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Direct and accurate miRNA detection based on CRISPR/Cas13a-triggered exonuclease-iii-assisted colorimetric assay

Yunxiao Li[1†], Qiuxia Wang[1†] and Yali Wang[1*]

Abstract

The abnormally expressed microRNAs (miRNAs) serve as crucial indicators for disease diagnosis and are strongly associated with the progression of various diseases. The quantification of miRNAs is highly significant for the clinical diagnosis and treatment of various types of cancers. This study utilizes the CRISPR/Cas13a system, which combines CRISPR RNA (crRNA) and CRISPR-associated Cas13a, to recognize the miRNA directly and specifically, thus activating the *trans*-cleavage activity of Cas13a. By integrating the CRISPR-Cas13a system with an exonuclease-iii (Exo-iii)-assisted chain cleavage of silver ions (Ag^{+})-aptamer and an Ag+ -based color reaction, a method for the detection of miRNA that is specific and sensitive is developed. This approach demonstrated exceptional sensitivity in detecting miRNA, with a low detection limit of 5.12 fM. Moreover, the suggested method was effectively utilized to precisely measure the amount of miRNA in intricate biological samples, demonstrating its practical applicability. Furthermore, due to its elevated sensitivity and simple probe design and fluorophore labeling, the suggested colorimetric technique holds great potential for use in clinical diagnostics.

Keywords microRNAs (miRNAs), Exonuclease-iii (Exo-iii), Clustered regularly interspaced short palindromic repeats (CRISPR), Color reaction

Introduction

MicroRNAs, which consist of 18–23 nucleotides, are a category of small noncoding RNA that exerts a substantial influence on numerous cellular biological processes (Abdalla et al. 2020), including but not limited to cellular differentiation, development, and apoptosis (Wu et al. 2021; Zhang et al. 2022). Abnormally expressed microRNAs have been identified as key biomarkers for disease diagnosis because they are intimately linked to the progression of numerous types of diseases (Suarez et al. 2022; Pahlavan et al. 2020). Hence, the precise identification of miRNA is crucial for early diagnosis and treatment of disease, as well as for fundamental biological investigation. Nevertheless, the precise and delicate identification of miRNAs is currently encountering obstacles due to low abundance and the interference from homologous sequences.

Various signal amplification techniques have been developed to enhance the accuracy and sensitivity of miRNA detection (Jet et al. 2021; Granados-Riveron and Aquino-Jarquin 2021). These techniques include quantitative reverse transcription-polymerase chain reaction (qRT-PCR) (Deng et al. 2017; Cheng et al. 2018), exponential isothermal amplification reaction (EXPAR) (Reid et al. 2018; Kim et al. 2021), rolling circle amplification (RCA) (Wang et al. 2020; Zhang et al. 2020), and hybridization chain reaction (HCR) (Cao et al. 2023; Qiu et al. 2023). Among them, the use of endonuclease

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or exonuclease enzymes to regenerate the analyte is particularly attractive for developing miRNA detection methods. Exonuclease-iii (Exo-iii) is an enzyme that gradually eliminates individual nucleotides from the blunt or recessed -OH ends of double-stranded DNA (Yan et al. 2022; Zhou et al. 2020). However, it has only limited effectiveness on single-stranded DNA or double-stranded DNA with a projecting 3'-end. Exo-iii facilitates target regeneration without the need for a specific recognition site, distinguishing it from nicking endonuclease (Liu et al. 2018; Huang et al. 2014). This characteristic renders the Exo-iii-assisted approaches an adaptable and potent amplification strategy suitable for a wide range of biosensing applications. Nevertheless, these techniques typically necessitate well-optimized amplification settings or fluorophore tagging in order to obtain sensitive detection of miRNA. Additionally, they lack sufficient accuracy for miRNA detection, hence restricting their broad utilization. Hence, there is still a strong need to create a simple, effective, and sensitive platform for the miRNA detection.

The clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) adaptive immune system can effectively identify and cleave exogenous genetic material from invading viruses and phages by using RNA-guided endonucleases (Zhao et al. 2020; Zhang et al. 2023). CRISPR-Cas12, 13, 14 have been demonstrated to indiscriminately cleaves (*trans*-cleave) non-target molecules after specifically identifying nucleic acids. This indicates that the CRISPR/Cas system is a highly effective tool for editing gene sites, with significant potential not only in the field of genome editing but also in nucleic acid detection. CRISPR/Cas13a enzymes can form complexes with CRISPR RNA (crRNA) and activate the function of crRNA-guided Cas13a, leading to the precise cleavage of non-specific RNA substrates upon specific identification of the target RNA (Zhao et al. 2022, 2023). CRISPR/Cas13a has emerged as a highly promising and optimal tool for nucleotide acid detection due to its exceptionally specific recognition of target RNA.

We developed here a highly sensitive and precise approach for detecting miRNA using CRISPR/Cas13a-triggered exonuclease-iii-assisted color reaction. To demonstrate the concept, miRNA-155 is selected as a detection target due to its elevated expression in cancers, such as leukemia. When miRNA-155 is present, it activates the *trans*-cleavage activity of Cas13a to cleave the H1 probe and release the "a" strand. The "a" strand, once released, causes the H2 probe to unfold and reveal the "e" portion. This section then binds with the silver ions (Ag^+)-aptamer, resulting in the formation of a protruding 3′-terminus. The Exo-iii enzyme specifically recognizes and cleaves the Ag^+-aptamer at its 3′-terminus. The Ag^+ can be chelated by the Ag^+-aptamer via the interaction between Ag^+ and the N3 of cytosine (C), which forms a robust and stable "C–Ag^+–C" hairpin structure by bridging two cytosine residues. Sediment consists entirely of magnetically separated magnetic bead (MB) following the Exo-iii-assisted aptamer cleavage, and the supernate containing Ag^+ was collected. Afterward, 3,3′,5,5′-tetramethylbenzidine (TMB), an organic dye that may be oxidized by Ag^+, was added to the supernate as a visualization probe. On this occasion, Ag^+ interacted with TMB to produce color rather than binding to the aptamer. Therefore, the blue color appeared and distinctive UV–vis absorption peak at 652 nm was recorded in the solution containing oxidized TMB (TMBox). In the absent of target miRNA, Ag^+ chelated with the aptamer, which would subsequently have enriched magnetically. As a result, the Ag^+ in the supernate was removed; subsequently, the solution loses its color and TMBox is no longer present (Fig. 1).

Results and discussion

Feasibility of miRNA-155 detection

The cleavage of the activated Cas13a enzyme toward the H1 probe was initially confirmed (Fig. 2A). The successful assembly of the H1 probe is indicated in Fig. 2B, where the fluorophore labeled "a" section (FAM-"a") attaches to the quencher-labeled "b" section (BHQ1-"b"). The decrease in fluorescence signal indicates the close proximity of the fluorophore to the quencher and the successful assembly of the "stem" section in H1 probe. Additionally, the foiled signal is restored upon incubation of the assembled H1 probe with the activated Cas13a-crRNA13a and H2 probe. The outcome illustrates the effective cleavage of Cas13a at the rUrU site in the "c" section, and the "a" section can detach from the double-stranded structure once the H1 probe is cleaved with the aid of the H2 probe. We subsequently confirmed the accuracy of the CRISPR-Cas13 system in identifying and binding to specific miRNA targets, as demonstrated in Fig. 2C. The findings indicated that the combination of Cas13a-crRNA13a with target miRNA resulted in a noticeable increase in fluorescence signal, indicating that the *trans*-cleavage activity of Cas13a-crRNA13a was activated by the target miRNA. Conversely, when non-specific miRNA is present, there is a minimal alteration in the fluorescence signal. This indicates the exceptional selectivity of the Cas13a-crRNA13a system, as shown in Fig. 2D. Color reaction was used to verify the feasibility of the method. When the target miRNA, Cas13a-crRNA13a, H2 probe, and Exo-iii were present, significant color changes (from colorless to blue) were observed. In the absence of any of the experimental components, no significant color changes were observed, suggesting that

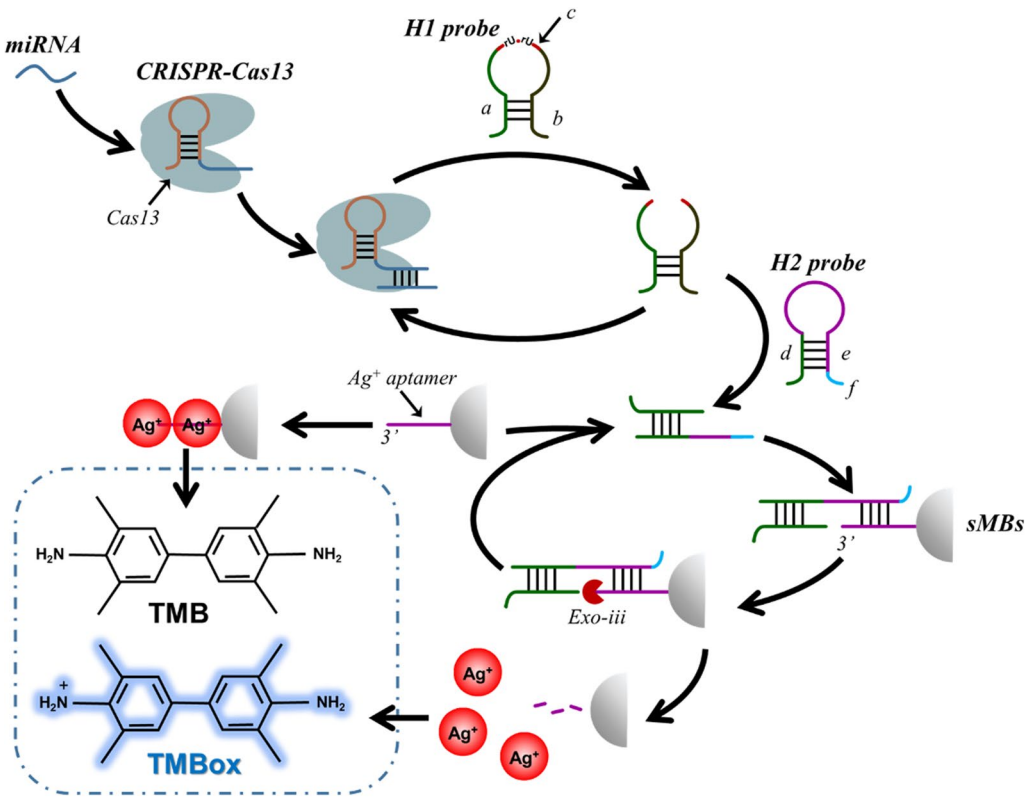

Fig. 1 The working mechanism of the approach for sensitive miRNA detection

these components are of great significance to the feasibility of the method (Fig. 2E).

Optimization of reaction conditions for improving analytical performance

By comparing absorbance under different reaction conditions, the optimal parameters for this method were determined. The reaction parameters, such as reaction duration and temperature, have been optimized based on the detection of 100 pM miRNA-155. In order to minimize the duration of the test, the reaction times of Cas13a and Exo-iii were initially optimized. As demonstrated in Fig. 3A, there is a rising trend in the absorbance with the growth of the reaction time of Cas13a from 0 to 45 min, which reached an equilibrium with a longer period. The results demonstrate that the activated Cas13a enzyme may completely cleave the H1 probe in 45 min. The optimal reaction time of Cas13a is 45 min. Figure 3B demonstrates a progressive enhancement in absorbance as the reaction time of Exo-iii increases from 0 to 30 min, after which it remains constant. Hence, the most favorable response time of Exo-iii is 30 min. The investigation focused on determining the ideal reaction temperatures for achieving maximum *trans*-cleavage activities of Cas13a and Exo-iii. In Fig. 3C, the absorbance progressively increases as the reaction temperature rises from 4 to 37 °C. However, it then sharply decreases, suggesting that temperatures above 37 °C negatively affect the *trans*-cleavage activity of Cas13a. Thus, the reaction temperature of Cas13a is 37 °C.

Ultra-high sensitivity of this method for synthetic miRNA-155 detection

Due to the relatively low abundance of miRNAs, particularly during the initial phases of disease, the biosensor's sensitivity is critical. Therefore, we evaluated the sensitivity of the colorimetric method facilitated by CRISPR/Cas13a-triggered Exo-iii under optimal reaction conditions. The absorbance values exhibit a progressive enhancement as the concentration of synthetic miRNA-155 escalates from 0 to 500 pM, as illustrated in Fig. 4A. The linear correlation between absorbance values and logarithmic values of miRNA-155 concentrations ranging from 5 fM to 100 pM is illustrated in Fig. 4B. The equation representing this relationship is $Y = 0.1244 \times \lg C_{target}$ (fM) + 0.2184, and correlation coefficient (R^2) = 0.9943. The estimated limit of detection (LOD) is 3.12 fM according to the triple deviation rule, which is superior to or comparable to the LODs of numerous colorimetric techniques.

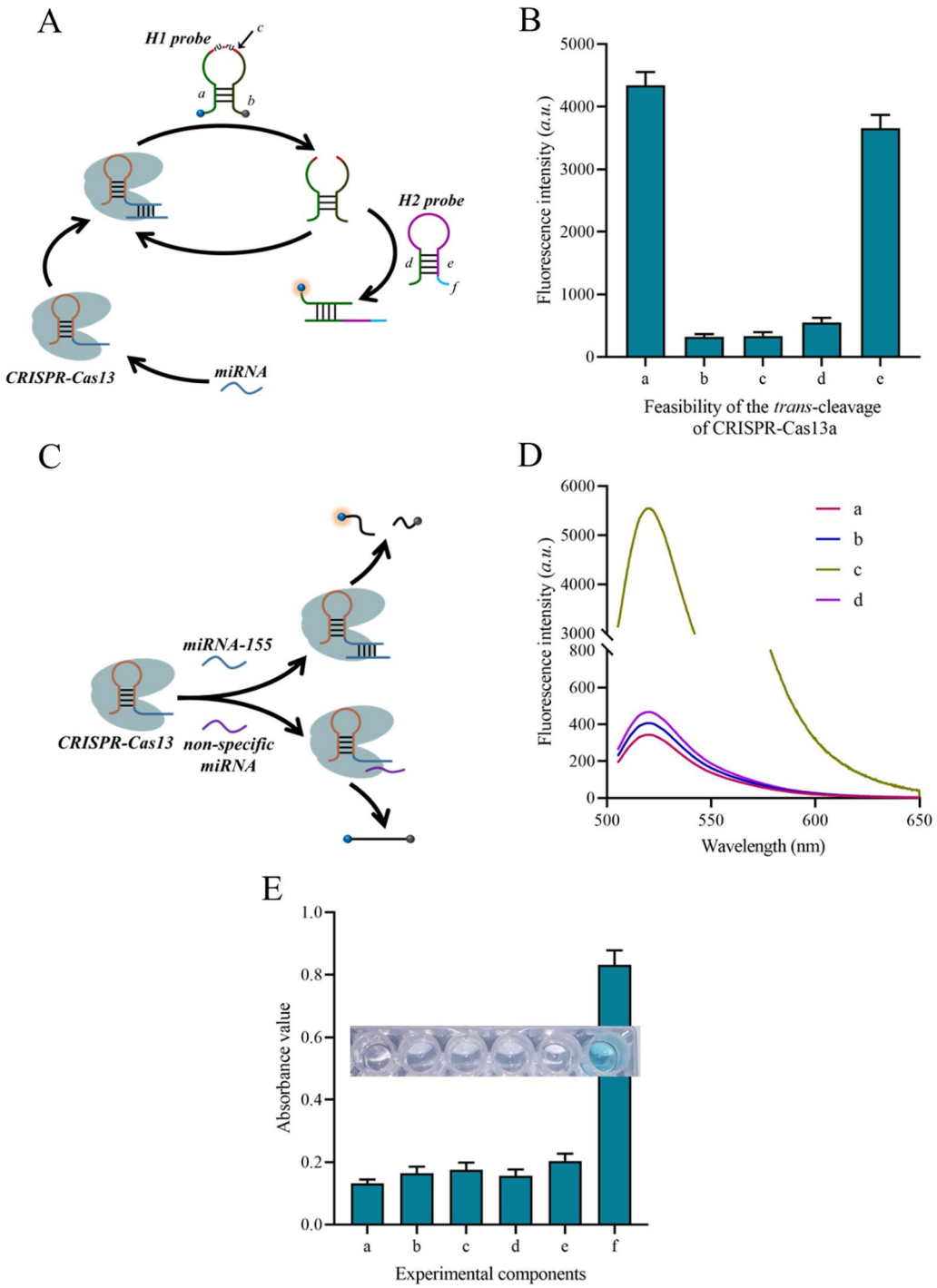

Fig. 2 Feasibility of the approach. **A** Schematic illustration of the fluorescent assay to test the *trans*-cleavage activity of CRISPR-Cas13a system. **B** Fluorescence intensity of the FAM labeled H1 probe during the *trans*-cleavage process of Cas13a-crRNA13a system. a, H1 probe (before assembly); b, H1 probe; c, H1 probe + miRNA-155; d, H1 probe + miRNA-155 + Cas13a-crRNA13a; e, H1 probe + miRNA-155 + Cas13a-crRNA13a + H2 probe. **C** Schematic illustration of the fluorescent assay to test the selectivity of CRISPR-Cas13a system. **D** Fluorescence intensity of the FAM labeled H1 probe when Cas13a-crRNA13a system was incubated with miRNA-155 and non-specific miRNA. a, reporter probe (FAM and BHQ were labeled on both terminus of reporter probe); b, reporter probe + Cas13a-crRNA13a; c, reporter probe + Cas13a-crRNA13a + miRNA-155; d, reporter probe + Cas13a-crRNA13a + non-specific miRNA. **E** Absorbance of the approach under different experimental conditions

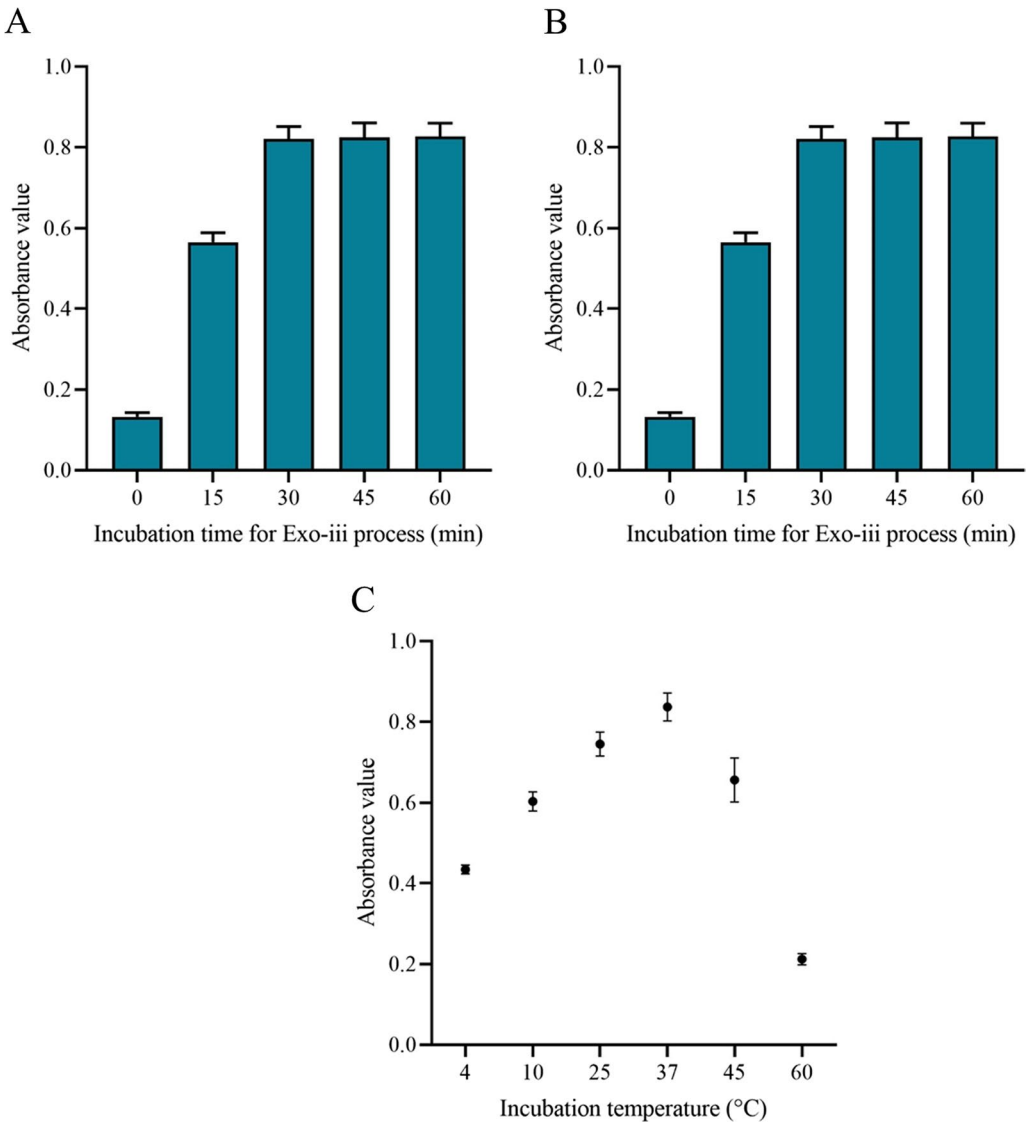

Fig. 3 Optimization of experimental parameters. Absorbance value of the approach with different incubation time for Cas13a protein (**A**), Exo-iii (**B**), and incubation temperature (**C**)

Specificity and reproducibility of this method

In order to assess the specificity of the proposed CRISPR/Cas13a-triggered exonuclease-iii-assisted colorimetric method, an experiment performed in which five distinct miRNAs were introduced into the system: two unmatched miRNAs (let-7a and miRNA-211), single-base mismatched (SM), double-base mismatched (DM), and three-base mismatched (TM). The findings (Fig. 5A) indicated that the color changes of the interfering miRNAs were all insignificant in comparison to the blank control. However, a significant increase in absorbance values was observed exclusively for miRNA-155. The aforementioned outcomes served as evidence of the proposed approach's exceptional specificity in detecting miRNA. In order to provide additional evidence for the stability of the method that was devised (Fig. 5B), miRNA-155 in six sample duplicates were measured by the proposed approach. Consequently, a relative standard deviation (RSD) of 3.442% was achieved, indicating that the developed approach had adequate repeatability.

Clinical sample detection and methodology comparison

Triple parallel analyses of miRNA-155 at concentrations of 100 fM and 1 pM, respectively, were performed as part of the recovery study in order to validate the stability of the method. As shown in Table 1, the recoveries ranged

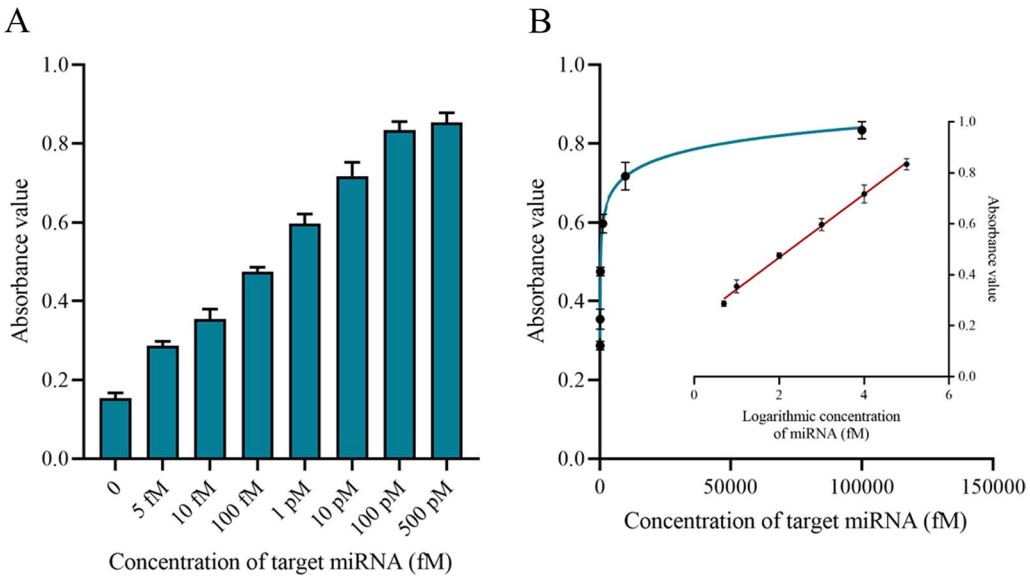

Fig. 4 Sensitivity of the approach for miRNA-155 detection. **A** Absorbance value of the approach when detecting different concentrations of miRNA. **B** Correlation between the absorbance value and the concentration of miRNA-155

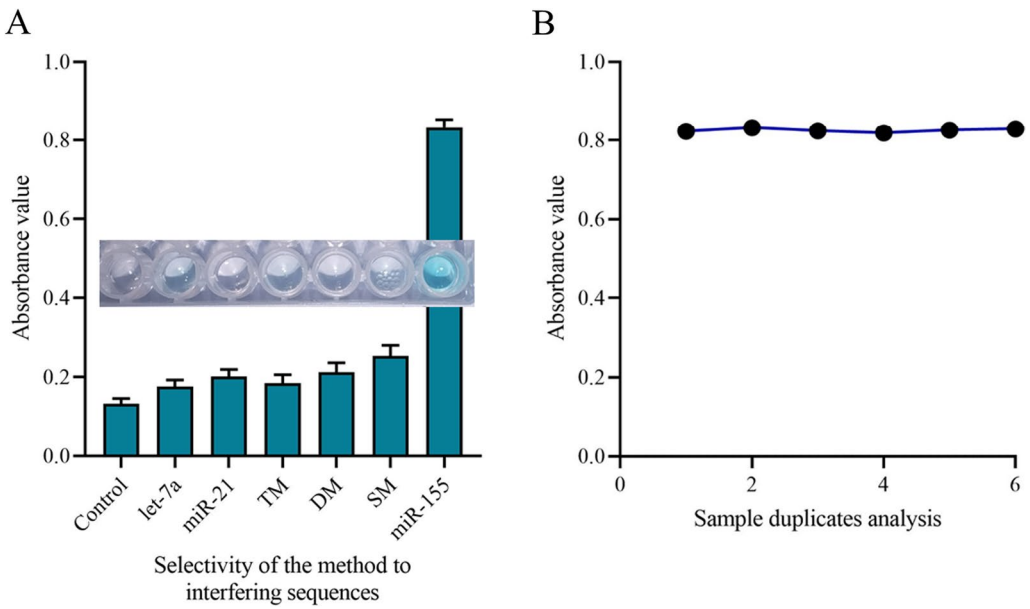

Fig. 5 Specificity of the approach for miRNA-155 detection. **A** Absorbance value of the approach when detecting different miRNAs. **B** Absorbance value of the approach when detecting six sample duplicate

Table 1 Recovery rate of the colorimetric approach

Sample	Added	Detected	Recovery rate (%)
1	100 pM	98.12 pM	98.12
2	10 pM	10.32 pM	103.2
3	5 pM	4.94 pM	98.8

from 98.21 to 103.2%, with RSDs between 3.21 and 4.12%. Additionally, the suggested approach was compared to the quantitative result obtained with the commercial stem-loop RT-qPCR reagent. To prepare the synthetic samples, various doses of miRNA-155 were diluted with commercial serum solution. Figure 6 illustrates the quantitative outcome. Our method yielded results that

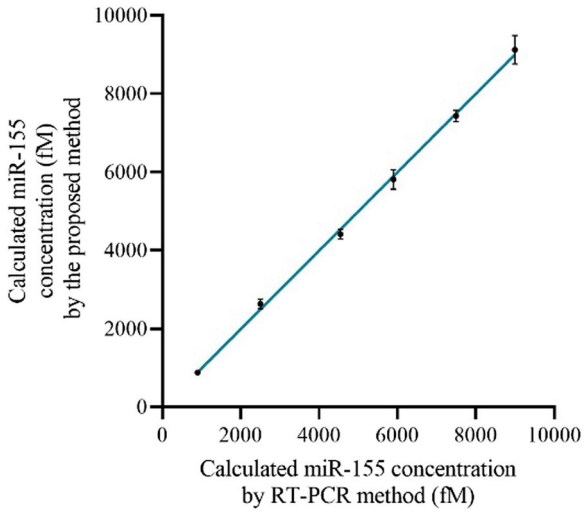

Fig. 6 Correlation between the calculated miR-155 concentration (fM) by the proposed method and by RT-PCR method

were highly consistent with the quantities of miRNA-155 calculated by RT-PCR. The finding suggests that the assay exhibited satisfactory performance in quantifying miRNA in an authentic biological sample. Consequently, it holds promise as a tool for identifying miRNA originating from cancer cells.

Conclusion

In summary, a simple yet exceptionally sensitive colorimetric approach for miRNA detection has been devised, which relies on an Ag^+-aptamer-based color reaction in conjunction with CRISPR/Cas13a-triggered exonuclease-iii-assisted signal amplification. The proposed method exhibits exceptional specificity in miRNA detection due to the high-fidelity signal amplification facilitated by CRISPR/Cas13a and the complementary base pairing between crRNA and target miRNA. Furthermore, through the integration of Exo-iii-assisted cleavage of Ag^+-aptamer and the high collateral cleavage efficiency of CRISPR/Cas13a, the proposed colorimetric method for miRNA detection has achieved a significantly enhanced sensitivity, as evidenced by its low detection limit of 5.12 fM. Furthermore, the reaction system is exceedingly simple; the detection platform can be broadly employed to detect other RNA molecules with sensitivity simply by programming the crRNA. The potential of the proposed strategy for clinical diagnostic applications is substantial, in our opinion.

Experimental procedure

Reagents and materials

Nucleic acid sequences listed in Table S1 were purchased from Sangon Biotechnology Co. Ltd. (Shanghai, China). LwaCas13a was purchased from Shanghai HuicH Biotech Co., Ltd. (Shanghai, China). The storage buffer (pH 7.5) of LwaCas13a contained 600 mM NaCl, 50 mM Tris–HCl, 5% glycerol, and 2 mM DTT. Exo-iii and NEBuffer 2.1 were obtained from New England Biolabs (Ipswich, MA UK). Diethyl pyrocarbonate (DEPC)-treated water was used throughout the experiment. Streptavidin-coated magnetic beads (MBs) were obtained from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China).

Preparation of H1 probe

A volume of 20 μL of H probe solution with a concentration of 5 5 μM was subjected to a water bath at a temperature of 95 °C for a duration of 5 min. Subsequently, it was gradually cooled to room temperature to facilitate the formation of the hairpin structure. The H1 probe duplexes that were made were stored at a temperature of 4 °C until they were used.

Modification of Ag^+-aptamer on MBs (Ag+ -aptamer-MB complex)

A solution containing 40 μL of Ag^+-aptamer (2.5 μM) was combined with 20 μL of MBs (5 mg/mL) and allowed to react for 1 h. This resulted in the formation of H1 probe that attached to the surface of the MBs. Subsequently, the acquired MB probes were subjected to three rounds of washing using 10 mM PBS (pH 7.4) with 0.05% Tween 20 through magnetic separation. Subsequently, the acquired MB probes were evenly distributed in 20 μL of PBS and preserved at 4 °C for subsequent utilization.

Detection of miRNA-155 using the developed method

Cas13a solution and Exo-iii solution are the two types of reaction solutions contained in the sensing system. A 2 μL of Cas13a guide RNA (crRNA13a, 22.5 nM), 2 μL of H1 probes, and 10 μL of reaction buffer (40 mM Tris–HCl, 60 mM NaCl, 6 mM $MgCl_2$, pH 7.3) comprise the Cas13a solution; 5 μL of NEBuffer 2.1, 20 μL of Exo-iii (50 nM), and 10 μL of Ag^+-aptamer-MB complex comprise the Exo-iii solution. In order to employ the methodology that we devised, 2 μL of miRNA-155 was introduced into the Cas13a reagent, followed by a 30-min incubation at 37 °C. Following that, the collected supernatant solution was incubated at 37 °C for 45 min with the Exo-iii solution. Before magnetic separation and two ultrapure water washes, 25 μL of 5 mM $AgNO_3$ was introduced and the sample was reacted at 37 °C for 10 min. Following this, 35 μL of 5 mM TMB was added to the mixture, which reacted with light avoidance at room temperature for an additional 25 min. Absorbance at 652 nm was subsequently determined, whereas coloration is readily apparent to the unaided eye.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40543-024-00434-4.

Additional file 1. Table S1. Sequence information of nucleic acid.

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Author contributions
YW is the supervisor of the team in all research steps including designing, data analysis and manuscript writing. YL and QW, as the first author, have the main role for experimental data collection, data gathering, preparation of results, and data analysis. All authors have read and approved the manuscript.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article [and its Additional file 1].

Declarations

Ethics approval and consent to participate
Chongqing University Cancer Hospital Ethics Committee approved the research. All participants were written consent informed.

Consent for publication
Study participants were written consent to publish.

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

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