence intensity, indicating that the primer was successfully elongated using the target miRNA as a template. This allowed the primer sequence to self-hybridize and release the miRNA from the miRNA/primer duplex (Fig. 2B). The above-mentioned result showed the practicability of the target miRNA-based CRT procedure. In order to confirm if the amplification products (3-2-1-2*-3*) could induce the *trans*-cleavage activity of Cas12a, a fluorescence experiment was conducted. Upon observation, it was found that the combination of miRNA-21, reverse transcriptase, and primer effectively stimulated the *trans*-cleavage function

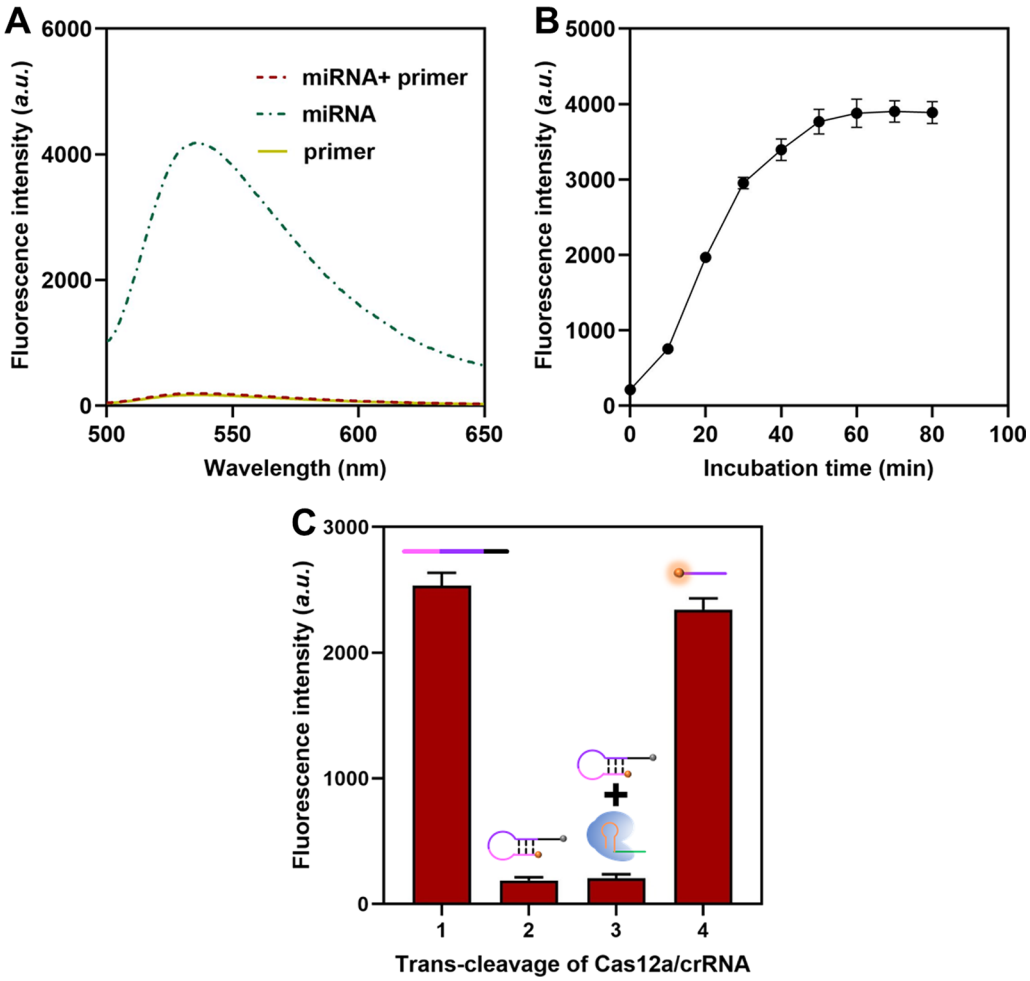

Fig. 2 Feasibility of the CRT and the *trans*-cleavage activity of Cas12a/crRNA. **A** fluorescence spectrum of the FAM labeled-miRNA before and after hybridizing with the primer sequences. **B** Fluorescence intensities of the FAM labeled-miRNA during the CRT process when the reverse transcriptase existed. **C** Fluorescence intensity of the H probe during the *trans*-cleavage activity of the Cas12a/crRNA. 1, H probe (linear); 2, H probe (hairpin structure); 3, H probe (hairpin structure) + Cas12a/crRNA; 4, H probe (hairpin structure) + Cas12a/crRNA + CRT product

of Cas12a. This led to the breakdown of the "5" fragment in the H probe (as shown in Fig. 2C) and triggered a significant increase in fluorescence signal.

Optimization of experimental parameters

Then, we maximized signal-generation potential of the method by optimizing the reaction conditions for both CRT and CRISPR-Cas12a. Primers with a concentration of 50 nM produced the strongest fluorescence signal, as seen in Fig. 3A. The most intense fluorescence signal was then seen at 100 nM of Cas12a (Fig. 3B). Earlier research has shown that the limit of detection and the signal-to-background ratio are both affected by the reporters used. As a result, we evaluated the ssDNA reporter's fluorescence response signal. Figure 3C shows that the reporter produced a stronger fluorescence response at 100 nM. Figure 3D shows that a fluorescent signal of the highest intensity was achieved with a CRT reaction time of 60 min and Cas12a reaction time of 30 min.

Analytical performance of the approach for miRNA analysis

Detection of miRNAs requires exceptionally high sensitivity and specificity. Subsequently, an assessment was conducted to determine the sensitivity and specificity of the platform. We utilized this platform to identify targets by subjecting them to a range of doses spanning from 10 nM to 10 fM (Fig. 4A). The fluorescence signals of the technique augmented according to the escalation of target concentration. A strong linear correlation existed between the logarithm of the target concentrations and the intensity of the luminescence signal. The correlation equation was $Y = 983.8^{*}\lg C + 127.5$, with an R^2 value of 0.9896. The detection limit, as determined in Fig. 4B, was calculated to be 3.2 fM. The sensitivity of miRNA detection based on CRISPR/Cas12a was much enhanced compared to prior studies. Four distinct microRNAs (miR-155, miR-221, miR-149, and miR-21) were introduced to the platform in order to assess the method's specificity. The results demonstrated that only

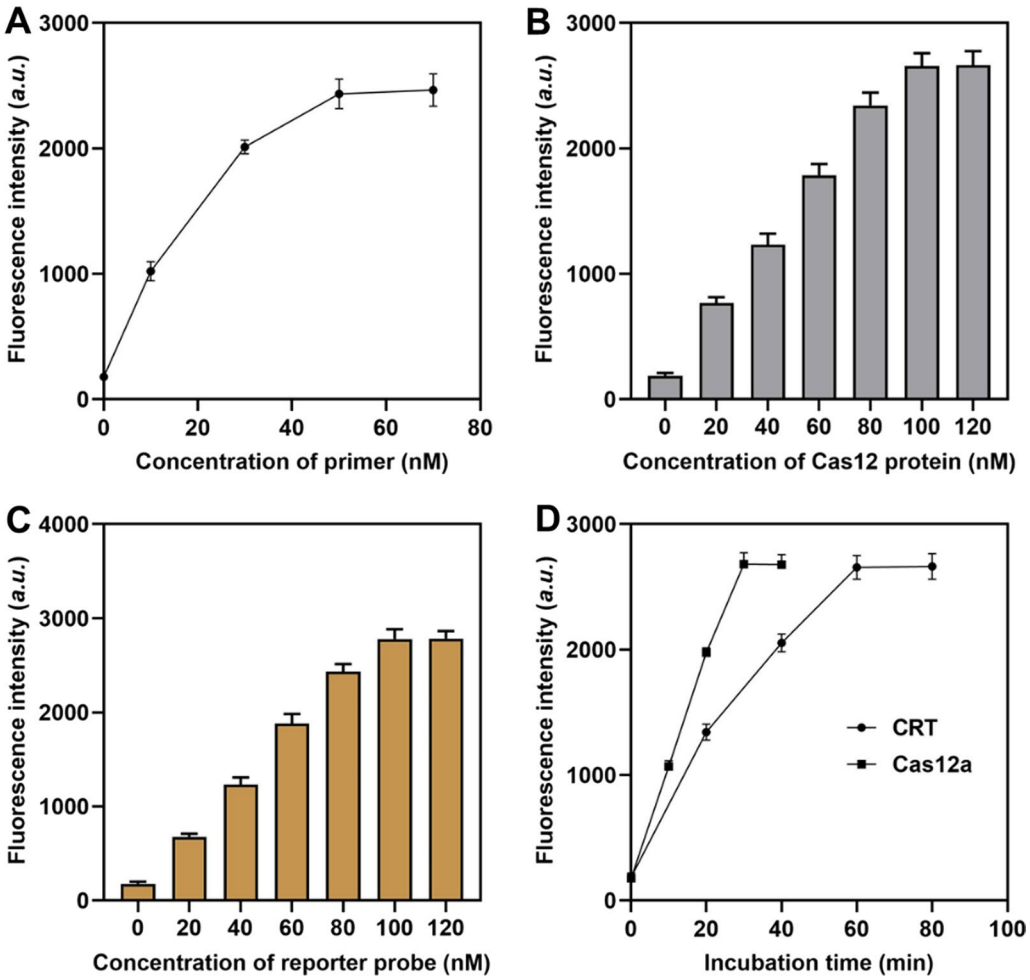

Fig. 3 Optimizations of experimental parameters. Fluorescence intensity of the approach when detecting miRNA with different concentration of primer (**A**), different concentration of Cas12a protein (**B**), different concentration of "reporter" probe (**C**), and different incubation time (**D**)

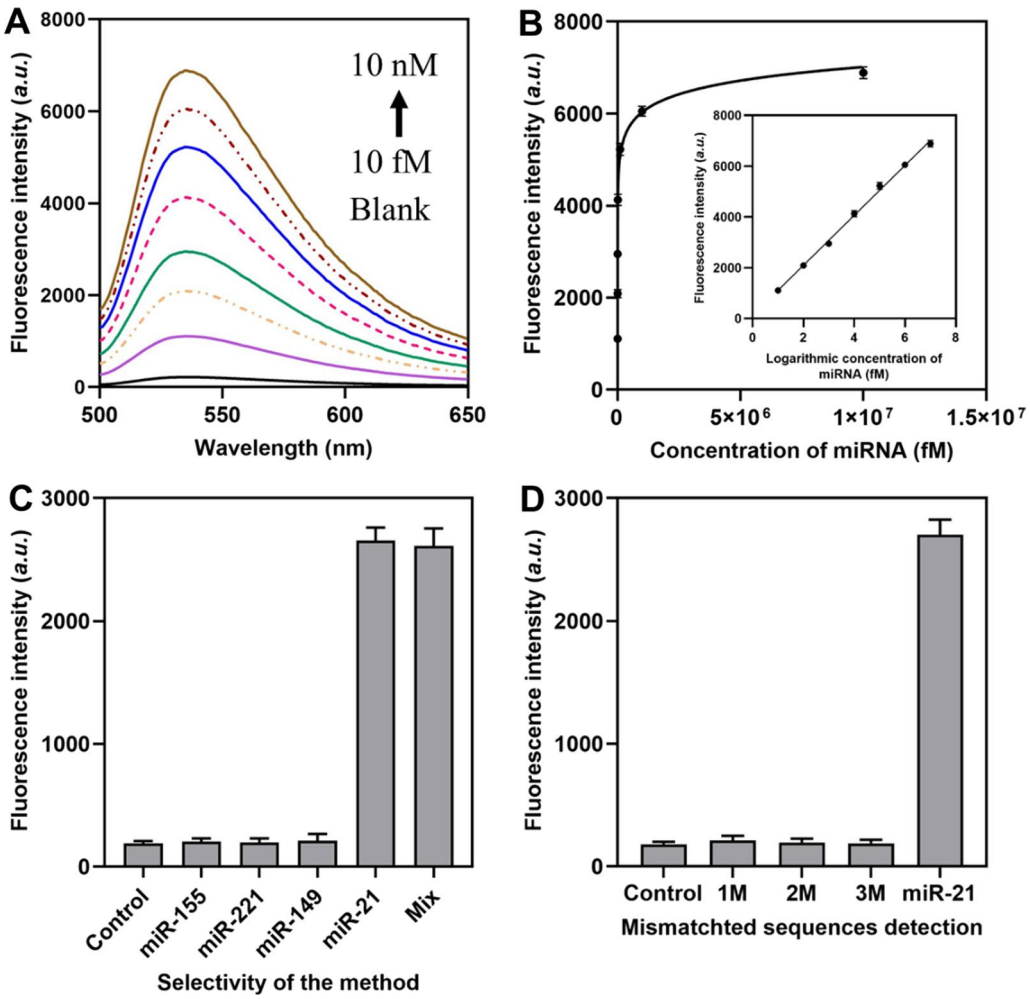

Fig. 4 Analytical performance of the approach for miRNA detection. **A** Fluorescence spectrum of the approach when detecting different concentrations of miRNA. **B** Calibration curve between the obtained fluorescence signals and the concentration of miRNA. **C** Fluorescence intensities of the approach when detecting different concentrations of miRNA. **D** Fluorescence intensities of the approach when detecting mismatched sequences

the experimental group containing the target miRNA-21 and the mixed group (Mix) had a substantial signal response. In contrast, the fluorescence signals of the other experimental groups were faint, showing the platform's high specificity (Fig. 4C). To further examine the specificity of the platform, we also performed an experiment on mismatched sequences. The results indicated that the signal response was stronger only for the target miRNA detection, while no significant signal responses could be observed for the single-base mismatched sequence (1 M), two-base mismatched sequence (2 M), and three-base mismatched sequence (3 M). The outcome aligned with the prior report, indicating the high selectivity of the approach.

Complex sample analysis by the proposed method

We assessed the platform's capability to detect miRNAs in an intricate setting. To provide a complicated environment for detection, a 10% concentration of fetal bovine serum (FBS) was introduced into the reaction solution. The findings demonstrated that the platform was able to effectively differentiate between the target and non-target in the complicated environment with the presence of 10% FBS, as indicated by the data shown in Fig. 5A. In addition, we employed the platform to identify artificially created serum samples, thereby showcasing its efficacy in analyzing serum samples. Based on the data presented in Fig. 5B, the platform effectively identified the contaminated samples. A spiked recovery experiment was conducted to test the stability of the approach (Table 1). The

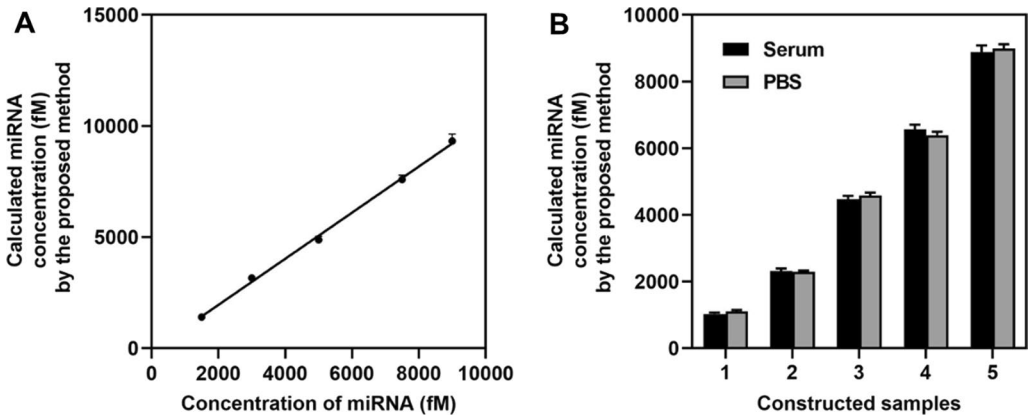

Fig. 5 Clinical application potential of the approach. **A** Calculated miRNA concentration by the proposed method from the samples with 10% FBS. **B** Calculated miRNA concentration by the proposed method from the samples with commercial serum and PBS

Table 1 Recovery rate of the approach

Samples	Added (fM)	Detected (fM)	Rate (%)
1	500	493	98.60
2	2500	2612	104.48
3	5000	4912	98.24

recovery rate ranged from 98.24 to 104.48%, which is sufficient for clinical application.

Conclusion

To summarize, we have developed a highly precise and sensitive platform for detecting miRNA. This platform combines the use of the CRT with the CRISPR-Cas12a system, which assists in generating signals with the help of DSN. The existence of the miRNA target can trigger the production of a substantial quantity of hairpin type products by the CRT. These products can function as activators, binding with crRNAs to activate Cas12. By combining the signal conversion and amplification capabilities of CRT with the extremely efficient catalytic properties of CRISPR-Cas12a, we have developed a two-stage amplified detection method for miRNA targets that offers a reasonably high level of sensitivity. Furthermore, the platform demonstrated a high level of specificity as a result of the dual assessment of targets: initially in the CRT and subsequently in the CRISPR system. When the settings were improved, the detection limit reached a remarkably low value of 3.2 fM. Crucially, the specificity of the system was enhanced by introducing a single-base mismatch. Additionally, the system was able to achieve a reasonable recovery rate even in the presence of a complex environment including 10% FBS. In summary, these findings clearly indicate that the platform has exhibited exceptional molecular diagnostic proficiency in detecting miRNA, hence presenting extensive potential for many applications (Additional file 1: Table S2). Our next goal is to integrate this platform with photonic crystals and portable equipment in order to accomplish point-of-care diagnostics.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-024-00430-8.

Additional file1: **Table S1**. Nucleic acid sequence used in the experiments. **Table S2**. A brief comparison of the approach with former methods.

Author contributions

XY is the supervisor of the team in all research steps including designing, data analysis and manuscript writing. JG, as the co-first author, has the main role for experimental data collection, data gathering, preparation of results, and 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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